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Yiping Qi *Editor*

Plant Genome Editing with CRISPR Systems

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Plant Genome Editing with CRISPR Systems

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Edited by

Yiping Qi

Department of Plant Science and Landscape Architecture, University of Maryland College Park, College Park, MD, USA

Institute for Bioscience and Biotechnology Research, University of Maryland, Rockville, MD, USA

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Editor
Yiping Qi
Department of Plant Science and Landscape Architecture
University of Maryland College Park
College Park, MD, USA

Institute for Bioscience and Biotechnology Research
University of Maryland
Rockville, MD, USA

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Preface

The world has witnessed a great period of food crop productivity growth in the past 50 years. Notably, the introduction of crop genetic improvement technologies into the developing world has resulted in drastic yield increases for major staple crops such as wheat and rice. This achievement is remembered as the Green Revolution (1966–1985). Afterwards, recombinant DNA-based biotechnology contributed to the development of highly efficient genetically modified (GM) crops, thanks to pioneers like Mary-Dell Chilton who co-developed Agrobacterium-mediated plant transformation technology. However, GM crops are expensive to develop, and they also face public acceptance problems in many countries. Meanwhile, conventional breeding cannot keep pace with global population growth and climate change. For example, the current rate of annual yield increases for four major crops (wheat, rice, maize, and soybean) must be doubled to meet the future demand in 2050. All these challenges call for the development of new breeding technologies that can potentially revolutionize agriculture. Genome editing is one such technology.

Genome editing enables rewriting the DNA sequence in a genome, which in most cases relies on the ability to make DNA double strand breaks (DSBs) in a sequence-specific manner. Sequence-specific nucleases (SSNs) are molecular scissors that are engineered to make targeted DNA DSBs. SSNs such as zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and CRISPR (clustered regularly interspaced short palindromic repeats)-Cas systems have been successfully applied in many plant species to achieve efficient genome editing. Because CRISPR-Cas is guided by a custom-designed guide RNA to recognize and cleave the target DNA, this mechanism drastically simplifies the engineering process of a customized SSN, making CRISPR-Cas the top choice for plant genome editing.

Developed in 2012 and applied to eukaryotic cells in 2013, CRISPR-Cas genome editing technology has since been revolutionizing plant biology. It boosts reverse genetics research in non-model plants and represents an efficient breeding technology for crop improvement. In recent years, the number of peer-reviewed papers utilizing CRISPR in plants has skyrocketed. Yet, it can be difficult and confusing for new users to choose a CRISPR system in order to achieve a specific genome editing outcome in a plant of interest. To help readers who are interested in learning and using CRISPR systems in plants, this book series provides comprehensive coverage of CRISPR systems and applications in different plant species.

The book starts with a review on plant DNA repair and genome editing by Qiudeng Que, Mary-Dell Chilton, and their colleagues (Chapter 1). The remaining chapters document methods and protocols on analysis of CRISPR-induced mutations (Chapters 2 and 3), multiplexed CRISPR-Cas9 systems (Chapters 4–7), CRISPR-Cas9 editing in monocots (e.g., rice and maize; Chapters 8–10), CRISPR-Cas9 editing in dicots (e.g., Arabidopsis, Brassica oleracea, tomato, potato, carrot, soybean, and citrus; Chapters 11–17), CRISPR-Cas12a (Cpf1) editing systems (Chapters 18–20), precise gene editing (e.g., gene replacement and base editing; Chapters 21 and 22), and non-Agrobacterium-based CRISPR delivery systems (e.g., virus delivery, ribonucleoprotein (RNP) delivery to calli or protoplasts, and automated protoplast transformation; Chapters 23–26).

I thank Aimee Malzahn for making the artistic cover picture and all the authors for making great contributions to this book. I would like to give special thanks to my wife, Hong Chen, for her generous support to my work and also to my former postdoc mentor, Daniel Voytas, for his introduction of my career into plant genome editing, an important and exciting field.

College Park, MD, USA ***Yiping Qi***

Contents

Contributors

Zahir Ali • *Laboratory for Genome Engineering, Division of Environmental and Biological Sciences and Engineering, King Abdullah University of Science and Technology, Thuwal, Saudi Arabia*

Abhishek Anand • *International Rice Research Institute, Manila, Philippines*

Tohru Ariizumi • *Faculty of Life and Environmental Sciences, Gene Research Center, University of Tsukuba, Tsukuba, Ibaraki, Japan*

Anindya Bandyopadhyay • *International Rice Research Institute, Manila, Philippines; Syngenta Beijing Innovation Center, Beijing, China*

Rafal Baranski • *Faculty of Biotechnology and Horticulture, Institute of Plant Biology and Biotechnology, University of Agriculture in Krakow, Krakow, Poland*

C. Robin Buell • *Department of Plant Biology, Michigan State University, East Lansing, MI, USA; Plant Resilience Institute, Michigan State University, East Lansing, MI, USA; Michigan State University AgBioResearch, Michigan State University, East Lansing, MI, USA*

Si Nian Char • *Department of Genetics, Development and Cell Biology, Iowa State University, Ames, IA, USA*

Hongqiao Chen • *Department of Biotechnology, School of Life Science and Technology, Center for Informational Biology, University of Electronic Science and Technology of China, Chengdu, China*

Kunling Chen • *State Key Laboratory of Plant Cell and Chromosome Engineering, Center for Genome Editing, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China*

Zhongying Chen • *Seeds Research, Syngenta Crop Protection, LLC, Research Triangle Park, NC, USA*

Mary-Dell Chilton • *Seeds Research, Syngenta Crop Protection, LLC, Research Triangle Park, NC, USA*

Sunghwa Choe • *Naturegenic Inc., West Lafayette, IN, USA; G+FLAS Life Sciences, Seoul, South Korea; School of Biological Sciences, College of Natural Sciences, Seoul National University, Seoul, South Korea*

Sunmee Choi • *G+FLAS Life Sciences, Seoul, South Korea*

Shujie Dong • *Seeds Research, Syngenta Crop Protection, LLC, Research Triangle Park, NC, USA*

David S. Douches • *Department of Plant, Soil and Microbial Sciences, Michigan State University, East Lansing, MI, USA; Michigan State University AgBioResearch, Michigan State University, East Lansing, MI, USA*

Akira Endo • *Plant Genome Engineering Research Unit, Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan*

Hiroshi Ezura • *Faculty of Life and Environmental Sciences, Gene Research Center, University of Tsukuba, Tsukuba, Ibaraki, Japan*

Ushio Fujikura • *Graduate School of Science, Technology and Innovation, Kobe University, Kobe, Hyogo, Japan*

Caixia Gao • *State Key Laboratory of Plant Cell and Chromosome Engineering, Center for Genome Editing, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China*
Weihang Gu • *State Key Laboratory of Hybrid Rice, Shanghai Jiao Tong University–University of Adelaide Joint Centre for Agriculture and Health, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai, China*
Samatha Gunapati • *Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN, USA*
Wendy Harwood • *John Innes Centre, Norwich Research Park, Norwich, UK*
Penny Hundleby • *John Innes Centre, Norwich Research Park, Norwich, UK*
Hongge Jia • *Citrus Research and Education Center, Department of Microbiology and Cell Science, Institute of Food and Agricultural Sciences (IFAS), University of Florida, Lake Alfred, FL, USA*
Tim Kelliher • *Seeds Research, Syngenta Crop Protection, LLC, Research Triangle Park, NC, USA*
Magdalena Klimek-Chodacka • *Faculty of Biotechnology and Horticulture, Institute of Plant Biology and Biotechnology, University of Agriculture in Krakow, Krakow, Poland*
Akihiko Kondo • *Graduate School of Science, Technology and Innovation, Kobe University, Kobe, Hyogo, Japan; Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, Kobe, Hyogo, Japan*
Tom Lawrenson • *John Innes Centre, Norwich Research Park, Norwich, UK*
Keunsub Lee • *Crop Bioengineering Center, Iowa State University, Ames, IA, USA*
Scott C. Lenaghan • *Department of Food Science, University of Tennessee, Knoxville, TN, USA; Department of Mechanical, Aerospace, and Biomedical Engineering, University of Tennessee, Knoxville, TN, USA*
Jiayang Li • *University of Chinese Academy of Sciences, Beijing, China; State Key Laboratory of Plant Genomics, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China*
Jun Li • *State Key Laboratory of Plant Cell and Chromosome Engineering, Center for Genome Editing, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China; University of Chinese Academy of Sciences, Beijing, China*
Qian Li • *Department of Biotechnology, School of Life Science and Technology, Center for Informational Biology, University of Electronic Science and Technology of China, Chengdu, China*
Riqing Li • *Department of Genetics, Development and Cell Biology, Iowa State University, Ames, IA, USA*
Zhen Liang • *State Key Laboratory of Plant Cell and Chromosome Engineering, Center for Genome Editing, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China; University of Chinese Academy of Sciences, Beijing, China*
Binglin Liu • *Department of Biotechnology, School of Life Science and Technology, Center for Informational Biology, University of Electronic Science and Technology of China, Chengdu, China*
Junqi Liu • *Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN, USA*
Yao-Guang Liu • *College of Life Sciences, South China Agricultural University, Guangzhou, China*
Xingliang Ma • *College of Life Sciences, South China Agricultural University, Guangzhou, China*

Ahmed Mahas • *Laboratory for Genome Engineering, Division of Environmental and Biological Sciences and Engineering, King Abdullah University of Science and Technology, Thuwal, Saudi Arabia*
Magdy M. Mahfouz • *Laboratory for Genome Engineering, Division of Environmental and Biological Sciences and Engineering, King Abdullah University of Science and Technology, Thuwal, Saudi Arabia*
Aimee Malzahn • *Department of Plant Science and Landscape Architecture, University of Maryland College Park, College Park, MD, USA*
Xiangbing Meng • *State Key Laboratory of Plant Genomics, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China*
Jean-Michel Michno • *Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN, USA*
Nicole T. Mihelich • *Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN, USA*
Satya Swathi Nadakuduti • *Department of Plant, Soil and Microbial Sciences, Michigan State University, East Lansing, MI, USA*
C. Neal Stewart Jr. • *Department of Plant Sciences, University of Tennessee, Knoxville, TN, USA*
Keiji Nishida • *Graduate School of Science, Technology and Innovation, Kobe University, Kobe, Hyogo, Japan*
Tomasz Oleszkiewicz • *Faculty of Biotechnology and Horticulture, Institute of Plant Biology and Biotechnology, University of Agriculture in Krakow, Krakow, Poland*
Vladimir Orbovic • *Citrus Research and Education Center, Institute of Food and Agricultural Sciences (IFAS), University of Florida, Lake Alfred, FL, USA*
Aiden Y. Park • *G+FLAS Life Sciences, Seoul, South Korea; School of Biological Sciences, College of Natural Sciences, Seoul National University, Seoul, South Korea*
Jongjin Park • *Naturegenic Inc., West Lafayette, IN, USA*
Slki Park • *G+FLAS Life Sciences, Seoul, South Korea; School of Biological Sciences, College of Natural Sciences, Seoul National University, Seoul, South Korea*
Yiping Qi • *Department of Plant Science and Landscape Architecture, University of Maryland College Park, College Park, MD, USA; Institute for Bioscience and Biotechnology Research, University of Maryland, Rockville, MD, USA*
Qiudeng Que • *Seeds Research, Syngenta Crop Protection, LLC, Research Triangle Park, NC, USA*
Paul Quick • *International Rice Research Institute, Manila, Philippines*
Nathan T. Reem • *Boyce Thompson Institute, Ithaca, NY, USA*
Qiurong Ren • *Department of Biotechnology, School of Life Science and Technology, Center for Informational Biology, University of Electronic Science and Technology of China, Chengdu, China*
Zenpei Shimatani • *Graduate School of Science, Technology and Innovation, Kobe University, Kobe, Hyogo, Japan*
David Skibbe • *Seeds Research, Syngenta Crop Protection, LLC, Research Triangle Park, NC, USA*
Colby G. Starker • *Department of Genetics, Cell Biology and Development and Center for Genome Engineering, University of Minnesota, Minneapolis, MN, USA*
Adrian O. Stec • *Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN, USA*

Robert M. Stupar • *Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN, USA*

Xu Tang • *Department of Biotechnology, School of Life Science and Technology, Center for Informational Biology, University of Electronic Science and Technology of China, Chengdu, China*

Manal Tashkandi • *Laboratory for Genome Engineering, Division of Environmental and Biological Sciences and Engineering, King Abdullah University of Science and Technology, Thuwal, Saudi Arabia*

Seiichi Toki • *Plant Genome Engineering Research Unit, Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, Tsukuba, Ibaraki, Japan; Kihara Institute for Biological Research, Yokohama City University, Yokohama, Japan*

Joyce Van Eck • *Boyce Thompson Institute, Ithaca, NY, USA; Plant Breeding and Genetics Section, School of Integrative Plant Science, Cornell University, Ithaca, NY, USA*

Daniel F. Voytas • *Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN, USA; Center for Genome Engineering, University of Minnesota, Minneapolis, MN, USA*

Chun Wang • *State Key Laboratory of Rice Biology, China National Rice Research Institute, Chinese Academy of Agricultural Sciences, Hangzhou, China*

Kan Wang • *Crop Bioengineering Center, Iowa State University, Ames, IA, USA*

Kejian Wang • *State Key Laboratory of Rice Biology, China National Rice Research Institute, Chinese Academy of Agricultural Sciences, Hangzhou, China*

Nian Wang • *Citrus Research and Education Center, Department of Microbiology and Cell Science, Institute of Food and Agricultural Sciences (IFAS), University of Florida, Lake Alfred, FL, USA*

Kabin Xie • *National Key Laboratory for Crop Genetic Improvement and Plant Gene Research Center (Wuhan), Huazhong Agricultural University, Wuhan, China*

Xianrong Xie • *College of Life Sciences, South China Agricultural University, Guangzhou, China*

Bing Yang • *Department of Genetics, Development and Cell Biology, Iowa State University, Ames, IA, USA; Crop Bioengineering Center, Iowa State University, Ames, IA, USA*

Yinong Yang • *Department of Plant Pathology and Environmental Microbiology, The Huck Institutes of the Life Sciences, The Pennsylvania State University, Pennsylvania, PA, USA*

Xiaojia Yin • *International Rice Research Institute, Manila, Philippines*

Jiyoung Yoon • *G+FLAS Life Sciences, Seoul, South Korea*

Hanchuanzhi Yu • *Section of Cell and Developmental Biology, University of California San Diego, La Jolla, CA, USA*

Zheng Yuan • *State Key Laboratory of Hybrid Rice, Shanghai Jiao Tong University–University of Adelaide Joint Centre for Agriculture and Health, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai, China*

Dabing Zhang • *State Key Laboratory of Hybrid Rice, Shanghai Jiao Tong University–University of Adelaide Joint Centre for Agriculture and Health, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai, China; Plant Genomics Center, School of Agriculture, Food and Wine, University of Adelaide, Adelaide, SA, Australia*

Yingxiao Zhang • *Department of Plant Science and Landscape Architecture, University of Maryland, College Park, MD, USA*

YONG ZHANG • *Department of Biotechnology, School of Life Science and Technology, Center for Informational Biology, University of Electronic Science and Technology of China, Chengdu, China*

YUNDE ZHAO • *Section of Cell and Developmental Biology, University of California San Diego, La Jolla, CA, USA*

XUELIAN ZHENG • *Department of Biotechnology, School of Life Science and Technology, Center for Informational Biology, University of Electronic Science and Technology of China, Chengdu, China*

ZHAOHUI ZHONG • *Department of Biotechnology, School of Life Science and Technology, Center for Informational Biology, University of Electronic Science and Technology of China, Chengdu, China*

JIANPING ZHOU • *Department of Biotechnology, School of Life Science and Technology, Center for Informational Biology, University of Electronic Science and Technology of China, Chengdu, China*

HUILAN ZHU • *Crop Bioengineering Center, Iowa State University, Ames, IA, USA*

XIUPING ZOU • *Citrus Research and Education Center, Department of Microbiology and Cell Science, Institute of Food and Agricultural Sciences (IFAS), University of Florida, Lake Alfred, FL, USA*

Part I

Review on Plant DNA Repair and Genome Editing

Chapter 1

Plant DNA Repair Pathways and Their Applications in Genome Engineering

Qiudeng Que, Zhongying Chen, Tim Kelliher, David Skibbe, Shujie Dong, and Mary-Dell Chilton

Abstract

Remarkable progress in the development of technologies for sequence-specific modification of primary DNA sequences has enabled the precise engineering of crops with novel characteristics. These programmable sequence-specific modifiers include site-directed nucleases (SDNs) and base editors (BEs). Currently, these genome editing machineries can be targeted to specific chromosomal locations to induce sequence changes. However, the sequence mutation outcomes are often greatly influenced by the type of DNA damage being generated, the status of host DNA repair machinery, and the presence and structure of DNA repair donor molecule. The outcome of sequence modification from repair of DNA double-strand breaks (DSBs) is often uncontrollable, resulting in unpredictable sequence insertions or deletions of various sizes. For base editing, the precision of intended edits is much higher, but the efficiency can vary greatly depending on the type of BE used or the activity of the endogenous DNA repair systems. This article will briefly review the possible DNA repair pathways present in the plant cells commonly used for generating edited variants for genome engineering applications. We will discuss the potential use of DNA repair mechanisms for developing and improving methodologies to enhance genome engineering efficiency and to direct DNA repair processes toward the desired outcomes.

Key words DNA repair, Genome engineering, Site-directed nuclease (SDN), Base editor (BE), Single-strand break (SSB), Double-strand break (DSB), Nonhomologous end joining (NHEJ), Alternative end joining (altEJ), Homology-directed repair (HDR)

1 Plant DNA Repair and Recombination Machineries

Plants are exposed to many biological and environmental conditions that can cause genomic DNA damages. For example, when leaf cells are exposed to sunlight, their genomic DNA is constantly subjected to highly damaging UV radiation [1]. Some essential biological processes including DNA replication, recombination, and transcription also generate mis-incorporated nucleotides or DNA breaks [2, 3]. Normal metabolic processes and genotoxic stresses such as heat and pathogen infection also generate free radicals that can cause DNA base damages. These diverse kinds of

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genomic DNA damages need to be repaired properly and promptly to maintain genome stability. If too much DNA damage is present in cells, the cell death process is triggered. There are several major pathways in plant cells for repair of different types of DNA damage [2–4] (Table 1). Repair of damaged bases and nucleotides is accomplished by photoreactivation, base excision repair (BER), and nucleotide excision repair (NER) pathways. Recognition and correction of mis-incorporated nucleotides and unpaired nucleotides are processed through mismatch repair (MMR) pathways. Recognition and repair of single-strand breaks (SSBs) or nicks can be accomplished through the BER pathway and homology-directed repair (HDR) pathways if homologous donors are provided. Repair of the most damaging DSBs employs both nonhomologous end joining (NHEJ) and homology recombination (HR) pathways [2, 3, 19]. DNA damage detection, repair pathways, and their regulation in plants have been reviewed in detail, and the reader is referred to these recent articles [2–4].

One of the most critical roles for plant DNA repair machineries is to repair base damages caused by the constantly present UV light during the daytime. The most common forms of DNA damage caused by UV light are cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidones (6-4 photoproducts). As in other organisms, in plants the photoreactivation process is responsible for the removal of such pyrimidine dimers [5]. Two different but related photolyases, CPD photolyase and 6-4 photolyase, are involved in the removal of these damaged bases [1, 2, 5]. All photolyases contain a highly conserved photolyase-homologous region (PHR) that binds the chromophore flavin adenine dinucleotide (FAD) which absorbs blue or visible light and uses the energy for cleavage of the pyrimidine dimer lesion and generation of two repaired pyrimidines [20].

Nucleotide excision repair (NER) is another major mechanism for repairing the bulky helix-distorting CPDs and pyrimidine (6–4) pyrimidones induced by UV radiation [6]. In addition, NER is responsible for detecting and removing a very wide range of structurally unrelated DNA lesions [7]. There are two different mechanisms of lesion detection to initiate NER of the bulky helix-distorting lesions: the global genome NER (GG-NER) and transcription-coupled NER (TC-NER) [2, 6, 7]. In animal cells, the GG-NER can be initiated anywhere in the genome, whereas TC-NER is involved in the repair of lesions in the transcribed strand of active genes [6, 7]. In GG-NER pathway, DNA damage is detected through the heterotrimeric RAD4/XPC-RAD23-CEN2 complex in collaboration with the heterodimeric damaged DNA-binding (DDB) protein complex [2, 6, 7]. In TC-NER, the recognition is initiated by a stalled RNA polymerase with the help of CSA, CSB, and XAB2 protein [2, 6, 7]. After DNA recognition, GG-NER and TC-NER converge into the same pathway in

Table 1
DNA damage lesions, repair pathways, and major components of the respective DNA repair machineries [2, 3, 5–18]

DNA lesions	Repair pathways	Major lesion recognition and repair components
UV-induced base adducts	Photoreactivation	Photolyases: CPD photolyases and 6-4 photolyase
Base damages: deaminated and alkylated bases, interstrand crosslinks	Nucleotide excision repair (NER): Global genome NER (GG-NER) and transcription-coupled NER (TC-NER) subpathways	XPC(RAD4)-HR23B(RAD23)-CEN2 complex, damaged DNA-binding protein (DDB), CSA, CSB, XPA binding protein 2 (XAB2), TFIIH complex (XPB and other factors) and cyclin-dependent kinase (CDK)-activating kinase (CAK) complex, DNA helicase XPD, XPA, RPA, endonucleases (XPG and ERCC1/XPF), PCNA, replication factor C (RFC), DNA polymerase δ, ε and κ, ligase 1
Base damages: deaminated, oxidized, methylated, and alkylated bases, AP sites	Base excision repair (BER): "Short"-patch and "long"-patch repair subpathways	DNA glycosylases/AP lyase, AP endonucleases (APE), polynucleotide kinase 3′ phosphatase (PNKP), aprataxin, tyrosyl DNA phosphodiesterase (TDP), XRCC1, poly(ADP-ribose) polymerase 1 (PARP1), FEN1, PCNA and DNA polymerase δ and ε, DNA ligase 1
Deaminated bases, replication errors, insertion/deletion loops (IDLs)	Mismatch repair (MMR)	MutS protein complexes (MutSα, MutSβ, MutSγ), PCNA, MutL heterodimers, PMS1 endonuclease, replication fork complex (RFC), exonuclease 1 (Exo1), PCNA, RPA, DNA polymerase δ, DNA ligase 1
Single-strand break (SSB)	Single-strand break repair (SSBR)	Extensive overlap with BER, NER, and MMR machineries. PARP1, XRCC1, PNKP, RPA, FEN1, DNA polymerase β, δ and ε, ligase 1 and 3
Double-strand break (DSB)	Canonical nonhomologous end joining (cNHEJ)	Ku70-Ku80, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), Artemis nuclease, XRCC4, XRCC4 like factor (XLF/Cernunnos), PAXX, polγ and μ, DNA ligase 4
	Alternative end joining (altEJ)	MRN complex (nuclease), CtIP/COM1, PARP-1, Exo1, BLM/DNA2 helicase/nuclease, XRCC1, DNA polymerase θ, DNA ligase 3
	Single-strand annealing (SSA)	MRN complex (nuclease), CtIP/COM1, FANCM, RAD52, Exo1, XPF/ERCC1, RPA,DNA polymerase δ, DNA ligase 1

(continued)

Table 1
(continued)

DNA lesions	Repair pathways	Major lesion recognition and repair components
	Homologous recombination (HR)	MRN complex (nuclease), CtIP/COM1, FANCM, BLM/DNA2 helicase/nuclease, BRCA1, PALB2, BRCA2, Exo1, RAD54, RPA, RAD51/XRCC3, FANCM, PCNA, RFC, resolvases (GEN1 endonuclease, MUS81-EME1, SLX1-SLX4), SEND1 (ssDNA endonuclease 1), DNA polymerase δ, DNA ligase 1

recruiting other components for the formation of stable preincision complex that includes transcriptional factor II H (TFIIH), XPA (xeroderma pigmentosum group A), RPA (replication protein A), XPG, and ERCC1 (excision repair cross-complementing 1)-XPF. After the preincision complex formation, endonucleases in the complex, ERCC1/XPF and XPG, work together to excise a single-strand oligonucleotide fragment of 24–32 nucleotide long containing the damaged site. Repair is completed by DNA synthesis via DNA polymerases δ, ε, or κ depending on chromatin accessibility of the damaged site, followed by nick sealing by DNA ligase 1 or 3α [2, 6].

Base excision repair (BER) is responsible for recognizing and repairing several different kinds of lesions including base damages from deamination, oxidation, and alkylation and also the abasic (apurinic or apyrimidinic, AP) sites [8]. The damaged base is excised by DNA glycosylase to generate an AP site. There are different DNA glycosylases in the cell that act specifically on particular kinds of damaged bases. The sugar-phosphate backbone at the AP site is then cleaved by an AP endonuclease or the AP lyase activity of the DNA glycosylase [2, 3, 8]. The nick in the DNA backbone is then processed and gap filled by DNA polymerase and ligase. Gap repair is completed through two mechanisms in mammalian cells: (1) the "short"-patch repair for single nucleotide gap through the activity of DNA polymerase β with XRCC1 (X-ray repair cross-complementing protein 1) and DNA ligase 3 (LIG3) and (2) the "long"-patch repair for gaps of more than two nucleotides via DNA polymerases δ and ε with the help of proliferating cell nuclear antigen (PCNA), flap endonuclease (FEN1), and DNA ligase 1 [3]. Because plants do not have DNA polymerase β and ligase 3 homologs, it is likely that ligase 1 is involved in both "short"- and "long"-patch modes of BER [3]. In plants, BER has another important role in epigenetic regulation of gene expression through DNA

demethylation in which 5-methylcytosine (5-meC) is directly removed by a dedicated glycosylase/lyase mechanism [21].

Mismatch repair (MMR) is responsible for correcting mismatches of normal or damaged bases or insertion/deletion loops due to strand misalignment [9, 10]. These include single base-base mismatches and unpaired nucleotides that result from replication errors, deamination of 5-methylcytosine, and recombination between divergent sequences [10]. MMR plays an important role in suppressing insertion/deletion (indel) loops (IDL) that are usually the result of slipped mispairing [9]. MMR is also involved in preventing recombination between homoeologous sequences as a speciation and rearrangement barrier in both bacteria and plant cells [2, 9]. DNA mismatch is recognized by MutS proteins comprised of related but distinct heterodimeric MutS homolog (MSH) subunits. In plants, these MSH subunits form functionally distinct complexes such as MutSα (MSH2-MSH6), MutSβ (MSH2-MSH3), and MutSγ (MSH2-MSH7), recognizing different types of lesions [2, 3]. Lesion recognition by MutS proteins is followed by assembly of a DNA repair complex through recruitment of heterodimeric MutL and endonuclease PMS1, producing a nick in the DNA strand with the lesion. The nicked DNA strand is further resected by exonuclease I (ExoI) for subsequent repair involving PCNA, replication protein A (RPA), replication fork complex (RFC), and DNA polymerase δ [2, 3].

Single-strand breaks (SSBs) are the most common form of DNA damage present in the cell. They may result directly from spontaneous DNA decay, attack by intracellular metabolites such as reactive oxygen species (ROS), or abortive activity of DNA topoisomerase 1; they may also arise indirectly from repair of damaged or mis-incorporated ribonucleotides or erroneous base modification by APOBEC and TET family proteins [11, 12]. Repair of SSBs is carried out efficiently as part of the other DNA repair pathways including BER, NER, MMR, and DNA ribonucleotide excision repair (RER) [11, 12, 22]. The study of repair mechanisms for genomic SSB has been greatly facilitated by the easily available Cas9 nickase [23–26]. Deep sequencing analysis showed that repair of nicked target sequences in *Arabidopsis* only generated background level of insertion/deletions (indels) [23]. SSB also uses homologous sequences present both *in cis* and *in trans* for HDR [23–25]. Since site-specific SSB generated by nickase can be used for directing targeted editing with a homologous DNA template [23–25] and also impacts the repair outcomes of base editing [26], it is important to understand the SSB repair mechanisms in plant cells to improve the frequency of desirable editing.

Double-strand breaks (DSBs) present in the cells are potentially the most damaging and mutagenic. Double-strand breaks can be repaired through several mechanisms: the classical or canonical nonhomologous end joining (cNHEJ), alternative end joining

(altEJ), single-strand annealing (SSA), and homologous recombination (HR) pathways [2, 3, 13, 14, 16, 19, 27–30]. It has been suggested that an early event in the selection of end processing determines the DSB repair pathways and outcomes [28]. In plants, DSBs are rapidly repaired through the Ku-dependent cNHEJ pathway and the highly error-prone Ku-independent altEJ pathway, especially in the somatic tissues or cells that are most often used as target materials for genome engineering studies [2–4, 19]. AltEJ has also been referred to as backup NHEJ (b-NHEJ) or microhomology-mediated end joining (MMEJ) [16]. In cNHEJ, DSBs are recognized and bound tightly by the Ku70-Ku80 heterodimer. Other cNHEJ factors including DNA-PKcs, XRCC4-ligase IV-XLF (XRCC4-like factor, also called *Cernunnos*), and Artemis nuclease are then recruited to the broken ends along with DNA polymerases γ and μ to carry out end processing and ligation [14, 16, 28]. The cNHEJ pathway involves minimal end processing, thus resulting in minimal DNA loss in the form of small indels (1–4 nucleotides) [14, 16, 28], whereas in altEJ pathway, the DSB is bound by the polyADP-ribose polymerase (PARP) proteins. PARP's binding to the broken ends triggers recruitment of MRE11-RAD50-NBS1 (MRN) complex to initiate end resection which facilitates generation of microhomology between the two DNA strands with free ends. MRN then interacts with the DNA ligase 3/XRCC1 complex to process the microhomology for end joining [14, 16, 28]. In the altEJ pathway, the broken DNA ends are more extensively resected and then extended by the error-prone DNA polymerase θ using templates with microhomology both in *cis* and *trans*, thus generating both larger size deletions and insertions of filler sequences, sometimes leading to sequence inversion and chromosomal translocation [14, 16, 28].

When long DNA homology is present, DSBs can also be repaired at low frequency through HDR mediated by a multiprotein complex [17, 19, 27, 29, 30]. In animal cells, HDR requires extensive resection of the broken DNA ends by MRN complex and BLM/Exo1 to generate free 3'-ends for initiating homology search and strand annealing. When the homologous sister chromatid is used as template, HDR results in conservative synthesis of DNA and accurate repair of DSB by the HR pathway. In plants there are two intermolecular HR subpathways: (1) the canonical DSB repair (DSBR) pathway and (2) synthesis-dependent strand annealing (SDSA) [3, 13, 19, 27]. DSBR and SDSA pathways share common steps in the beginning but differ in the way the displacement loop (D-loop) is resolved [27]. In the DSBR subpathway, strand exchange results in double Holliday junction (dHJ) formation, and resolution of dHJ leads to crossover (CO) between homologous chromosomes in meiotic recombination. In the SDSA subpathway, the HJ is dissolved, resulting in noncrossover (NCO) gene conversion. In plant somatic cells, all HDR of DSBs is

through the noncrossover SDSA subpathway [27, 29]. In plants, there is another HDR subpathway called SSA which uses homologous sequences within the same DNA sequence for DSB repair. In the SSA pathway, the two resected free ends of the break anneal at the neighboring region of complementarity, and the noncomplementary ends are trimmed off. Therefore, SSA results in deletion of the intervening sequences between the two repeat regions [13, 27]. It should be noted that the DSB repair pathways in plant somatic cells might be significantly different from those in the animal cells. It has been shown that MRE11 and COM1 were not required for SSA and SDSA in *Arabidopsis* recombination assays, whereas FANCM was required for both [15].

2 Role of DNA Recombination and Repair Machineries in Plant Development

In addition to lesion types, the choice of DNA repair pathways in a particular cell depends on its developmental stage and cell cycle phase. The DNA repair systems in somatic tissues are different from those in reproductive tissues. During the reproductive phase, there is an active meiosis in which the genome of each mother cell is replicated only once, but the cell goes through two rounds of division (meiosis I and II) to produce haploid gametes with a single set of chromosomes. In order for meiosis I to proceed, homologous chromosomes must pair and join to enable formation of chiasmata, which are required for crossover between non-sister homologous chromatids and subsequent proper segregation of chromosomes [31, 32]. Meiotic recombination is initiated in early meiotic prophase by DSB formation through cleavage by the Spo11 complex [32]. Spo11 protein has sequence similarity with Top6A, the catalytic subunit of archaebacterial type 2 topoismerase [31]. Following DSB formation, DNA ends are resected by the CtIP/COM1/Sae2 protein and the MRE11-RAD50-NDS1/XRS2 (MRX) complex, excising the Spo11-bound oligonucleotide and generating ssDNA with a free 3'-end [31]. The resulting free 3'-end then recruits DMC1 and RAD51 recombinases and initiates strand invasion and pairing between homologous chromatids [31]. Repair of these inter-chromatid homologs can lead to either COs or NCOs. It should be noted that the number of DSBs generated by Spo11 is much higher than the number of COs in *Arabidopsis* as in yeast and mouse [33]. However, at least one CO per homolog pair (known as obligate CO) is required for accurate segregation of homologous chromosomes in meiosis [32]. CO formation and strand exchange steps involve the second end capture and double Holliday junction (dHJ) formation mediated by ZMM proteins (Zip1, Zip2, Zip3 Zip4, Spo16, Msh4, Msh5, and Mer3). The junction is then resolved through nuclease cleavage

mediated by MLH1/3 complexes and MUS81 proteins followed by ligation [31].

There are active mechanisms in plant cells to promote NCOs, and the proteins involved are FANCM helicase and its two cofactors, MHF1 and MHF2 [32]. Despite the active meiotic recombination activities present in almost all higher plants, the meiotic recombination machinery has not been exploited for targeted genome engineering due to the difficulties in delivering reagents into the meiotic mother cells at the right stage. However, genes involved in meiotic recombination or their homologs are also expressed in other plant tissues, and many are probably shared with other DNA repair pathways which can be used to facilitate genome editing through HDR mechanisms. A good example has been shown in that the *Arabidopsis* FANCM is involved in HR in both somatic and meiotic cells [34]. Also, the break end-binding and resection complexes, MRE11-RAD50-NBS (MRN complex), ssDNA-binding proteins replication protein A (RPA) complex, RAD51, and its paralogs, are also involved in DNA repair in both somatic and reproductive tissues [3]. It has been shown that *Arabidopsis* lines with mutations in MRE11 and RAD50 genes are hypersensitive to DSB-inducing agents and are sterile, suggesting that these genes are required for the general DSB repair in somatic tissues and the meiotic recombination in reproductive tissues [35, 36]. The RPA protein family members also play overlapping roles in DNA repair, meiosis, and DNA replication [37].

Cell cycle plays a critical role in repair pathway selection [18]. There are different cell types in somatic tissues. Meristems have actively dividing cells, but differentiated tissues have only nondividing cells that have exited the cell cycle. Actively dividing cells in different phases of mitosis may possess very different DNA repair machineries from those of differentiated cells. In somatic cells of plants, HR mainly functions during the S and G2 phase of the cell cycle [3]. cNHEJ is active throughout the whole cell cycle but is dominant in the G1 and G2 phases, whereas altEJ is more active during the S phase [38, 39]. Both HDR (HR and SSA) and altEJ require end resection which is promoted by the cyclin-dependent kinase (CDK) [18]. Since differentiated cells have already exited the cell cycle, the dominant DSB repair mechanism is the cNHEJ pathway. It should also be noted that DNA repair efficiency and outcomes can also be influenced by environmental conditions that directly or indirectly impact the gene editing machinery and/or the cell's repair pathways. For example, pathogen infection has been shown to increase the somatic recombination frequency [40]. Heat treatment of *Arabidopsis* plants at 37 °C has produced much higher frequencies of Cas9-induced mutations than were found in control plants grown continuously at 22 °C, likely a result of higher Cas9 activity or perturbation of the plant DNA repair pathway at higher temperature [41].

3 Targeted Mutagenesis and Insertion Mediated by NHEJ Repair Pathways

Until recently, the majority of genome engineering tools used are SDNs that generate DSBs at the chromosomal target sequences. In the absence of homologous repair donor template, DSBs are repaired through the cNHEJ and altEJ pathways in plant somatic cells, leading to genomic changes such as deletions, insertions, and sometimes rearrangements [2, 3, 19]. DSBs can be generated using different types of SDNs including meganucleases, ZFNs, TALENs, CRISPR-Cas9, CRISPR-Cpf1 (Cas12a), paired dCas9-FokI, and Cas9 nickases [42–46]. DSB repair products have been analyzed extensively in many types of plant and animal cells [42–57]. Repair outcomes depend on many factors, including the nuclease used, the type of ends generated, the sequence context surrounding the target site, cell types, and the physiological status of target tissue [49]. For example, the CRISPR-Cas9 system generates blunt ends, but the Cas9 protein remains bound to the target sequence for a long time and thus may serve as an end protector to prevent extensive resection [58]. Probably due to its end protection property, Cas9 tends to produce a higher proportion of small indels [48, 51, 59], whereas meganucleases, ZFNs, and TALENs generate a higher proportion of larger deletions [45, 46, 49, 53, 55]. It is plausible that a DSB located within an actively transcribed region may result in Cas9 being quickly dislodged by the transcriptional machinery, exposing the ends for resection and triggering altEJ, thus causing deletions of larger size. If there are two adjacent DNA nicks in the chromosome, there can be formation of repair products with tandem duplications in addition to deletions, probably as a result of altEJ-mediated repair [60]. Unlike Cas9, most other SDNs generate sticky ends that may affect repair outcomes too. For example, meganuclease I-*Sce*I generates a 4 nucleotide 3′-overhang [42, 47], and Cpf1(Cas12a) cleavage results in a DSB break with 5′-overhang [43]. Deep sequencing analysis of LbCpf1 and AsCpf1 break repair products in rice showed that more than 90% of the mutations are deletions, mostly 6–13 bp in size, considerably larger than the 1–3 bp of most Cas9-mediated deletions [44]. DSB repair is also influenced by the sequence contexts surrounding the target cleavage sites, and the outcomes are nonrandom. It is shown that the occurrence of SSA-mediated repair depends on the presence and distance of repeats flanking the DSB; the frequency and size of insertions also increased if sequences with high similarity to the target site are present *in cis* [50].

The DSB repair outcome via NHEJ is impacted by plant source materials such as species, tissue types, and their physiological status. Repair of DSBs in *Arabidopsis* resulted in a significantly higher proportion of deletions and larger size deletions, while in tobacco and barley, many repair products have insertion of stuffer sequences [47, 51]. It has been proposed that DSB repair produces a clear net

DNA loss in organisms with small genomes such as *Arabidopsis*, mainly resulting from SSA repair pathway. However, in another study with single molecule sequencing of chemically induced I-*Sce*I expression, *Arabidopsis* and tobacco plants exhibited very similar NHEJ repair patterns [46]. In both species, the vast majority of I-*Sce*I break repair events had either no loss of sequence or small deletions at the repair junctions. In only a small percentage of junctions, repair was less conservative with large deletions or insertions [46]. These apparently inconsistent observations can probably be explained by the different experiment designs and selection conditions for recovery of the repaired products. Indeed, results from other studies suggested that DSB repair products in *Arabidopsis* also can have large size insertions [59, 61].

If, as has been suggested, early events in selection of end processing determine the DSB repair pathways and outcomes [28], desired outcomes may be manipulated through modulation of plant DNA repair machinery. For example, the cNHEJ pathway players, Ku70, Ku80, or DNA-PKcs, might be downregulated to shift DSB toward altEJ, SSA, or HR to favor larger size deletions or HDR. Likewise, suppression of end resection nucleases CtIP/COM1, MRE11, and other proteins in the altEJ, SSA, and HR pathways such as RAD50, NBS, and PARP-1 should shift repair toward cNHEJ, resulting in small indels. This idea is supported by studies in which the use of *ku70* and *lig4* mutants defective in cNHEJ repair resulted in enhancement of HDR-based gene targeting (GT), especially in the *ku70* background which also suggests that that Ku70 is a main player in the selection of end processing and the resulting DSB repair pathway [61]. Interestingly, the mutations formed in both *ku70* and *lig4* mutant lines were predominantly large deletions, consistent with the default use of microhomology-mediated altEJ when the cNHEJ pathway is defective [61]. In another study with a rice *lig4* mutant, the mutation frequency of all types of mutations was higher, and the ratio of large deletions and deletions repaired with altEJ (or MMEJ) was higher than in wildtype [62]. Unexpectedly, it was found that NHEJ mutagenesis at the *Adh1* locus was also enhanced in a line defective for *smc6b* which is implicated in sister chromatid recombination [61]. In contrast, the deletion size in the *Adh1* gene at the nuclease cleavage site in the *smc6b* line was similar to that of wildtype. It is possible that in *smc6b* background the chromatin is more accessible to the DNA repair machineries. DNA polymerase theta (Polθ) is involved in the generation of filler sequences in altEJ-mediated repair due to its ability to extend minimally paired 3′ ends and to switch templates during DNA synthesis. *TEBICHI/POLQ* mutant plants defective in DNA polymerase theta (Polθ) are resistant to T-DNA integration even though they are susceptible to *Agrobacterium* infection [63]. Therefore, it is possible that suppression of Polθ expression may result in fewer mutants with patchwork filler

sequence and a higher ratio of mutants with deletions resulting from cNHEJ and SSA.

NHEJ-mediated mechanisms have also been used to insert DNA sequences into DSBs generated with SDNs in both plant and animal cells [64–69]. Simultaneous cleavage of donor vector DNA and the chromosomal target site was found to increase significantly the targeted integration of donor DNA [66, 68, 70]. When there was microhomology between the free ends of the donor fragment and the DSBs of the target sites, altEJ (or MMEJ) appeared to mediate efficient integration of donor sequences, and a fraction of the integrants were found to carry precisely joined junctions [67]. In order to avoid disrupting the protein coding sequence, SDNs are targeted to noncoding or untranslated regions such as introns for protein coding sequence replacement efforts. The altEJ-mediated approach has been successfully employed to replace endogenous gene sequence with a donor template bearing mutations to confer glyphosate resistance using a pair of sgRNAs targeting adjacent introns of the rice *EPSPS* gene at a frequency of about 2.0% [68].

4 HDR-Mediated Sequence Replacement with Synthetic Oligodeoxyribonucleotide (ODN) Donors

Short single-stranded ODNs (ssODNs) have been used in CRISPR-Cas9-mediated GT studies with plant protoplasts or isolated immature embryos [71–73]. However, the efficiency of oligonucleotide-mediated replacement mutants was generally still low, and such efforts have succeeded only when the products could be selected with herbicide after editing created the herbicide resistant mutation [71, 73]. At very low frequency, the mutations may be difficult to distinguish from spontaneous background mutations. For non-selectable targets, in principle the recovery of oligonucleotide-mediated replacement mutants can also be accomplished by co-transformation with a selectable marker in conjunction with genotyping screening. In order to increase the efficiency of ODN-mediated genome engineering, it is critical to investigate what mechanism(s) are involved when ssODNs are used for replacing one or a few nucleotides in the target sequence in conjunction with SSB or DSB generated by SDNs. Typically, when ODNs are used as repair donors, the repair templates have homology arms of 25 to 50 bases flanking the mutant sequence. In theory, both altEJ (MMEJ) and HDR (SSA or SDSA) mechanisms can lead to ssODN integration and/or replacement at the genomic break site.

A recent study suggested that the DSB repair was likely to use the SDSA HDR mechanism when linear ssODN or dsDNA molecules with only short region of homology were provided as templates because the repair products were sensitive to ssODN polarity

and prone to template switching [74]. The study showed that ~35 nucleotides of homology with the targeted locus on each side of the dsDNA template molecule were sufficient to efficiently introduce edits ranging from 1 to 1000 nucleotides into DSBs introduced by Cas9 [74]. Interestingly, it is the insert size, not the overall size of the donors, which determines editing efficiency when dsDNA fragments are used as templates for HDR of DSB; also, insert sizes larger than 1 kb resulted in very low targeting efficiency [74]. In mammalian cells p53-binding protein 1 (53BP1) is a key DSB repair pathway regulator, promoting cNHEJ while preventing HR [16, 28, 75]. 53BP1 interferes with DNA end resection by preventing CtIP/COM1 from accessing DNA ends; 53BP1 also inhibits recruitment of BRCA1 protein to the DSB sites [16]. Inhibition of 53BP1 and thus cNHEJ greatly stimulated HDR when ssODN donors were provided. Also, when expression of the key end resection factor CtIP/COM1 was suppressed with siRNA, ssODN-mediated HDR was reduced. These results suggest that end resection plays an important role in ssODN-mediated HDR [75]. Because dissociation of Cas9 from the cleaved substrates is slow, the 3′ end of the cleaved nontarget strand is released first [58], and use of the optimal length asymmetric ssDNA donors complementary to the first released strand significantly increased the rate of HDR in human cells [58]. Also, the length of the donor homology arms can be optimized to improve targeting efficiency. Long ssDNA donors flanked by about 70 to 100 nucleotide homology arms were very efficient in replacement of endogenous sequences when co-delivered with CRISPR ribonucleoprotein with 2 sgRNAs in a process called Easi-CRISPR in mice zygotes [76]. Whether use of such long ssDNA donors will result in higher targeting efficiency in plant cells remains to be tested.

For HDR repair of SSB with ssODN donors, there appear to be two annealing-type pathways based on the repair outcomes of nicks generated with Cas9(D10A) nickase with different donors, one depending on annealing-driven strand synthesis (or synthesis-dependent strand annealing, SDSA) and the other depending on annealing-driven heteroduplex correction (or single-stranded DNA incorporation, SSDI) that acts only at nicks [24, 25]. SSB repair mediated by HDR with ssODN donors requires RPA, but it is suppressed by loading of RAD51 onto ssDNA at nicks [24]. The repair outcomes of SSB depended on the polarity of the ssODN donors; the ssODNs were found to be directly incorporated into the genome only in a bidirectional, but not in unidirectional, conversion pathway [25]. Improved GT efficiency was observed with ssODN donors designed based on the two different repair mechanisms [24, 25]. Editing with ssODN can be further enhanced by optimizing donor molecule design based on the ends generated after nuclease cleavage.

5 Targeted Insertion of Large Size Donor Sequence Through Homologous Recombination

Nucleases generating both SSB and DSB have been used to induce HDR for the purpose of GT or precise sequence replacement. HDR is a minor DNA repair pathway in higher plants in comparison with the dominant NHEJ pathways. Nevertheless, HDR-based targeted insertion into transgene reporter or native chromosomal loci has been successfully achieved in plants at low efficiency when various SDNs were used to cleave the target locus [60, 61, 77–80]. In eukaryotic cells, there are several mechanisms for carrying out DSB repair using homologous template, including the canonical DSBR, SDSA, and SSA. Theoretically, the efficiency of GT mediated by SDNs can be improved by several approaches, including overexpression of key genes involved in HR pathways, downregulation of NHEJ pathways, improved donor template configuration, improved delivery methodology, and increased availability of donor templates.

On the delivery side, large size donors were usually delivered with either a physical method or through *Agrobacterium*-mediated transformation for GT or targeted insertion studies in plants. In physical delivery methods such as biolistic bombardment, DNA templates are provided in double-stranded (ds) form [78–80]. When *Agrobacterium*-mediated transformation is used, the donor DNA is delivered into plant cells as single-stranded T-DNA with its 5′-end blocked by a covalently linked VirD2 protein [60, 61, 77]. Direct delivery of DNA by biolistic bombardment has been found to yield higher targeted insertion frequency than *Agrobacterium*-mediated delivery [80]. Perhaps T-DNA, if covered with VirE2 protein to protect it from nuclease degradation, is not available for RAD51/RPA binding to initiate homology search and HR. Alternatively, it is possible that there are much higher number of donor DNA molecules available for homology searching when DNA is delivered physically, thus resulting in higher targeted insertion rate. The suggestion that the number of template donor molecules is limiting is supported by studies showing the use of viral replicons based on a geminivirus, bean yellow dwarf virus (BeYDV), resulted in one to two orders of magnitude higher GT in both tobacco and tomato plants [81, 82]. However, the possibility that the viral replicon in ssDNA form is more accessible for HDR cannot be ruled out.

In recent years, there has been great progress in understanding the mechanisms governing DSB signaling and repair pathway choices [28–30]. Many studies have been carried out in efforts to enhance homology-dependent GT through manipulating (1) the DNA repair pathway choices, either by suppressing the endogenous NHEJ pathways or upregulating the HDR pathways, and (2) the

DNA repair pathway components involved in different steps of NHEJ or HR. Knockout of the 53BP1 gene or inhibition of 53BP1 activity greatly improved GT and chromosomal gene conversion with either dsDNA or ssODN donors [75]. Inhibition of NHEJ factor DNA ligase 4 with a chemical inhibitor SCR7 or silencing of Ku70 and ligase 4 also increased HDR efficiency [83, 84]. Interestingly, overexpression of adenovirus E1B55K and E4orf6 proteins which mediate the ubiquitination and degradation of DNA ligase 4 also significantly enhanced the efficiency of HDR and almost completely abolished the NHEJ activity [83]. In plants, it has also been shown that GT was increased by knocking out Ku70 and ligase 4 and SMC6B [61]. Since random DNA integration in the genome is dependent on Polθ-dependent altEJ mechanism whereas HR does not require Polθ, abolishing Polθ and other altEJ-specific components in target tissues may greatly reduce unwanted random integration, leading to enrichment of HDR-mediated GT products [63]. GT is also enhanced in *Arabidopsis* by knocking out altEJ component RAD50 [85]. It is also possible that other mutants and genes that lead to increased HR frequency can be used to improve targeted insertion. For example, HR frequency was increased in mutants defective in chromatin assembly factor 1 (CAF1) that is involved in nucleosome assembly following DNA replication and NER [86]. Intrachromosomal recombination frequency was also enhanced in transgenic lines overexpressing a MIM gene which has extensive homology with the SMC family proteins [87]. In addition to DSB, SSB generated by nickase can be employed to initiate efficient HR for GT in plants. For example, the Cas9(D10A) nickase induced HDR to a similar extent as the wild-type (WT) Cas9 nuclease or the homing endonuclease I-*Sce*I in both SSA and SDSA pathways [23]. Also, repair of SSB does not cause a considerable number of indel mutations in comparison with DSB [23]; perhaps more future GT studies will be done through the use of nickases, due to the fewer potential mutant lines that have to be screened to recover targeted events.

6 Site-Directed Base Editing

Recently, a new class of gene editing tool called base editors has been developed based on direct deamination of cytidine and adenine bases using chimeric fusion proteins between Cas9 and deaminases [26, 88–90]. One or both nuclease active sites (RuvC and HNH) in the Cas9 protein can be inactivated to create a nickase (nCas9) or deactivated Cas9 (dCas9) with RuvC domain D10A and HNH domain H840A mutations. Since both nCas9 and dCas9 mutant proteins still retain the crRNA binding activity, engineered single-guide RNAs (sgRNAs) can be used to target the Cas9-deaminase fusion proteins to specific chromosomal sequences as

in the normal CRISPR-Cas9 system. Pairing of the target sequence with gRNA mediated by Cas9 is thought to open up the nontarget strand as substrate for the deaminase which can only act on ssDNA [26]. Cytosine (C) deamination is catalyzed by cytidine deaminases and results in formation of uracil (U), which pairs with adenine (A), thus resulting in C:G to T:A mutation during DNA replication. On the other hand, adenine is deaminated to form inosine (I) which pairs with cytidine (C), resulting in A:T to G:C transition mutation [90]. The base-editing systems do not require DNA template or DSBs for generating intended mutations; the editing outcomes are determined by base modification through deaminase and BER pathway, rather than by the dominant error-prone cNHEJ and altEJ DSB repair pathways present in most cells. Therefore, the outcomes from base editing are much more predictable and precise [26].

The base deamination efficiency, position, and activity window within the target sequence are influenced by the property of deaminase and also the linker length between Cas9 and deaminase [26, 89, 91]. Under normal cellular environment, DNA BER pathway actively removes the cytidine deamination product, uracil, by uracil DNA glycosylase (UNG), thus reverting the mutation back to the WT and effectively reducing the base-editing efficiency. This problem was solved by directly incorporating a small bacteriophage uracil glycosylase inhibitor (UGI) into the fusion protein to block the UNG activity. To further increase the efficiency of base editing, nCas9, the Cas9(D10A) nickase with mutation in the RuvC active site, was used to preferentially nick the target strand to induce the long-patch base excision repair to remove the WT target sequence to favor the desired repair outcome [26]. One of the main drawbacks of the first generation BEs was that they were very processive and efficiently converted most or all Cs and As within the five-base activity window on the target DNA strand [26, 89]. It is also suggested that UNG activity causes formation of unexpected base-editing product and such unexpected products are more likely to occur at target sites that only contains a single C within the base-editing window [89]. By mutating the cytidine deaminase domains, the width of the editing window was narrowed from ~5 nucleotides to as little as 1–2 nucleotides [91]. Alternatively, different variants of deaminase with additional target sequence context requirements can be used, so only a subset of C or A residues are deaminated and edited. This is similar to the use of restriction enzymes with different sequence specificity requirements to generate sequence-specific cleavage of DNA.

Current systems of BEs are mostly limited to transition-type mutations (C to T, G to A, A to G, and T to C). It would be very useful if the frequency of transition-type mutations could be increased significantly without causing indel so that BE becomes a more flexible base mutagenesis tool. If this can be done efficiently,

direct base editing can be used to generate a diverse library of coding or regulatory region mutations in native chromosomal context for gene function studies and crop breeding. In addition, a wider mutagenesis window is desirable, since some regions may not be targetable due to the PAM-site restriction of Cas9. Along this line, translational fusions of MS2-binding protein with activation-induced cytidine deaminase (AIDs) variants have been generated [92]. This base-editing fusion protein is tethered to dCas9 non-covalently for carrying out targeted base editing through binding to the chimeric sgRNA bearing the MS2 hairpin sequences [92]. This resulting somatic hypermutation system, named CRISPR-X, with its non-covalently tethered hyperactive AID (hAID*Δ) configuration, produced base-edited mutations in a wider window spanning from +20 to +40 bp downstream of sgRNA's PAM sites relative to the direction of transcription independent of the strand of sgRNA targeting [92]. With the CRISPR-X system, edited mutations occurred downstream of the PAM site, a region likely to be double stranded. Since AID-mediated somatic mutagenesis in B lymphocytes requires transcription, it is possible that base editing mediated by dCas9-tethered deaminase fusions also happens when the target gene region becomes transiently single stranded while being transcribed [93]. In addition to the wider editing window, more mutation types (transition and transversion) were also obtained with CRISPR-X [92].

The cytidine and adenine deaminase fusions have been applied to site-directed mutagenesis in plant cells [94–100]. Similar to the results in mammalian cells [26], fusions of nCas9(D10A) nickase with rat cytidine deaminase (APOBEC1) or sea lamprey cytidine deaminase (PmCDA) resulted in higher cytidine editing efficiency than the dCas9 (deactivated Cas9)-APOBEC1 or PmCDA fusions in rice and wheat protoplasts, based on fluorescent protein reporter assays [96, 97]. The editing efficiency mediated by nCas9-APOBEC1 editor can be target dependent, e.g., efficiency on gene target *NRT1.1B* was lower compared with that on *SLR1* [94]. High efficiency of targeted base editing at chromosomal gene sequences was also observed in rice, wheat, and maize plants regenerated from tissues that had been transformed with the nCas9-APOBEC1-UGI (uracil glycosylase inhibitor) fusion protein expression vectors [94, 96]. Interestingly, it was observed that the indel frequency was much higher in plants (~10%) in comparison with the mammalian base-editing system (typically <1%) [26, 94, 95, 97]. This may be caused by stably integrated T-DNA that continuously generates nCas9 nickase activity in plants, whereas in the mammalian cells, the Cas9(D10A) was transiently expressed [95]. However, lower indel frequency was reported in a deep sequencing study with DNA isolated from protoplasts where UGI was also a component of the BE fusion protein [96]. It is thus possible that inclusion of UGI as part of

the BE fusion protein may help to reduce the number of indels in addition to decreasing the offtype base-editing products. It was shown that the unwanted indel formation could be greatly reduced by adding a DSB end-binding protein Gam to the BE fusion in mammalian cells [89]. Such measure of blocking DSB free ends with an end-binding protein should also be effective in reducing the indel frequency in plant cells. Recently, hyperactive AID mutant (AID*Δ) has also been applied to increase the editing efficiency in GC-rich rice genome [98].

Adenine base editors have also been shown to work efficiently in plants [99, 100]. In one study, two different adenine base editors were tested in rice; one editor (ABE-P1) had adenine deaminase TadA*7.10 [90] fused to the *Streptococcus pyogenes* Cas9 nickase (nSpCas9), and another (APE-P2) had TadA*7.10 fused to the *Staphylococcus aureus* Cas9 nickase (nSaCas9) which has a different PAM site requirement [99]. Interestingly, APE-P1 had somewhat wider editing window in rice in comparison with that of ABE7-10 editor in mammalian cells even though the editor fusion proteins were very similar except that ABE-P1 had a different nuclear localization signal from VirD2 protein [99]. All tested plant adenine base editors were highly specific; no off-target editing and indel formation at the on-target sites were detected in the edited rice mutant plants [99, 100]. With the remarkable development of both cytidine and adenine BEs, we now have a broad selection of tools for making targeted sequence changes in plants. Since there is a tremendous diversity in the bacterial RNA-guided CRISPR systems, it is expected that more CRISPR-based systems will be engineered into other types of genome editing tools for performing various kinds of DNA modifications.

7 Perspectives

One challenge to crop genome editing is their large genome size, the paleopolyploid and/or polyploid nature of many important crop species. It is possible that homoeologs and other homologs in the same gene family have redundant functions that should be considered when designing studies. Even though it is possible to disrupt several homoeologs simultaneously, the generated sequence variation in these edited homoeologs is usually quite heterogeneous. It is necessary to screen many edited plants to identify the lines with desirable mutant variants. Often, subsequent intercrossing is needed to bring desirable variants in different homoeologs from several edited lines together to achieve proper trait efficiency, as shown by an example in editing wheat for achieving powdery mildew resistance [101]. In addition, there are many varieties in widely cultivated crops such as corn and wheat. It is important to sequence the genomes of the target varieties to ensure

that gene editing machinery will result in desired edited outcomes across different genetic background. Attention needs to be given for the methodologies used to generate edited variants for vegetatively propagated crops such as potato and sugarcane, to ensure that transgenes are not integrated into the genome. Mutant plants with heritable edits have been generated by transient delivery of DNA vectors in potato [102], RNA in wheat [103], and ribonucleoprotein complexes in lettuce, corn, and wheat crops [104–106]. Since many of the important agronomic traits are multigenic, editing of several genes might be needed to achieve trait efficacy. For these complex trait engineering purposes, it is possible that precise edits, rather than simple indel of target sequences, will be needed. Therefore, there is still an unmet need to develop efficient tools that can modify multiple target sequences with predictable outcomes, either in the form of BEs or SDNs.

One area where genome editing tools may have big potential in breeding is targeted meiotic recombination. Breeding for certain traits is limited by lack of recombination in certain regions of chromosomes called cold spots [107]. Targeted enhancement of meiotic recombination can be used to accelerate breeding and trait introgression. It is estimated that targeted recombination in maize could double the selection gains for quantitative traits [108]. In yeast, local stimulation of meiotic recombination at a number of chromosomal sites has been achieved with Spo11 protein fused to various DNA-binding modules including dCas9 [109]. Another potential application of genome editing tools is for targeted chromosomal rearrangements such as deletion, translocation, and homoeologous recombination. Regions of chromosome harboring undesirable traits such as allergens can be removed from the crop [110]. Cleavage of chromosome by CRISPR-Cas9 also resulted in efficient translocation and inversion [111]. This kind of targeted chromosomal rearrangement can be used to move one or more desirable trait loci from a wild species' chromosome to a cultivated species' chromosome in wide cross progeny where there is little or no possibility of recombination between these chromosomes.

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Part II

CRISPR Design and Mutation Analysis

Chapter 2

Rapid Screening of CRISPR/Cas9-Induced Mutants Using the ACT-PCR Method

Chun Wang and Kejian Wang

Abstract

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system provides a technological breakthrough in targeted mutagenesis. However, a significant amount of time and cost is required to screen for the CRISPR/Cas9-induced mutants from a typically large number of initial samples. Here, we describe a cost-effective and sensitive screening technique based on conventional polymerase chain reaction (PCR), termed "annealing at critical temperature PCR" (ACT-PCR), for identifying mutants. ACT-PCR requires only a single PCR step followed by agarose gel electrophoresis. The simplicity of ACT-PCR makes it particularly suitable for rapid, large-scale screening of CRISPR/Cas9-induced mutants.

Key words ACT-PCR, CRISPR/Cas9, Genome editing, Mutant screening, Rapid, Large-scale, Cost-effective

1 Introduction

The CRISPR/Cas9 system employs the CRISPR-associated endonuclease, Cas9, along with a single-guide RNA (sgRNA) to generate double-strand breaks (DSBs) at the target DNA site. Genetic mutations are subsequently formed through nonhomologous end-joining (NHEJ) repair [1–3]. Insertion or deletion (indel) mutations induced by the CRISPR/Cas9 system usually occur proximate to the DSB site, 3 bp upstream of the protospacer-adjacent motif (PAM) [2]. The number of investigations regarding mutant generation by CRISPR/Cas9 has significantly increased in recent years, particularly for large-scale mutant screening, owing to the rapidly increasing popularity of genome editing in biological research.

PCR is a widely used technique that is capable of screening a large number of samples in a short time and with high specificity. A single PCR cycle consists of three steps: denaturation, annealing, and extension. The appropriate annealing temperature is critical for

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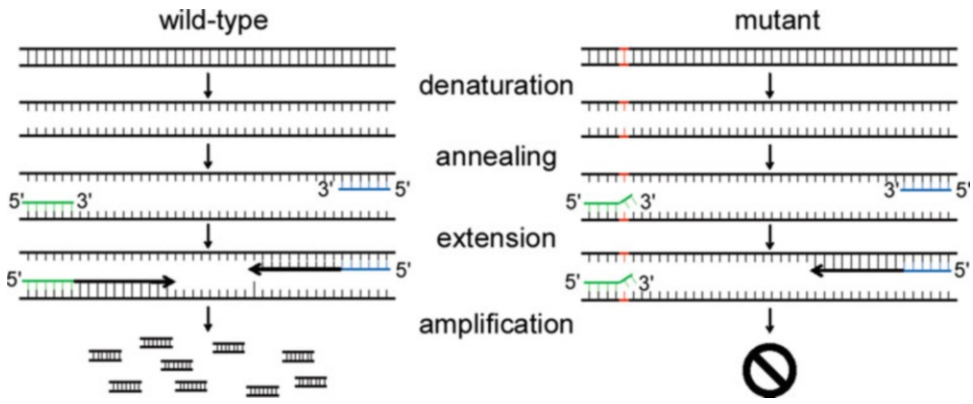

Fig. 1 Schematic of the ACT-PCR method. The primer DS, primer R, and the mutation site are labelled in green, blue, and red, respectively. At the critical annealing temperature, amplicons are obtained from the wild-type (WT) gene but not from the CRISPR/Cas9-induced mutant owing to the introduction of mismatches at the target site. (Reproduced from Ref. [4] with permission from the Journal of Genetics and Genomics)

successful PCR, as it determines effective primer–template pairing. An optimal temperature suppresses mismatched annealing, thereby reducing the generation of non-specific products.

On the basis of this theory, we developed the "annealing at critical temperature PCR" (ACT-PCR) method to detect CRISPR/Cas9-induced mutants easily, accurately, rapidly, and inexpensively [4]. This method consists of three steps: (1) design of primers, (2) detection of the critical annealing temperature by preliminary gradient PCR, and (3) the screening of mutants. First, primer pairs specific to the target genes are designed. The forward primer, named the DSB site-specific primer (primer DS), flanks the DSB site with its 3′ end containing a 4-bp overhang relative to the DSB site to ensure specificity and sensitivity for wild-type (WT) gene binding and PCR amplification. The reverse primer (primer R) is located outside the DSB site and has a higher T_m value than the DS primer to ensure DNA template binding at the critical annealing temperature. Next, preliminary gradient PCR is performed to determine the critical annealing temperature. Finally, conventional PCR is performed at the previously determined critical annealing temperature. If a mutation is present, the DS primer does not bind to the mutated sequence, and no amplicons are produced (Fig. 1). Thus, mutants are identified based on the absence of amplicons, which are reliably produced in the wild-type (WT) samples. We note this method is only good for identifying homozygous or biallelic mutants.

2 Materials

1. 2× Taq Master Mix (*Taq* DNA polymerase, dNTPs, and $MgCl_2$) or other similar PCR reagents.
2. Gradient PCR instrument.
3. Purified WT and mutant sample genomic DNA.
4. Agarose electrophoresis system.
5. 1.5% agarose gel.

3 Methods

For this chapter, we used the T_1 generation of CRISPR/Cas9-edited rice with the *OsPDS* gene as an example of a target gene, while the rice variety "Nipponbare" (*Oryza sativa* L. ssp. *japonica*) was used as the WT. Genomic DNA was extracted from both samples using the cetyltrimethyl ammonium bromide (CTAB) method (*see* **Note 1**).

3.1 Primer Design

The primer pairs were designed according to the principles outlined in Fig. 2. Primer DS was 20 bp in length, with the 3′ end having a 4-bp overhang relative to the DSB site. Primer R was designed to have a higher T_m value compared with the DS primer by increasing the length of the primer or choosing a GC-rich region as the PCR target (*see* **Note 2**). The T_m value of primer was calculated using an online tool (e.g., https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

In our example, the CRISPR/Cas9 target site of *OsPDS* is 5′-GTTGGTCTTTGCTCCTGCAGAGG-3′ (the PAM is underlined). Thus, the primer PDS-DS was designed as 5′-TTGGTCTTTGCTCCTGCAGA-3′ (T_m, 59.53 °C; Fig. 3a), and the primer PDS-R was designed as 5′-CTCCACTACAGACTGAGCACAAAGCTTC-3′ (T_m, 65.36 °C).

3.2 Detection of the Critical Annealing Temperature by Preliminary Gradient PCR

The PCR reaction for detecting the critical annealing temperature is set up as follows:

2× Taq Master Mix	7.5 μL
Primer DS (10 μM)	0.5 μL
Primer R (10 μM)	0.5 μL
WT genomic DNA	1 μL (about 50–100 ng)
ddH_2O	5.5 μL
Total volume	15 μL

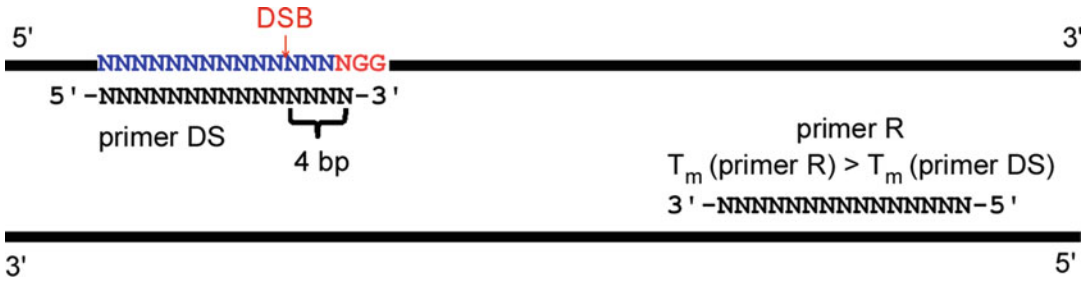

Fig. 2 Primer design. The forward primer, which is a double-strand break site-specific (DS) primer, is designed with a 4-bp overhang relative to the DSB site. The reverse primer, primer R, is designed with a higher melting temperature (T_m) value compared with the DS primer. (Reproduced from Ref. [4] with permission from the Journal of Genetics and Genomics)

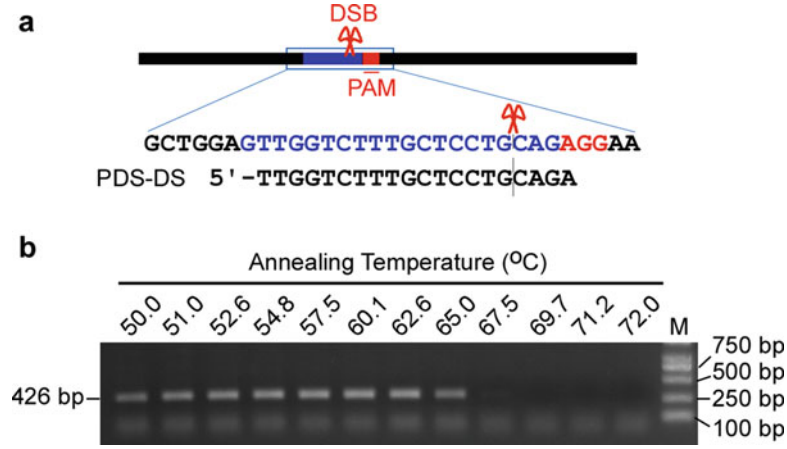

Fig. 3 The critical annealing temperatures detected by preliminary gradient PCR. (**a**) The wild-type sequence of *OsPDS* is shown at the top. The target sequence is labelled in blue, and the PAM is labelled in red. The DSB site is indicated with a scissor. The sequence of the PDS-DS primer is listed under the WT sequence. (**b**) The critical annealing temperature detected by gradient PCR using primers PDS-DS and PDS-R for the *OsPDS* target. The critical annealing temperature of PDS-DS/PDS-R is 65 °C, which is the highest temperature at which PCR products were still detected for the depicted sample. (Reproduced from Ref. [4] with permission from the Journal of Genetics and Genomics)

The PCR program is set as follows:

1. 94 °C, 2 min
2. 94 °C, 30 s
3. 50 to 72 °C, 30 s (gradient annealing temperature)
4. 72 °C, 1 kb/min

   Go to **step 2** for 29 cycles
5. 72 °C, 5 min

The PCR products were detected using 1.5% agarose gels. The critical annealing temperature is defined as the highest temperature at which PCR products can be detected (*see* **Note 3**). The critical annealing temperature of PDS-DS/PDS-R was 65 °C (Fig. 3b).

3.3 Mutant Screening

The PCR reaction for identifying the mutants was set up as follows:

2× Taq Master Mix	7.5 μL
Primer DS (10 μM)	0.5 μL
Primer R (10 μM)	0.5 μL
Sample DNA	1 μL (about 50–100 ng)
ddH_2O	5.5 μL
Total volume	15 μL

The PCR program is set as follows:

1. 94 °C, 2 min
2. 94 °C, 30 s
3. Critical annealing temperature, 30 s
4. 72 °C, 1 kb/min

   Go to **step 2**, 29 cycles
5. 72 °C, 5 min

The PCR products were detected using 1.5% agarose gels (*see* **Note 4**). Mutants were identified based on the absence of WT amplicons (*see* **Note 5**). In our example, samples #4, #7, #15, #16, and #19 were identified as mutants, as these samples failed to yield any PCR products (Fig. 4).

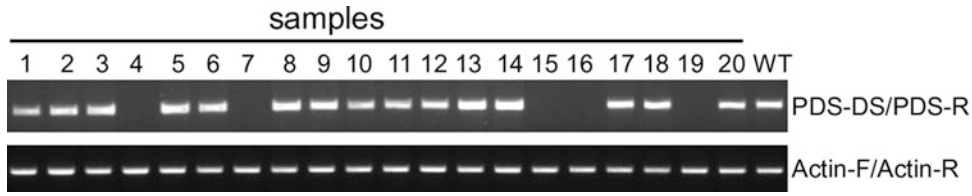

Fig. 4 Isolation of homozygous *Ospds1* mutants from the CRISPR/Cas9-induced rice using ACT-PCR. Samples #4, #7, #15, #16, and #19 were identified as mutants. In the bottom gel, *OsActin* was amplified as the control

4 Notes

1. Choosing a species-appropriate method for extracting the genomic DNA is critical to ensure that the concentration and quality of the sample genomic DNA remains consistent with that of the WT.
2. It is important to avoid choosing a homologous region when designing the ACT-PCR primers.
3. When the gradient PCR is performed, it is noted that the annealing temperatures of the lowest and the highest are set by the users, while the temperatures of at middle points are automatically calculated and set by the PCR instrument. The temperatures in the gradient are therefore slightly irregularly spaced.
4. To ensure the consistency of the genomic DNA of samples with that of the WT, a reference gene should be amplified as a control. In this example, *OsActin* is chosen as the control.
5. The limitation of ACT-PCR is that heterozygous or chimeric mutations cannot be detected in the screening process.

Acknowledgments

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Chapter 3

Decoding Sanger Sequencing Chromatograms from CRISPR-Induced Mutations

Xianrong Xie, Xingliang Ma, and Yao-Guang Liu

Abstract

In many diploid organisms, the majority mutations induced by clustered regularly interspaced short palindromic repeats (CRISPR)-mediated genome editing are non- chimeric, including biallelic, homozygous, and heterozygous mutations. Direct Sanger sequencing of the PCR amplicons containing non-homozygous mutations superimposes sequencing chromatograms, displaying overlapping peaks beginning from the mutation sites. In this chapter we describe the degenerate sequence decoding (DSD) strategy and its automatic web-based tool, DSDecodeM, for decoding the Sanger sequencing chromatograms from different types of targeted mutations. DSDecodeM, as a convenient and versatile tool, can considerably facilitate the genotyping work of CRISPR-induced mutants.

Key words CRISPR, Genome editing, Sanger sequencing, Superimposed chromatogram, Decoding, DSD, DSDecodeM

1 Introduction

To validate the outcome of CRISPR-mediated genome editing, it is necessary to confirm the targeted mutations and determine the mutated sequences for further study. A variety of methods, including T7E1 [1], qPCR assay [2], restriction enzyme site-based assay [3], high-resolution melting curve assay [4], and Surveyor nuclease assay [5], have been applied to screen mutations in target sites and measure the editing efficiency. However, these methods are not applicable for all targeted mutation types neither can resolve the mutation sequences [6]. Although deep sequencing-based methods is high-throughput and capable of detecting rare mutations in chimeric mutants, it is costly and time-consuming, thus not convenient enough for routine use [7, 8].

In many diploid organisms, for instance, rice, CRISPR/Cas9-based genome editing generates mostly uniform mutations (i.e., non-chimeric mutations, including biallelic, homozygous, and heterozygous mutations) [9]. However, Sanger sequencing of PCR

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amplicons from two distinct alleles results in superimposed signal peaks beginning from the mutant nucleotide position in the chromatogram. Although sequencing multiple *Escherichia coli* colonies obtained from cloning of each amplicon is a solution, this strategy is costly, tedious, and time-consuming. In this chapter, we describe a method termed degenerate sequence decoding (DSD) for decoding of such superimposed sequencing chromatograms from direct sequencing of PCR products with heterozygous and biallelic mutations [10]. Considering DSD is still time-consuming (about 3–4 min for each sequencing file) for a large number of samples, we have developed the web-based automatic tool DSDecode and its updated version DSDecodeM [11, 12]. DSDecodeM can automatically and rapidly analyze sequencing chromatograms of various mutation types, including biallelic, heterozygous, and homozygous mutations. To obtain high-quality sequencing chromatograms for DSDecodeM, guidelines for preparing the sequencing data are also provided, including the manual method and the web-based tool primerDesign-A. Using these tools, the preparation and analysis processes for amplification of target site-containing sequences, sequencing, and decoding of the targeted mutations can be implemented efficiently and automatically. All of these programs have been integrated into the web-based software toolkit CRISPR-GE (http://skl.scau.edu.cn/) [12].

2 Materials

1. Genomic DNA samples from plants transformed with CRISPR constructs.
2. PCR reagents including *Taq* DNA polymerase, dNTPs, primers, and PCR buffer.
3. DNA gel purification kit.

3 Methods

3.1 Manual Design of Primers for PCR and Sequencing

1. Design specific PCR primers: Generally, a primer pair should be about 200–400 bp flanking the targeted sites in the genome. For two or more closely positioned target sites, design PCR primers to produce longer (e.g., about 2–3 kb) amplicons containing these target sites (Fig. 1).
2. Design nest sequencing primers to obtain high-quality sequencing chromatograms; nest primers, rather than reusing the PCR primers (*see* **Note 1**), are designed as the sequencing primers, which are located about 150–300 bp away from the targeted sites. If the amplicons contain multiple target sites, design an independent sequencing primer for each site (Fig. 1).

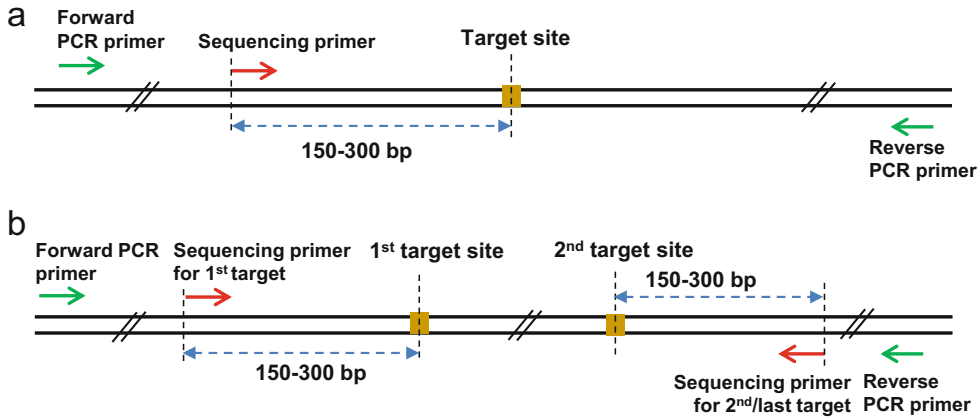

Fig. 1 The strategy of designing specific primers for PCR and sequencing. (**a**) For an amplicon containing one target site, an internal sequencing primer is designed. (**b**) For an amplicon containing two or more target sites, an independent sequencing primer for each target site is designed

3.2 Design-Specific Primers for PCR and Sequencing Using the primerDesign-A Tool (An Alternative to the Manual Design as in Subheading 3.1)

1. Visit the website of primerDesign-A (http://skl.scau.edu.cn/primerdesign/amp/) (Fig. 2).
2. Select a target (reference) genome listed in primerDesign-A (*see* **Note 2**). The reference genome is subjected for evaluating the specificity of all candidate primer pairs and output high-specific primers for the designated genome.
3. Paste a PCR template sequence containing the target site(s), or input a gene locus ID.
4. Enter the target site sequence(s). If the amplicon contains two or more target sites, paste the first and the second/last target site sequences in the input boxes (*see* **Note 3**).
5. Set the minimum and maximum distances of the primers from the target site(s). Generally, it is not needed to change the default settings.
6. Run the program by clicking the "Design" button.
7. The result table lists 5–10 pairs of credible and specific primers for amplification, with their positions in the template sequence and their melting temperature (Tm) values (*see* **Note 4**). The locations of target site(s) and primers can be directly displayed in the template sequence by clicking the "ID number" in the first column. When clicking the "Generate Sequencing Primer" button, primer(s) for sequencing are generated and displayed in the sequence (Fig. 3).

3.3 PCR and Sequencing

1. Amplify the target site(s)-containing genomic fragment (s) from candidate genome-edited individuals with the designed specific primers following a regular PCR procedure.
2. Purify the PCR products by agarose gel electrophoresis and the use of a gel purification kit.

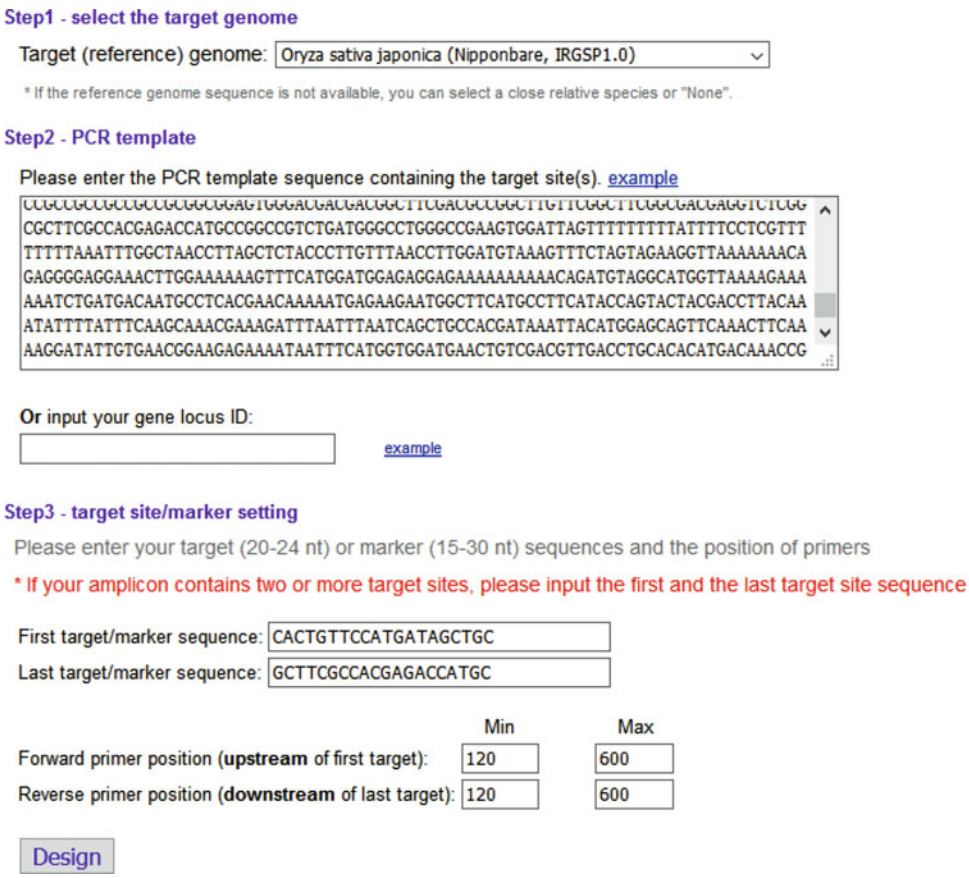

Fig. 2 The submission page of primerDesign-A. The steps to initiate a design task include selecting the target/reference (or closely related) genome, pasting the PCR template sequence or inputting a gene locus ID, entering the target site sequence (if two or more target sites, inputting the first and the last target site sequences), and setting the forward and reverse primer positions at upstream and downstream of the target site(s). Usually, no need to change the default settings

3. Subject the purified PCR products for Sanger sequencing using internal sequencing primer(s).

3.4 Decoding Mutant Allelic Sequences Using the DSD Method

1. Open the sequencing chromatogram in a chromatogram (trace) viewer such as Chromas (http://technelysium.com.au/wp/chromas/) or BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).
2. Mark an anchor sequence (AS) (10–15 nt) adjacent to the first overlapping peaks (Fig. 4).
3. Read a short degenerate sequence (DS) (12–15 nt) starting from the first nucleotide position with overlapping peaks according to the deduction from the color peaks of the chromatogram. The nucleotides are represented by the standard degenerate IUPAC/IUB symbols (R, A/G; Y, C/T; S,

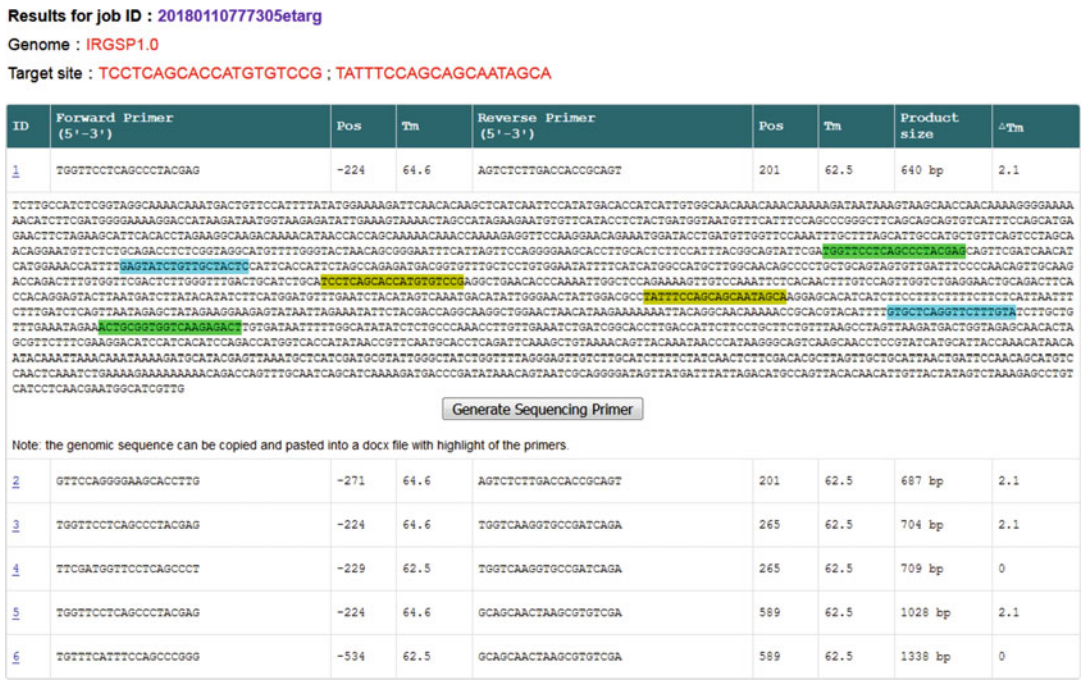

Fig. 3 The result page of primerDesign-A. The result table lists position, melting temperature (Tm), and product size for each primer pair. The locations of selected PCR primers (green), target site(s) (brown), and sequencing primer(s) (cyan) are directly displayed in the target genomic sequence

G/C; W, A/T; K, G/T; M, A/C; H, A/T/C; B, T/G/C; V, A/G/C; D, A/T/G; N, G/C/T/A).

4. Search the DS sequence against the intact (wild-type) reference sequence containing the target site with a DNA analysis program such as EditSeq (http://www.dnastar.com/) or Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).
5. Decode the first allele sequence (allele 1) by linking the matched reference sequence with the AS sequence (Fig. 4).
6. Carry out a second searching of the DS sequence against the reference sequence to decode the second allele sequence (allele 2) by linking the newly matched reference sequence to the AS sequence. If there is no a new hit in the second searching, obtain the allele 2 sequence by subtracting the allele 1 nucleotides from the degenerate nucleotides and linking the subtracted sequence to the AS sequence (*see* **Notes 5** and **6**).

3.5 Decoding Targeted Mutations Using the DSDecodeM Tool

1. Load the submission page of DSDecodeM (http://skl.scau.edu.cn/dsdecode/) (Fig. 5).
2. Input the intact wild-type reference sequence in the reference text area. The reference sequence must be identical to that of the parental individual for targeting, and completely cover the

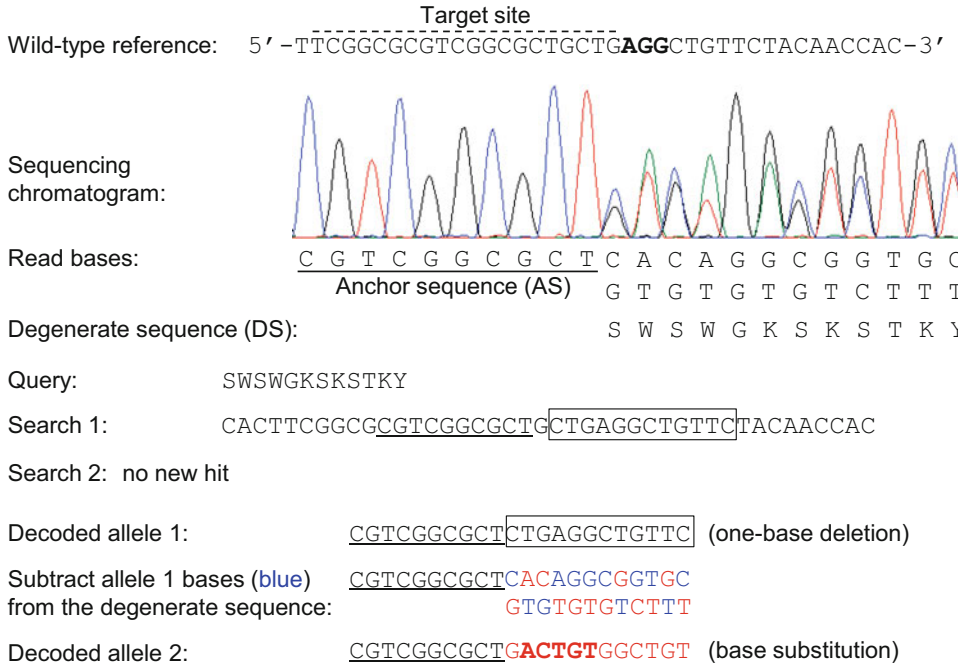

Fig. 4 An example of manual decoding of a Sanger sequencing file based on the DSD method. Nucleotides are read according to the peak colors, and overlapping nucleotides are converted into degenerate base symbols based on the standard degenerate nucleotide symbols. The DS sequence is used to search against the wild-type reference sequence using a DNA analysis program (such as EditSeq), and the allele 1 is decoded by linking the matched sequence (boxed) to the AS sequence. In this case the second search does not find a new hit for allele 2 (due to the presence of nucleotide substitution shown in bold red). So, the allele 2 bases (red) are obtained by subtracting the allele 1 bases (blue) from the DS sequence

range of the sequencing file(s) for one or multiple target sites (*see* **Note 7**).

3. Upload the sequencing chromatogram file(s) in ab1 format (up to 20 files for one round decoding) from the same or different closely located targets.
4. Reset some optional parameters if necessary, including the cutoff signal ratio (noise-peak/base-peak signal ratio), length of anchor and degenerate sequences, and inputting a target sequence. Generally it is not necessary to change the default settings or input the target sequence unless the decoding fails at first time. By adjusting these parameters, it is possible to exclude or reduce the interference from the sequencing noise signals (*see* **Note 8**).
5. Click the "Decode" button to run the program. The result page of DSDecodeM displays all decoding result(s) of the uploaded sequencing file(s), including the AS and DS, and decoded allele 1 and allele 2 sequences, with their alignments with the reference sequence and indication of the mutation

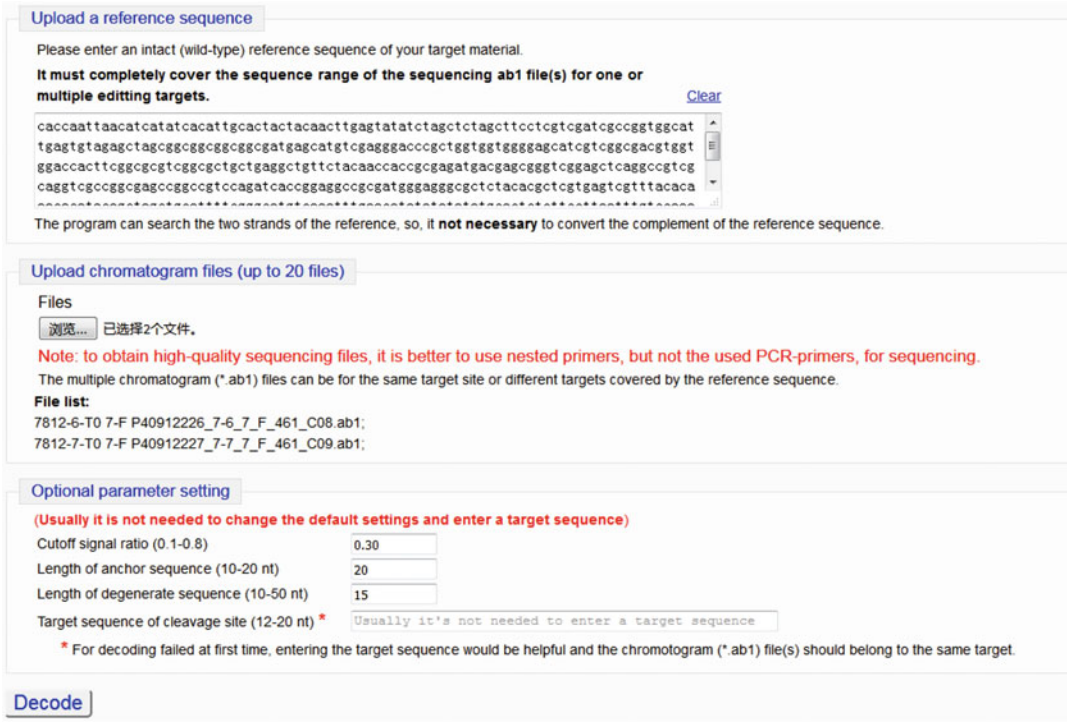

Fig. 5 The submission page of DSDecodeM, including the inputting of a wild-type reference sequence, uploading sequencing file(s), and optional settings for cutoff signal ratio, length of AS and DS, and inputting a target sequence if the primary decoding fails

types (*see* **Note 9**) (Fig. 6). The decoding results can be downloaded into a txt format file or copied into a local document file.

6. If a decoding task fails, try to run the decoding process again after resetting the parameters setting (*see* **Note 10**), or turn to a subprogram "Semi-automatic DSDecode" (SaDSDecode), which is for decoding low-quality sequencing files (with higher levels of noise signal).

3.6 Decoding Low-Quality Sequencing Files Using SaDSDecode

1. Load the SaDSDecode program linked from the decoding failed page or by visiting the submission page directly via http://skl.scau.edu.cn/sadsdecode/.
2. Input an intact wild-type reference sequence in the text area.
3. Input the manually read AS and DS sequences from a sequencing chromatogram as described earlier in Subheading 3.4 into the corresponding boxes.
4. Run the program by clicking the "Decode" button.
5. The result page of SaDSDecode displays decoding sequence of allele 1, allele 2, and their alignments with reference as described in DSDecodeM.

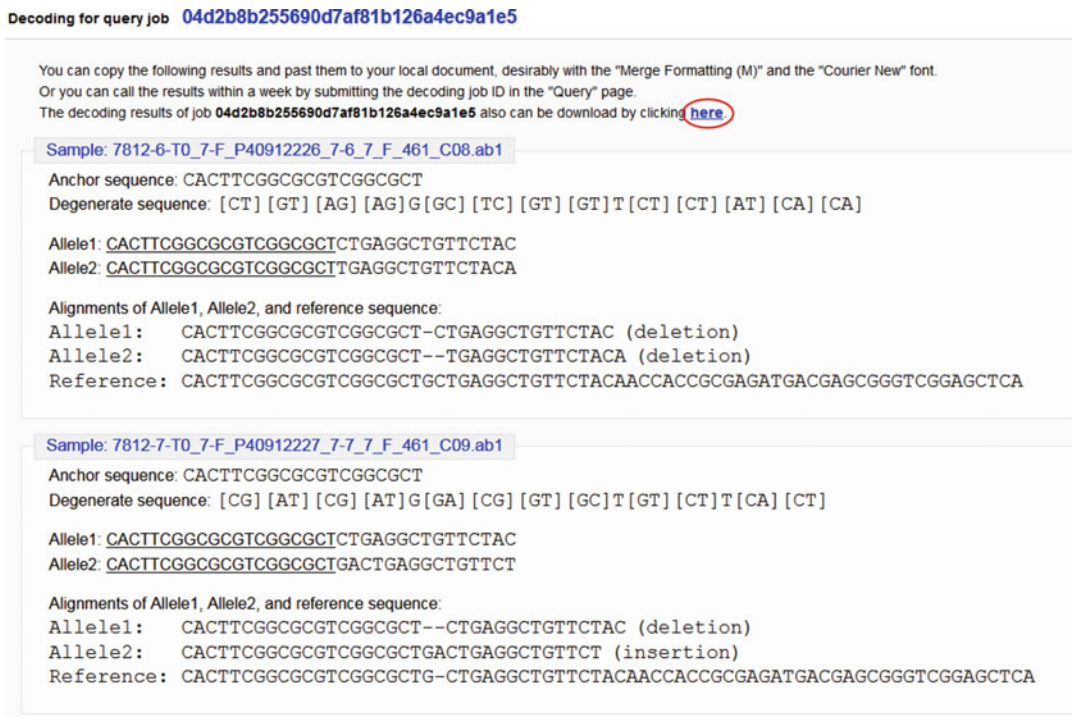

Fig. 6 The result page of DSDecodeM. Additional "-" indicates a base deletion in the decoded allele(s) or the corresponding position of a base insertion in the reference sequence. Clicking "here" (indicated by red ellipse) can download the decoding results in a txt format file

4 Notes

1. The accuracy and efficiency of decoding are sensitive to the quality of sequencing files. To obtain high-quality sequencing files, it is highly recommended that the purified PCR products are sequenced with internal (nested) specific primers rather than with the PCR primers.
2. Currently, 34 plant genomes and 5 genomes of non-plant organisms, including human, mouse, zebra fish, fruit fly, and nematode, are provided as the targets/references in CRISPR-GE. If no reference or genome of close relative species is available in the tool, users can contact with the developer for adding more genome(s) to the database of the tool.
3. The application of primerDesign-A can also be extended to design high-specificity primers for conventional PCR amplification of genomic sequences, by entering two short flanking sequences of a target genomic region.
4. In some instances, there is no specific primers output in the result page of primerDesign-A; due to that the target region is

highly homologous to other sequence(s) in the reference genome. To solve this problem, users can try to enter a longer PCR template sequence and appropriately adjust the minimum and maximum positions of forward and reverse primers upstream/downstream of the target site(s).

5. If the mutation type contains nucleotide substitution, the second searching cannot find a new hit, so the allele 2 can only be decoded by subtracting the allele 1 nucleotides from the degenerate nucleotides.
6. Both the DSD method and DSDecodeM tool are incapable of decoding complicated chimeric mutations or some rare biallelic mutations with insertion of two or more bases in both alleles. These mutations can be decoded by cloning the amplicons in a vector and sequencing multiple clones. Alternatively, chimeric mutations may be inherited into their progenies to generate simple, uniform mutations, many of which are in heterozygous and biallelic statuses. Then these mutation sites in the progenies can be directly sequenced and decoded using DSD or DSDecodeM.
7. DSDecodeM can search the two strands of the reference sequence. Hence, it is not necessary to convert the reverse complement of the reference sequence.
8. Noise signal peaks before the target may deceive the program to recognize an incorrect beginning of DS. If the primary decoding fails, inputting the target sequence with excluded noise peaks can help decode the mutations correctly.
9. In some cases, one mutated allele may possess a relatively large deletion that is longer than the primary setting of the degenerate sequence length. In these cases, DSDecodeM will automatically reset a longer degenerate sequence length (the bases of the large deletion plus 5 nt) and run the decoding process again and output new result (Fig. 7).
10. For some low-quality sequencing files, some adjustments would be helpful to improve the chance of success and accuracy, including increasing the cutoff signal ratio to eliminate the noise peaks in the sequencing chromatogram and decreasing the DS length to exclude the noise signals in the DS region. However, it is important to check the quality of sequencing chromatogram before initiating a decoding task using DSDecodeM. Keep in mind that the mutations usually occur at positions adjacent to the cleavage site. If the decoded mutations are far away from the target(s), the results would be unreliable.

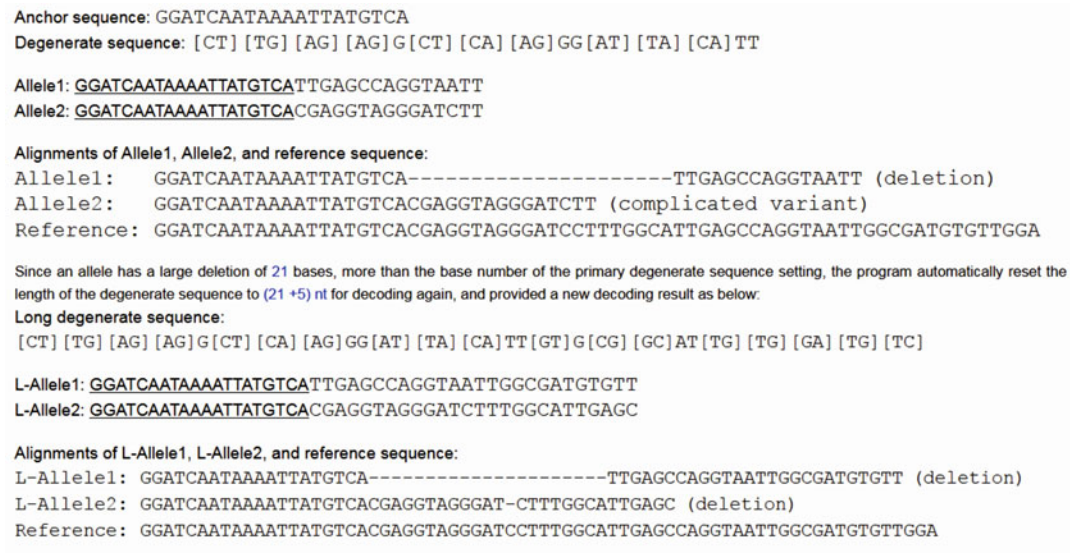

Fig. 7 An example of decoding of one mutated allele that has a relatively large deletion (21 nt), which is longer than the default setting for DS length (15 nt). Hence, allele 2 cannot be correctly displayed. For this case, the program automatically resets the DS length to 26 (21 + 5) nt and runs the decoding again to provide a new result

Acknowledgment

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Part III

Multiplexed CRISPR-Cas9 Systems

Chapter 4

Creating Large Chromosomal Deletions in Rice Using CRISPR/Cas9

Riqing Li, Si Nian Char, and Bing Yang

Abstract

Engineered CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat/CRISPR-associated protein 9) is an efficient and the most popularly used tool for genome engineering in eukaryotic organisms including plants, especially in crop plants. This system has been effectively used to introduce mutations in multiple genes simultaneously, create conditional alleles, and generate endogenously tagged proteins. CRISPR/Cas9 hence presents great value in basic and applied research for improving the performance of crop plants in various aspects such as increasing grain yields, improving nutritional content, and better combating biotic and abiotic stresses. Besides above applications, CRISPR/Cas9 system has been shown to be very effective in creating large chromosomal deletions in plants, which is useful for genetic analysis of chromosomal fragments, functional study of gene clusters in biological processes, and so on. Here, we present a protocol of creating large chromosomal deletions in rice using CRISPR/Cas9 system, including detailed information about single-guide RNA design, vector construction, plant transformation, and large deletion screening processes in rice.

Key words CRISPR/Cas9, Genome editing, Rice, *Agrobacterium*-mediated rice transformation, Targeted mutagenesis, Large chromosomal deletion

Abbreviations

CRISPR/Cas9	Clustered regularly interspaced short palindromic repeat/CRISPR-associated protein 9
DSB	Double-stranded DNA break
HDR	Homology-directed recombination
hptII	Hygromycin phosphotransferase gene for hygromycin B resistance
MCS	Multiple cloning sites
NHEJ	Nonhomologous end joining

Yiping Qi (ed.), *Plant Genome Editing with CRISPR Systems: Methods and Protocols*, Methods in Molecular Biology, vol. 1917, https://doi.org/10.1007/978-1-4939-8991-1_4,

1 Introduction

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated proteins (Cas), collectively called CRISPR/Cas immune systems of prokaryotes, have been adapted for genome editing [1] and applied in many organisms including plants [2–4]. Among Cas proteins, Cas9 from *Streptococcus pyogenes* (SpCas9) is the well-studied and most commonly used nuclease for genome editing [5], which contains NHN nuclease domain that cleaves the complementary strand and RuvC domain that cleaves the noncomplementary strand of the gRNA spacer region [6]. Thus, the genome-enabling CRISPR/Cas system consists of the nuclease Cas9 protein and the single chimeric guide RNA (gRNA) derived from the trans-acting crRNA (tracrRNA) and CRISPR RNA (crRNA) (referred to here as Cas9/gRNA) [1]. Site-specific double-stranded DNA breaks (DSBs) in vitro or in vivo are induced when gRNA-guided Cas9 cleaves the target sequence 3 base pairs (bp) upstream of the protospacer-adjacent motif (PAM, the most efficient site for SpCas9 is 5′-NGG-3′) [1], which subsequently triggers either nonhomologous end joining (NHEJ) or homology-directed recombination (HDR) DNA repair processes in targeted cells. HDR repair process is exploited for gene replacement or nucleotide-substitution-type gene editing with donor DNA template that contains the desired genetic change flanked by the DNA arms homologous to the target region of the genomic locus of interest [7, 8], while NHEJ is the predominant repair process in most, if not all, eukaryotes and is most frequently used to produce random nucleotide insertions, deletions, and substitutions at the repaired sites. With the established Cas9/gRNA system, various constructs can be made just through subcloning the spacer sequence (ca. 20 bp) at the multiple cloning site (MCS) preceding the scaffold of the single-guide RNA (gRNA), and multiple gRNAs can be made for multiplex targeting due to respective independent ribonucleoproteins with Cas9 [9]. Therefore, Cas9/gRNA system is an efficient and amenable tool for targeted mutagenesis in plants.

Besides mutagenesis at particular genomic sites, large chromosomal deletions are of interests in cases of studying genetic function of a whole chromosomal fragment and gene clusters in particular biological processes. In fact, via NHEJ large chromosomal deletion could also be obtained by inducing DSBs at both flanking sites of the target chromosomal region [9, 10]. Here, we present a protocol of creating both large chromosomal deletions and site-specific mutagenesis in rice using CRISPR/Cas9 system.

The major steps in this protocol includes the following: (1) design and construct a single plasmid expressing both Cas9 and at least two gRNAs based on the flanking sequences of target

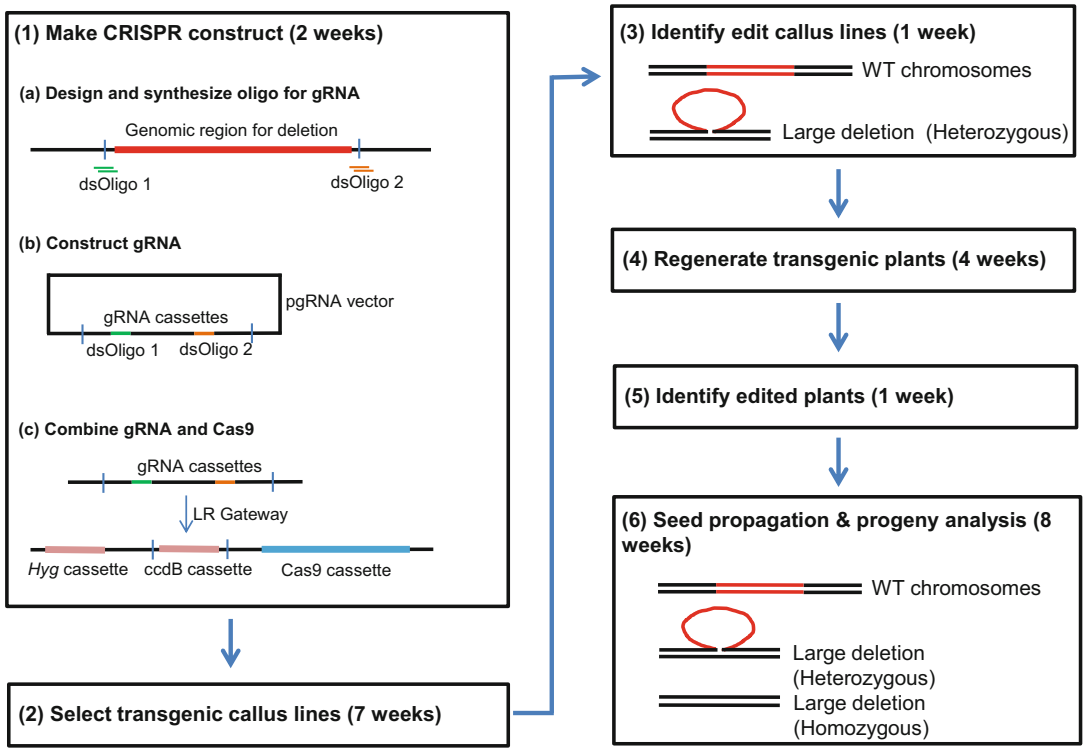

Fig. 1 Flowchart of a large chromosomal deletion project. Six major steps are needed from making CRISPR construct (step 1) to progeny analysis (step 6). The approximate time is indicated for each step

genomic region of interest for deletion, (2) transform the rice embryo-derived callus cells with the resultant construct through either *Agrobacterium*-mediated or biolistic particle-mediated DNA delivery method to produce transgenic callus lines and rice plants, (3) genotype the transgenic callus lines and rice plantlets to identify the intended large deletions using a PCR approach with deletion-specific primers, and (4) analyze inheritance of the chromosomal deletions and removal of the CRISPR transfer DNA (T-DNA). Here, we present a step-by-step protocol for each stage of the targeted large chromosomal deletions in rice (Fig. 1). This protocol has been successfully applied to delete many chromosomal fragments in various lengths in *japonica* rice cultivars (e.g., Kitaake) in our lab.

2 Materials

2.1 Molecular Cloning Reagents

1. Restriction enzymes (AatII, BamHI, BsaI, BtgZI, HindIII).
2. Calf intestinal alkaline phosphatase (CIAP).
3. RNase A.

4. ExoSAP-IT (a mixture of exonuclease I and alkaline phosphatase to remove excess primers and dNTPs in PCR products).
5. Polynucleotide kinase (PNK).
6. T4 DNA ligase.
7. LR Recombinant Clonase.
8. T7 Endonuclease I.
9. Adenosine triphosphate (ATP).
10. DNA ladder (e.g., 1 kb plus).
11. GENECLEAN III Kit (MP Biomedical) for DNA purification.
12. Bacterial competent cells (e.g., XL1-Blue, DH5α).
13. Luria-Bertani (LB) broth medium with appropriate antibiotics, e.g., kanamycin (50 mg/L), rifampicin (30 mg/L).
14. 30 and 37 °C incubators.
15. 30 and 37 °C shakers.
16. Gel electrophoresis apparatus.

2.2 Rice Tissue Culture and Transformation

1. pGW-rCas9 (available at the Yang lab upon request) (Fig. 2).
2. pgRNA1 (available at the Yang lab upon request) (Fig. 2).
3. *Agrobacterium tumefaciens* strain EHA105.

2.2.1 Plant Expression Vectors and Agrobacterium tumefaciens Strain

2.2.2 Rice Cultivar

The immature embryos of the *japonica* rice (*Oryza sativa* L.) cultivar Kitaake.

2.2.3 Reagents and Media for Rice Tissue Culture and Transformation

1. Medium for *Agrobacterium tumefaciens* strain EHA105.

   TY medium: Tryptone (5.0 g/L), yeast extract (3.0 g/L), kanamycin (50 mg/L), rifampicin (30 mg/L), or agar for solid medium (15 g/L), pH 5.5.
2. Callus induction.

   MSD medium: Murashige and Skoog (MS) basal medium with Gamborg's vitamins (4.4 g/L), sucrose (30 g/L), 2,4-dichlorophenoxyacetic acid (2 mg/L), and agar (8 g/L), pH 5.8.
3. Co-cultivation of calli with *Agrobacterium*.

   MSD + AS medium: MSD medium, acetosyringone (AS) (0.2 mM), or agar for solid medium (8 g/L), pH 5.2.
4. Callus selection.

   MSD + Hyg medium: MSD medium, Timentin (400 mg/L), hygromycin B (50 mg/L), and agar (8 g/L), pH 5.8.
5. Shoot regeneration.

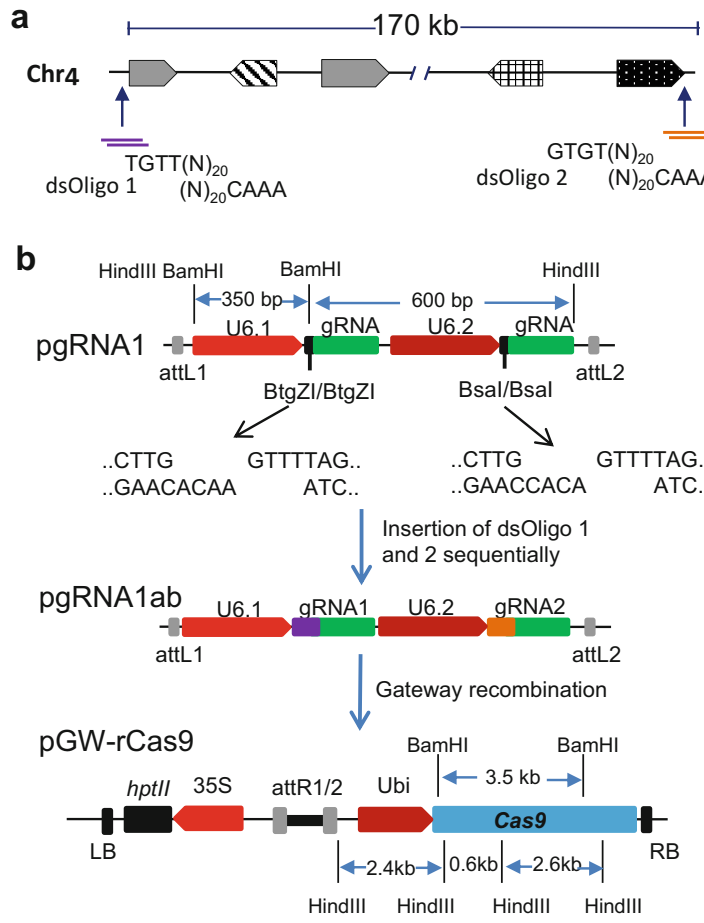

Fig. 2 Schematics of the target locus and CRISPR/Cas9 construct. (**a**) Structure of chromosomal region (170 kb), this is targeted for deletion by two gRNAs. (**b**) Stepwise construction of pGW-rCas9 vector with pgRNA1ab for multiplex editing

MSS medium: MS basal medium with Gamborg's vitamins (4.4 g/L), sucrose (30 g/L), sorbitol (50 g/L), 6-benzylaminopurine (BAP) (3 mg/L), 1-naphthaleneacetic acid (NAA) (0.5 mg/L), hygromycin B (25–50 mg/L), and agar (12 g/L), pH 5.8.

6. Root induction.
   MSR medium: MS basal medium with Gamborg's vitamins (2.2 g/L), sucrose (10 g/L), hygromycin B (25 mg/L), Agargellan (6 g/L), pH 5.8.

2.3 Analysis of Transgenic Rice Callus Lines or Plants

2.3.1 Plant Genomic DNA Extraction

1. DNA extraction buffer: CTAB (cetyltrimethyl ammonium bromide) (2%), NaCl (1.5 M), Tris–HCl (100 mM), EDTA (ethylenediaminetetraacetic acid) (20 mM), β-mercaptoethanol (1%, freshly added before use).
2. Chloroform: Isoamyl alcohol (24:1).
3. Isopropanol.
4. 70% ethanol.
5. RNase A.
6. TE buffer: Tris–HCl (10 mM), EDTA (1 mM), pH = 8.0.
7. Pestle.
8. Liquid nitrogen.
9. 65 °C water bath.

2.3.2 Genotyping

1. DNA polymerase.
2. T7 endonuclease I (T7EI).
3. Restriction enzymes.
4. Deoxynucleotide mixture (10 mM).
5. PCR buffers and primers.
6. Dimethyl sulfoxide (DMSO).
7. PCR thermocycler.

3 Methods

3.1 Select Cas9/gRNA Target Sites of Interest in Rice Genome

3.1.1 For Regular Gene Mutagenesis

The spacer sequence of guide RNA is chosen based on the Nipponbare reference genome sequence or sequences of interest using the CRISPR Genome Analysis Tool (CGAT) software (*see* **Note 1**). This publicly available tool is hosted at the Iowa State University Crop Bioengineering Center website (http://cbc.gdcb.iastate.edu/cgat/). The online tool was previously described [11]. The CGAT bioinformatics tool is user-friendly for identifying the potential target site in the genome of interest. The potential off-target sites will also be shown by using CGAT bioinformatics tool.

3.1.2 For Large Chromosomal Deletion

Determine the chromosomal fragment expected to be deleted, then select the target site sequences for design of guide RNA spacer sequences in both franking regions of the target chromosomal fragment. The gRNA spacer sequence design method is identical to Subheading 3.1.1. An example of a 170 kb fragment deletion in rice chromosome 4 is shown (Fig. 2a) (*see* **Note 1**).

3.2 Construct Cas9/gRNA Expression Plasmid

3.2.1 Design and Synthesize gRNA: Specific Oligonucleotides

1. To design oligos for gRNA gene construct, synthesize two reverse complementary oligonucleotides (22–25 nt) in a way that both strands are annealed to form a double-stranded fragment (dsOligo) with the sense strand consisting of the 4-nt overhang of TGTT at the 5′ end and the antisense strand consisting of an overhang of AAAC at the 5′ end for BtgZI-digested site.
2. Similarly, another two reverse complementary oligonucleotides are designed and synthesized to form a double-stranded DNA fragment with sense strand containing a 4-nt overhang of GTGT at the 5′ end and antisense strand containing AAAC at the 5′ end for BsaI-digested site.
3. The two oligo-derived fragments will be subcloned into the BtgZI- and BsaI-digested pgRNA1 vector sequentially, first through BtgZI and then BasI.

3.2.2 Clone the Synthesized Oligonucleotides into BtgZI-Digested Vector pgRNA1

1. Digest 1 μg of pgRNA1 DNA with BtgZI in a 30 μL of digestion reaction for 3 h at 60 °C in incubator or PCR thermocycler:

X μL (1 μg)	pgRNA1
1 μL	BtgZI
3 μL	10X buffer
0.2 μL	RNase A
Add to 30 μL	H_2O

2. Gel separate and purify BtgZI-digested pgRNA1 DNA using GENECLEAN III Kit.
3. Phosphorylate and anneal the pair of oligonucleotides synthesized for insertion at BtgZI site:

2 μL	Oligo 1 (10 μM)
2 μL	Oligo 2 (10 μM)
2 μL	PNK buffer
1 μL	ATP (1 mM)
1 μL	T4 PNK
12 μL	H_2O

4. Heat a beaker of water to boiling point and put the mixed 18 μL oligonucleotides into boiling water, let the water cool down to room temperature to allow oligonucleotides to anneal, then add ATP and T4 PNK, and incubate the reaction at 37 °C for 0.5 h before using the reaction for next step ligation.

3.2.3 Clone the Oligonucleotide-Derived Double-Stranded Fragment (dsOligo) into BtgZI-Digested pgRNA Vector

1. Set up ligation reaction:

X μL	BtgZI-digested pgRNA1 (50 ng)
5 μL	Annealed and phosphorylated dsOligo from **step 4** of Subheading 3.2.2
1 μL	10X ligation buffer
1 μL	T4 ligase
Add to 10 μL	H_2O

2. Incubate the ligation reaction at room temperature for 3 h, and transfer half of the reaction into the *E. coli* XL1-Blue competent cells.
3. Conduct colony PCR assay by using gRNA-F1 (oligonucleotides as forward primer for the spacer sequence of gRNA) and U6T-R (reverse primer complementary to the region downstream of gRNA gene in the backbone of pgRNA1).
4. Further confirm the plasmid DNA by digestion with BamHI. If the dsOligo is successfully inserted into the pgRNA1 vector, the BamHI site at the MCS will disappear, and clones lacking the 350 bp fragment in the BamHI digestion pattern are potential insertion-positive clones.
5. Sequence the positive candidate clone by using forward primer (U6P-F1b) or reverse primer (pENTR4-R) to confirm the accuracy of spacer sequence in the gRNA scaffold. The confirmed plasmid is named pgRNA1a.
6. After the first dsOligo is inserted into BtgZI restriction site, the second dsOligo is sequentially ligated at the BsaI restriction site.

3.2.4 Clone a Second gRNA-Specific Fragment into the BsaI-Digested gRNA Scaffold Vector

1. Digest 1 μg of pgRNA1a plasmid with BsaI in 30 μL for 3 h at 37 °C:

X μL (1 μg)	pgRNA1a
3 μL	10X buffer
1 μL	BasI
0.2 μL	RNase A
Add to 30 μL	H_2O

2. Ligate the annealed second dsOligo (prepared similarly to the first dsOligo) into the BsaI-digested pgRNA1a plasmid that contains the first dsOligo insertion for the first gRNA gene.

Set up a ligation reaction as follows:

X μL	BsaI-digested pgRNA1a (50 ng)
5 μL	Phosphorylated and annealed oligonucleotides
1 μL	10X ligation buffer
2 μL	T4 ligase
X μL	H_2O
10 μL	DNA ligation

3. Incubate the ligation reaction at room temperature for 3 h, and transfer half of the 10 μl of reaction into the *E. coli* XL1-Blue competent cells.
4. Conduct colony PCR assay by either using gRNA-F2 (oligonucleotides as forward primer for the spacer sequence of the second gRNA) and U6T-R (reverse primer complementary to the region downstream of gRNA gene in the backbone of pgRNA1) or gRNA-F1 and gRNA-R2 (oligonucleotides as the forward primer for the spacer sequence of the first gRNA and oligonucleotides as the reverse primer for the spacer sequence of the second gRNA, respectively).
5. Further confirm positive clones by digest plasmid DNA with BamHI and BsaI. There is no BsaI restriction site within the MCS if the dsOligo is successfully cloned into the vector, and the positive clones will lack the 790 bp fragment as compared to pgRNA1a.
6. Sequence the positive plasmid by using the forward primer (U6P-F1b) or reverse primer (pENTR4-R) to confirm the accuracy of the first and second spacer sequences. The confirmed plasmid is named pgRNA1ab.

3.2.5 Assembly of Four Guide RNA Cassettes for Multiplex Editing

Our intermediate vectors can also be used to construct up to four gRNAs for multiplex editing in rice or for targeting two sites within each flanking region of the intended chromosomal deletion locus to increase the success rate of deletion. To construct gRNA cassettes that contain four guide RNA genes, two gRNA constructs need to be made first by using pgRNA1 and pgRNA2 individually. pgRNA2 is almost identical to pgRNA1 except that it does not contain the second HindIII restriction site at the 3′ end of the second guide RNA scaffold.

1. Clone two dsOligo fragments into pgRNA2 similarly to the cloning of two dsOligo fragments into pgRNA1 as described above, resulting in pgRNA2ab.
2. Release the cassette of two gRNA genes from pgRNA1ab by digestion with HindIII. Gel purify the fragment of gRNA cassette of ~1 kb.

3. Subclone this gRNA cassette into Hind III-digested and subsequently CIAP-treated pgRNA2ab that already contains two guide RNA genes. Digest plasmid DNA from the cloning step with HindIII and BamHI to confirm the insertion of gRNA cassette, resulting in plasmid pgRNA1/2ab.
4. Assemble the four guide RNA cassettes into the destination vector pGW-rCas9 by using the Gateway LR Clonase kit with a reaction as follows (*see* **Notes 2** and **3**):

X μL (150 ng)	pGW-rCas9 (destination vector)
X μL (50–150 ng)	pgRNA1ab or pgRNA1/2ab (entry clone)
2 μL	LR Clonase
Add to 10 μL	TE buffer or H_2O

5. Confirm the resulting plasmid DNA from individual clones by digestions with BamHI or HindIII. A digestion pattern of 12 kb, 2.6 kb, 2.4 kb, 1 kb, 650 bp, and 125 bp from HindIII digestion or a pattern of 12 kb, 3.5 kb, and 2.8 kb due to BamHI digestion indicates a successful mobilization of gRNA cassettes into the destination Cas9 plasmid.

3.3 Transgenics of Rice with Cas9/gRNA

Rice transformation involves DNA delivery into callus cells through biolistic particle bombardment or *Agrobacterium* infection. Here, we describe the *Agrobacterium*-delivered CRISPR system for mutagenesis in rice by using calli induced from the scutella of immature seeds. The transformation protocol is modified from the method described by Hiei *et al.* [12].

3.3.1 Transform Rice Callus Cells with the Cas9/gRNA Construct for Chromosomal Deletion

1. Transfer the Cas9/gRNA plasmid into the *Agrobacterium* strain EHA105 through electroporation (*see* **Note 4**).
2. Callus initiation: Sterilize the dehusked immature seeds with 50% bleach (3% sodium hypochlorite) for 20 min, and rinse three times with sterilized water. Then place the seeds on MSD medium, and incubate at 30 °C with continuous light for 14 days to initiate actively growing calli.
3. *Agrobacterium* infection: Grow Cas9/gRNA containing *Agrobacterium* culture to 1.0–2.0 of OD600. Mix 2 mL *Agrobacterium* cells with 20 mL liquid MSD medium (with 0.2 mM acetosyringone, pH 5.2), and immerse the rice calli in the suspension for 30 min. Blot dry the calli with filter paper, and transfer the calli to MSD + AS plate. Keep the calli in the dark at room temperature for 2 to 3 days.
4. Transformed callus selection: Transfer the infected calli to selection medium (MSD supplemented with 50 mg/L of hygromycin B and 400 mg/L of Timentin) at an interval of

2 weeks for selection of transformed callus cells. The tissue culture and regeneration at this and subsequent stages are performed in growth chamber at 30 °C and under continuous light.

5. Plantlet regeneration: Transfer the individual hygromycin-resistant callus lines to regeneration medium to regenerate transgenic plantlets.
6. Root induction: Transfer the regenerated plantlets into ½MS medium for root induction.
7. Transfer the plantlets into soil, and grow them in a growth chamber with the temperature of 28 °C, relative humidity of 75%, and photoperiod of 12 h till plants are mature. Harvest seeds for further analysis.

3.4 Screening and Identifying CRISPR/Cas9-Induced Deletion Mutations

3.4.1 Extract Genomic DNA from Transformed Calli or Plants

1. Extract genomic DNA from rice callus cells or pooled leaves of individual transgenic plants by using the CTAB (cetyltrimethylammonium bromide) method [13].
2. Measure the concentration of the isolated genomic DNA by using the Nanodrop ND1000 spectrophotometer. The genomic DNA is used as the template for PCR amplification of target regions with deletion-specific primers designed to flank the target sites.

3.4.2 Screen for Putative Large Chromosomal Deletion Lines

The efficiency of large chromosomal deletions is much lower than regular mutations. For this reason, we usually identify deletion-positive transgenic calli and use those callus lines carrying large chromosomal deletions for regeneration of plants.

1. Design primers F1 and R1 around the left cleavage site of intended chromosomal deletion, and design primers F2 and R2 around the right site (Fig. 3a). F1 and R2 are outside of targeted deletion region, while R1 and F2 are within the intended deletion region.
2. Conduct PCR using F1&R2, F1&R1, and F2&R2 primer pairs.
3. Detect PCR amplicons by electrophoresis with 1–2% agarose gel. Successful PCR amplification with F1&R2 indicates at least one chromosome contains large deletion. Successful PCR amplification with F1&R1 and F2&R2 indicates non-deletion events (Fig. 3b).

3.4.3 Screen for Putative Site-Specific Mutations Through T7EI Assay

The PCR amplicons with primer pair F1&R1 or F2&R2 as indicated in Fig. 3a are tested for the targeted mutations by using T7 endonuclease 1 (T7E1) assay.

1. Mix PCR products originated from the transgenic tissues with respective amplicon derived from the wild-type plant. Denature

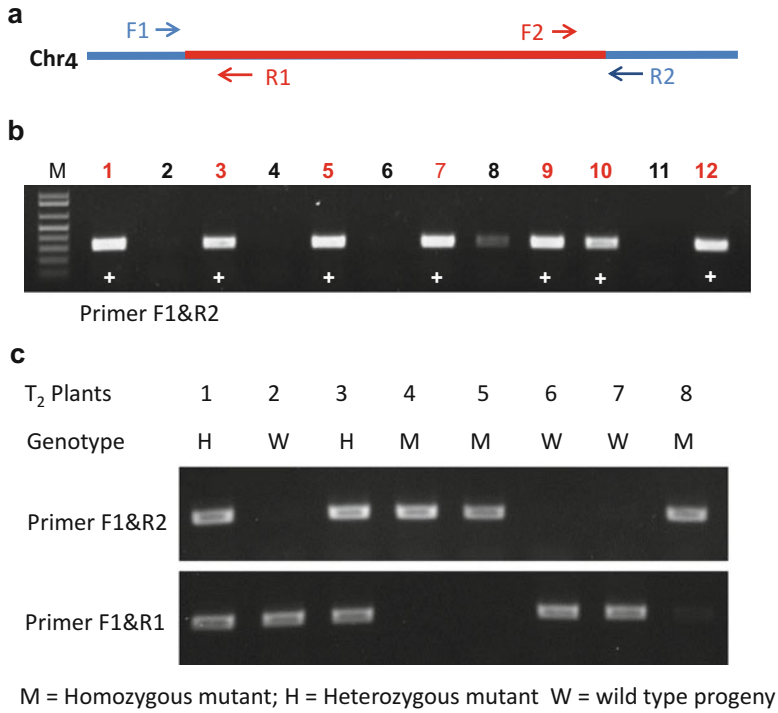

Fig. 3 Analysis of large chromosomal deletion in rice plants. (**a**) Schematics showing locations of two pairs of primers. Line in red indicates region for intended deletion. (**b**) Detection of large chromosomal deletion with the deletion-specific primers. (**c**) Detection of homozygosity or heterozygosity of progeny plants with two pairs of primers

(95 °C for 5 min) and reanneal (ramp down to 25 °C at 5 °C/min) the products.

2. Digest with T7EI enzyme.
3. Analyze the result in 1–2% agarose gel electrophoresis. The electrophoresis pattern with additional bands compared to the original PCR amplicon of transgenic plant indicates a mutant genotype, otherwise, a wild-type genotype.

3.4.4 Sequence PCR Amplicons to Confirm the Mutant Genotypes

1. Treat the PCR amplicons derived from the positive callus lines or plants with ExoSAP-IT to remove the excessive primers and single nucleotides.
2. Perform Sanger sequencing. Carefully examine the sequencing chromatograms for exact peak pattern that might contain mono-allelic or di-allelic mutations (for regular mutagenesis). For large chromosomal deletion lines, sequence of amplicons derived from primers F1 and R2 could help to find out precise boundary of the large deletion.

3.4.5 Identify the Heritable Mutations from Progeny

Grow the T_0 plants in growth chamber, and let individual T_0 plants be self-pollinated. Collect seeds from the individual T_0 plants, and put them in a 37 °C incubator for 1 to 2 weeks to dry and break the seed dormancy. Germinate the seeds in soil or the ½MS medium depending on the purpose of experimentation.

1. For large chromosomal deletion: Genotype the target locus to determine the heritability of deletion mutations from T_0 to T_1 and T_2 generation plants. PCR with both F1&R2 and F1&R1 primer pairs for each genomic DNA sample, and then run 1–2% agarose gel. PCR amplicons being both positive with F1&R2 and F1&R1 indicate the corresponding plants are heterozygous. Plants showing amplicons being positive only for F1&R2 are homozygous for large deletion, while plants only being positive for F1&R1 contain no large deletion (Fig. 3c).
2. For regular targeted mutagenesis: Conduct T7E1 assay on the individuals of T1 or T2 plants. The heterozygous plants are detected by the presence of digested bands. If only an uncut band is present, mix the PCR amplicons of this sample with the wild-type amplicons, and conduct T7E1 assay again. The homozygous plants are detected by the presence of digested bands. If only an uncut band is present, the plant is wild type.

3.4.6 Segregate Out the T-DNA from Progeny

1. Design primers to amplify selected regions of the Cas9/gRNA T-DNA (e.g., hygromycin resistance gene (*hptII*) or gRNA genes).
2. Screen the T_1 plants with PCR by using these primers. Include positive control (T_0 generation callus line or plant) and negative control (wild-type Kitaake plant). Plants that do not have detectable PCR products of T-DNA are considered as T-DNA-free plants (*see* **Note 5**).
3. Genotype T-DNA-free plants for targeted mutations or chromosomal deletions as described above.

4 Notes

1. A few requirements are needed for selecting the unique target sequences: (a) PAM sequence (5′-NGG-3′ or 5′-NAG-3′) is required following the target sequence of the guide RNA (PAM must not be included in the spacer sequence of gRNA); (b) off-target mutagenesis could be avoided by BLAST searching of the rice genome database; (c) an appropriate restriction enzyme site overlapping with the Cas9/gRNA cleavage site is preferable; (d) the 5′-G or 5′-A is required for the stability of gRNA expressed from U6 or U3 promoter, respectively.

2. The intermediate vector is linearized with AatII or EcoNI or other unique restriction sites outside the attL1 and attL2 cassettes before used for the LR recombination. Linearization of entry plasmid can increase the efficiency of Gateway LR recombination.
3. The bacterial colonies are selected on the LB plate supplemented with two antibiotics (50 mg/L kanamycin and 25 mg/L hygromycin B). Pick up the small colonies for further confirmation. We usually observed that correct colonies are small probably due to the negative effect of Cas9 on bacterial growth.
4. The binary vector pGW-rCas9 is kanamycin resistant. It is hence not suitable to use *Agrobacterium tumefaciens* strains (e.g., EHA101) that are already resistant to kanamycin.
5. Southern blot or ideally whole-genome sequencing can be used to further confirm the null segregates (transgene-free) if needed.

Acknowledgments

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Chapter 5

A Multiplexed CRISPR/Cas9 Editing System Based on the Endogenous tRNA Processing

Kabin Xie and Yinong Yang

Abstract

The CRISPR-Cas9 system has become a powerful and popular tool for genome editing due to its efficiency and simplicity. Multiplex genome editing is an important feature of the CRISPR-Cas9 system and requires simultaneous expression of multiple guide RNAs (gRNAs). Here we describe a general method to efficiently produce many gRNAs from a single gene transcript based on the endogenous tRNA-processing system. A step-by-step protocol is provided for the design and construction of the polycistronic tRNA-gRNA (PTG) gene. The PTG method has been demonstrated to be highly efficient for multiplex genome editing in various plant, animal, and microbial species.

Key words CRISPR-Cas9, tRNA, gRNA, Multiplex, Genome editing

1 Introduction

Since the initial demonstration of CRISPR-Cas9 from *Streptococcus pyogenes* for sequence-specific DNA cleavage [1], the CRISPR-Cas system has rapidly become the most popular tool for genome editing of human cells [2, 3] and many other organisms [4]. The RNA-guided genome editing only requires two components: Cas9 nuclease and guide RNA (gRNA). The 5′-end of gRNA contains a specific guide sequence (or spacer sequence) which recognizes the DNA target site with protospacer adjacent motif (PAM, 5′-NGG-3′) and directs Cas9 to make specific cleavage. Besides generating double or single strand breaks at the specific sites, the CRISPR-Cas9 system has many other innovative applications such as targeted transcriptional control [5] and specific base editing [6]. In addition, the Cas9 protein has been rationally redesigned to recognize variable PAM sequences and improve targeting fidelity [7–9]. These innovations have further elevated the CRISPR-Cas9 system as a powerful and versatile tool for genetic engineering.

Multiplex genome editing is an attractive feature of the CRISPR-Cas9 system. In principle, Cas9 is capable of editing

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many genomic sites if multiple specific gRNAs are provided. Indeed, advanced editing technologies often require simultaneous expression of many gRNAs in vivo. Multiple gRNAs could be co-expressed from a construct containing several promoters: gRNA cassettes [10, 11]. However, only few promoters could be used to express gRNAs, and most of them are derived from snoRNA U3 and U6 genes. These polymerase III (Pol III) promoters are suitable to express noncoding small RNAs, and their transcripts always start with a fixed nucleotide. A more sophisticated approach is to express many gRNAs from a single synthetic gene whose transcript could be processed precisely by RNases. For example, self-cleavable ribozyme and Csy4 RNase were engineered to produce multiple gRNAs from one RNA transcript [12, 13]. Interestingly, eukaryotic cells contain various endogenous RNA-processing machineries, and some of them could be engineered to produce gRNAs from one transcript. To this end, we demonstrated that the endogenous tRNA-processing system could be engineered as a general platform to express many gRNAs for CRISPR-Cas9 multiplex genome editing [14]. As shown in Fig. 1, multiple pre-tRNA-gRNA fusion units can be arrayed as a single polycistronic tRNA-gRNA (PTG) gene. The synthetic PTG transcript produced in vivo is precisely cleaved by the endogenous tRNA-processing enzymes, RNases P and Z (Fig. 1). As a result, many gRNAs could be readily co-expressed from a single synthetic PTG gene and direct Cas9 to edit many target sites simultaneously [14]. Due to the presence of the tRNA-processing enzymes in all living organisms, the PTG-mediated multiplex editing approach has been successfully demonstrated in various plants [14, 15], animals [16–18], and microbes [19].

The PTG approach has a number of important advantages for multiplex genome editing [14]. First, tRNA contains cis-elements to enhance Pol III transcription. In comparison with Pol III promoter (e.g., U3 promoter) alone, the use of tRNA can increase the gRNA expression by one order of magnitude and significantly improve the editing efficiency. Second, the synthetic PTG gene could be efficiently expressed by strong Pol II promoters (including inducible and tissue-specific promoters) instead of Pol III promoters. Third, the size of tRNA is quite small, which allows compact packing of many gRNAs in an average-size synthetic gene. For example, the rice glycine pre-tRNA is only 77 bp. Multiple tRNA-gRNA units could be packed within an intron which is fused with the Cas9-coding sequence as a single hybrid gene [20]. Fourth, tRNA can be used with the CRISPR-Cpf1 system for efficient gRNA expression and multiplex editing [20]. Fifth, RNases P and Z recognize conserved tRNA structure for accurate cleavage of tRNA and efficient production of gRNAs. This feature is important for high-fidelity Cas9 derivatives [21]. The rice glycine tRNA was efficiently cleaved by the endogenous tRNA-processing

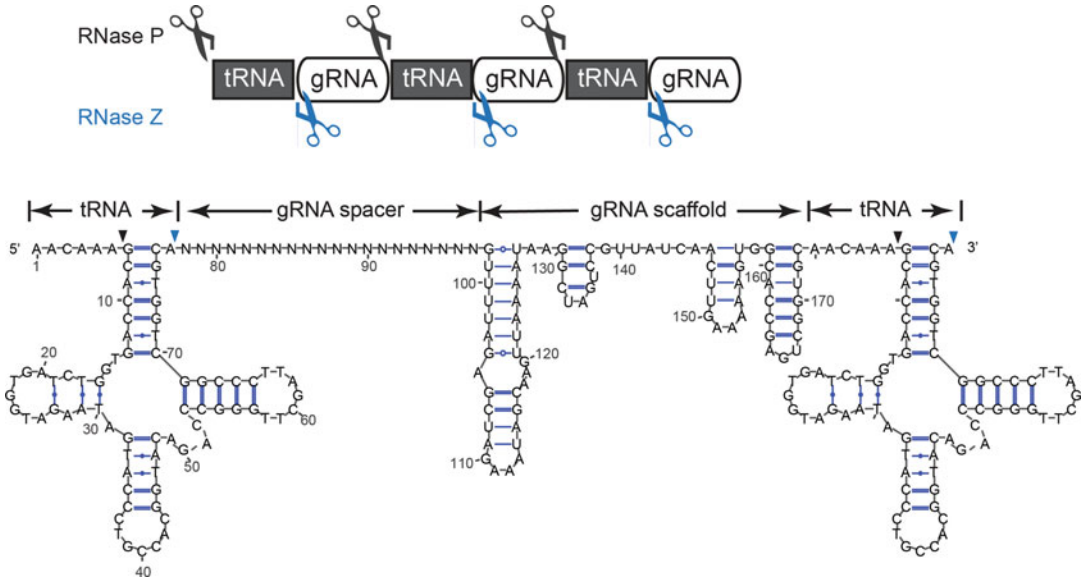

Fig. 1 Schematic illustration of polycistronic tRNA-gRNA structure and sequence. The endogenous RNases P and Z recognize and cleave at the tRNA ends (shown as black and blue scissors) in the tRNA-gRNA polycistron. The bottom panel shows the sequence and secondary structure of rice glycine tRNA fused with gRNA. RNases P and Z cut sites are labeled with black and blue triangles, respectively

enzymes in diverse organisms such as rice [14], human cells [17], fruit fly [18], and fungus [19].

This chapter presents a practical protocol to make PTG constructs for Cas9-mediated multiplex genome editing. The experimental procedure is flexible and allows the construction of various tRNA-gRNA units in a single PTG fragment using the same set of primers. Although the protocol is based on our CRISPR-Cas9 vectors for rice transformation and genome editing, it can be readily used to make synthetic PTG genes for other CRISPR vectors.

2 Materials

1. *Oryza sativa* L. ssp. *japonica*, Kitaake.
2. pGTR, pRGEB32, pRGEB33, and pRGEB34 vectors (Addgene, plasmids #63143 and #63142; also see Fig. 2).
3. *Bsa*I (New England Biolabs, catalog number: R0535S).
4. *Fok*I (New England Biolabs, catalog number: R0109S).
5. Phusion high-fidelity polymerase (Thermo Fisher Scientific, catalog number: F530S).
6. T7 DNA ligase (New England Biolabs, catalog number: M0318S).
7. GoTaq DNA polymerase (Promega Corporation, catalog number: M3001).

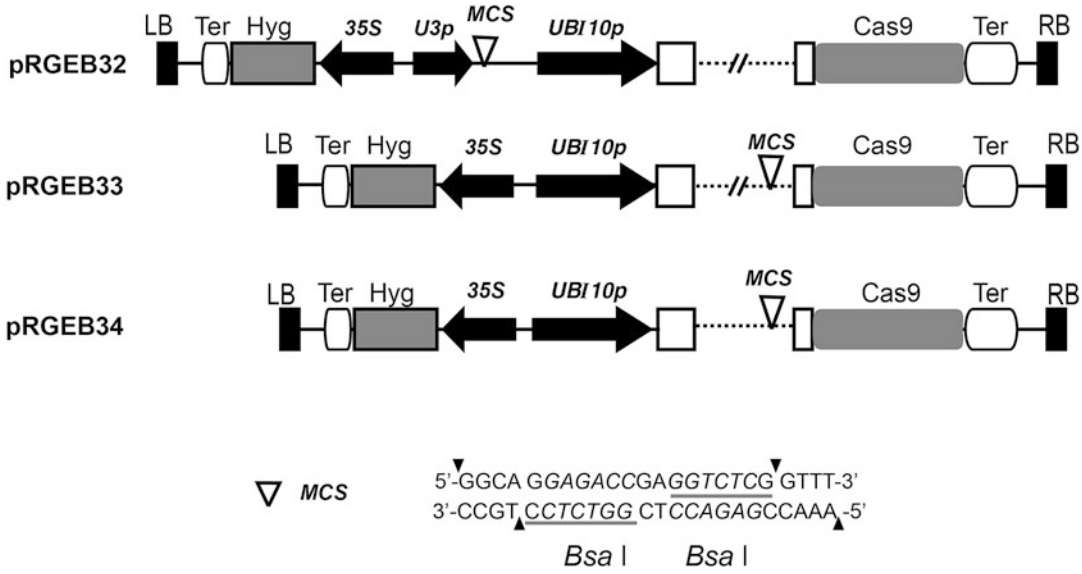

Fig. 2 Schematic illustration of plasmid vectors. All three vectors contain the same dual-BsaI site (indicated as MCS, multiple cloning site) for insertion of gRNA fragment. The pRGEB33 and pRGEB34 vectors allow the cloning of tRNA-gRNA polycistron within the intron at the 5′-UTR of the Cas9 gene. *LB* left border of T-DNA, *Ter* terminator, *Hyg* hygromycin B phosphotransferase gene, *35S* cauliflower 35S promoter, *U3* rice snoRNA U3 promoter, *UBI* rice ubiquitin gene promoter, *RB* right border of T-DNA

8. 5× Green GoTaq® reaction buffer (Promega Corporation, catalog number: M7911).
9. Alkaline phosphatase, calf intestinal (CIP) (New England Biolabs, catalog number: M0290S).
10. T4 DNA ligase (New England Biolabs, catalog number: M0202S).
11. QIAGEN plasmid midi kit (QIAGEN, catalog number: 12143).
12. Spin Column PCR Purification Kit (Bio Basic, catalog number: BS363).
13. Spin Column DNA Gel Extraction Kit (Bio Basic, catalog number: BS353).
14. Oligonucleotide primers. The design of specific gRNA sequence primers is described in Subheading 3. Below are the adaptor sequences used in this method.

L5AD-F	GCCA GGATG GGCAGTCTG GGCA acaaagcaccagtgg
L3AD-R	TAAC GGATG AGCGACAGC aaac aaaaaaaaaa gcaccgactcg
S5AD-F	GCCA GGATG GGCAGTCTG GGCA
S3AD-R	TAAC GGATG AGCGACAGC AAAC

3 Methods

3.1 Selection of Specific gRNA Spacer Sequences for Multiplex Genome Editing

Specific gRNA spacers are selected from a given genome sequence based on the PAM sequence (NGG) recognized by Cas9. In theory, any 20 bp DNA sequence before NGG could be used as a gRNA spacer. Due to the off-target risk of Cas9, however, bioinformatics prediction tools should be used for selection of highly specific gRNA spacer sequences. A number of bioinformatics programs such as CRISPR-Plant [22], CRISPR-P [23], and CRISPR-GE [24] have been developed and could be readily used to select specific spacer sequences for various plant species.

3.2 Design and Synthesis of Oligonucleotides for Polycistronic tRNA-gRNA Assembly

The Golden Gate (GG) cloning method is used to assemble PTG as illustrated in Fig. 3 (*see* **Note 1**). A pair of oligonucleotide primers is used to amplify each tRNA-gRNA unit for assembly of PTG. In this example, the 9th to 12th nucleotides of a 20 nt long spacer of gRNA[*x*] is chosen as the *Bsa*I overhang for GG cloning (Fig. 3a).

5′-N_1-N_2-N_3-N_4-N_5-N_6-N_7-N_8-[N_9-N_{10}-N_{11}-N_{12}]-N_{13}-N_{14}-N_{15}-N_{16}-N_{17}-N_{18}-N_{19}-N_{20}-3′
The primers should be:
gR[*x*]-F (Forward primer, anneal to 5′-end of gRNA scaffold):
5′-ta-*GGTCTC*-N-[$N_9N_{10}N_{11}N_{12}$]$N_{13}N_{14}N_{15}N_{16}N_{17}N_{18}N_{19}N_{20}$-gttttagagctagaa-3′
gR[*x*]-R (Reverse primer, anneal to 3′-end of pre-tRNA):
5′-cg-*GGTCTC*-N-[$N_{12}N_{11}N_{10}N_9$]$N_8N_7N_6N_5N_4N_3N_2N_1$-tgcaccagccggg-3′

Any four consecutive nucleotides in the spacer could be selected as overhangs after *Bsa*I digestion. This allows the selection of a specific overhang for each DNA part in GG assembly. The two 5′-terminal bases (shown in lowercase) are randomly added nucleotides to enhance *Bsa*I digestion of PCR products. The italic letters (*N*) indicate the reverse complementary sequence of targeting DNA site. The lowercase letters at the 3′-end indicate bases that anneal to gRNA (forward primer) or tRNA (reverse primer).

3.3 One-Step Assembly of PTG Containing Four tRNA-gRNA Units

A scalable Golden Gate cloning procedure is designed to assemble tRNA-gRNA fragments. In the following example, a total of four gRNAs are fused to tRNAs for generating a single PTG fragment. The same procedure could be used to generate PTG fragments with 1–6 gRNAs (*see* **Note 2**).

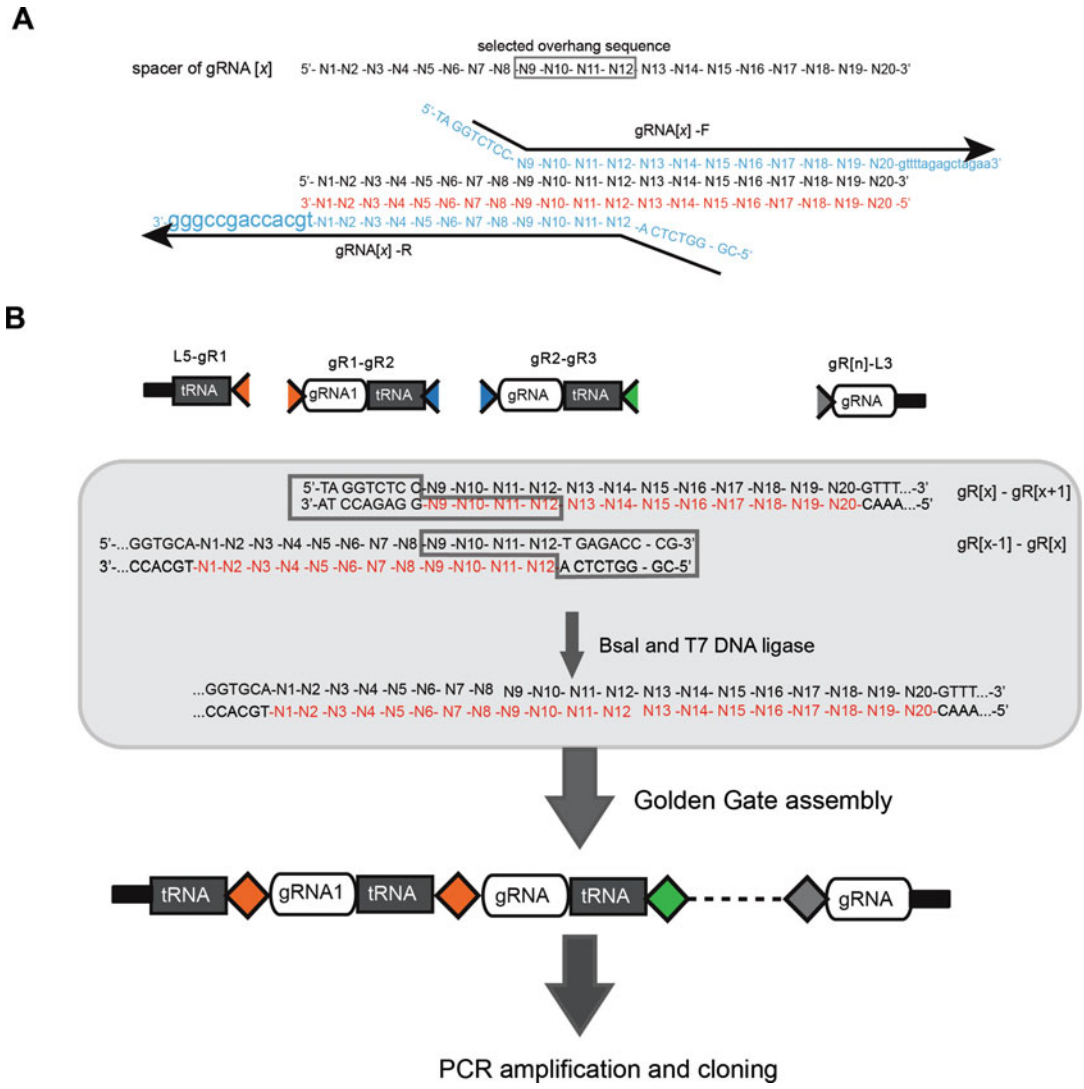

Fig. 3 Schematic illustration of Golden Gate cloning to assemble tRNA-gRNA fragment. (**a**) Design of specific gRNA sequence primers for Golden Gate assembly. (**b**) Golden Gate cloning steps to assemble the tRNA-gRNA fragment

1. Set up 50 μL PCR reactions to amplify DNA parts for PTG construction.

Reagent	Amount
pGTR (1 ng/μL)	1 μL
5× Phusion HF buffer	10 μL
dNTPs (10 mM)	1 μL
Forward primer (10 μM)	2.5 μL

(continued)

Reagent	Amount
Reverse primer (10 μM)	2.5 μL
Phusion polymerase (2 U/μL)	0.4 μL
H_2O	x μL
Total	50 μL

The primers for amplifying DNA parts are added as follows:

PCR ID	Forward primer	Reverse primer	Level 1 parts symbol
P1	L5AD5-F	gR1-R	L5-gR1
P2	gR1-F	gR2-R	gR1-gR2
P3	gR2-F	gR3-R	gR2-gR3
P4	gR3-F	gR4-R	gR3-gR4
P5	gR4-F	L3AD5-R	gR4-L3

Run PCR reactions with the following program:

Temperature	Time	Cycles
98 °C	2 min	1
98 °C	10 s	35
50 °C	20 s	
72 °C	20 s	
72 °C	2.5 min	1
4 °C	Hold	1

2. Check the PCR products by electrophoresis of 5 μL of PCR product in 2% agarose gel.
3. Purify the remaining PCR products with the Spin Column PCR Purification Kit. Determine the concentration of purified PCR products using a NanoDrop.
4. Prepare the GG reaction in a PCR tube as follows (*see* **Note 3**).

Level 1 parts	25–50 ng (add equal amount for each parts)
2 × T7 DNA ligase buffer	10 μL
Bovine serum albumin (1 mg/mL)	2 μL
*Bsa*I (10 U/μL)	0.5 μL

(continued)

T7 DNA ligase (3000 U/μL)	0.5 μL
Total volume	20 μL

5. Incubate the reaction mixture in a thermal cycler at 37 °C for 5 min and then 20 °C, 10 min for 50 cycles; finally, hold the reaction at 20 °C for 1 h.
6. Dilute the GG reaction product with 180 μL of H_2O, and then amplify the assembled PTG fragment using the terminal adaptor primers as follows (*see* **Note 4**).

GG reaction product (1:10 dilution)	1 μL
5× Go Green buffer (Promega)	10 μL
dNTPs (10 mM)	1 μL
S5AD5-F (10 μM)	1 μL
S3AD5-R (10 μM)	1 μL
GoTaq DNA polymerase (2 U/μL)	1 μL
H_2O	35 μL
Total	50 μL

Run PCR reaction in a thermal cycler with the following program:

Temperature	Time	Cycles
95 °C	2 min	1
95 °C	10 s	35
60 °C	20 s	
72 °C	1 min/kb	
72 °C	2.5 min	1
4 °C	Hold	1

7. Purify the PCR product with the Spin Column PCR Products Purification Kit.
8. Digest the amplified PCR product with *Fok*I.
9. Run electrophoresis of the *Fok*I-digested PCR product in 1% agarose gel. Excise the expected DNA fragment (approximate fragment size = no. of gRNA × 173 bp + 20 bp) from the gel, and purify it with the Spin Column DNA Gel Extraction Kit.

3.4 Preparation of PTG Constructs for Plant Transformation

1. Set up the *Bsa*I enzyme reaction for plasmid digestion as follows.

Plasmid (pRGEB32, pRGEB33, or pRGEB34)	5 μg
10× CutSmart Buffer (NEB)	5 μL
*Bsa*I (10 U/μL, NEB)	1 μL
Add H_2O to	50 L

2. Digest the plasmid at 37 °C for 3 h; then add 1 μL of CIP (10 U/μL) and incubate at 37 °C for 10 min.
3. Run the digested plasmid DNA in 0.8% agarose gel. Recover the digested plasmid vector using the Spin Column DNA Gel Extraction Kit.
4. Ligate the *Fok*I-digested GG fragment into the *Bsa*I-digested pRGEB32 (or pRGEB33) vectors with T4 DNA ligase.
5. Transform the ligation product to *E. coli* DH5α, purify the recombinant plasmids, and confirm the constructs by DNA sequencing.
6. The confirmed PTG constructs are prepared with the QIAGEN plasmid midi kit for transient gene expression using rice protoplasts or for stable transformation into rice cultivars such as Kitaake (*Oryza sativa* L. ssp. *japonica*) via the *Agrobacterium tumefaciens*-mediated method. The resulting transgenic rice lines are characterized using PCR, restriction enzyme digestion, DNA sequencing, and other genotyping methods. The expected editing efficiency for targeted mutagenesis in rice is about 50–100% in the primary transgenic lines.

4 Notes

1. The PTG fragment could also be synthesized using commercial gene synthesis service.
2. If the PTG construct contains more than six gRNAs, a two-step GG cloning approach is recommended to hierarchically assemble two PTG fragments.
3. The T7 DNA ligase has higher fidelity than T4 DNA ligase and is more efficient to ligate multiple DNA fragments in one reaction.
4. Based on our experience, regular Taq DNA polymerase is more efficient than high-fidelity DNA polymerase to amplify a PTG fragment containing more than three gRNAs.

Acknowledgments

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Chapter 6

A Single Transcript CRISPR-Cas9 System for Multiplex Genome Editing in Plants

Xu Tang, Zhaohui Zhong, Qiurong Ren, Binglin Liu, and Yong Zhang

Abstract

The CRISPR-Cas9 system has been widely adopted in genome editing. By changing the 20 bp guide sequence, it can easily edit any sequence adjacent to a protospacer adjacent motif (PAM) in a genome. Multiplex genome editing could be accomplished with simultaneous expression of multiple single-guide RNAs (sgRNA). Given that sgRNAs are expressed by Pol III promoters, multiplex genome editing is conventionally done by assembly of multiple complete sgRNA expression cassettes together, which can be a challenge in vector construction. Here, we described a multiplex genome editing system based on a single transcript unit CRISPR-Cas9 (STU CRISPR-Cas9) expression system driven by a single Pol II promoter. It represents a novel approach for multiplex genome editing.

Key words STU, CRISPR-Cas9, Multiplex genome editing

1 Introduction

The evolution in nature is fundamentally based on mutations of DNA. There are four basic ways to create mutations including physical and chemical mutagenesis, spontaneous mutagenesis, insertional mutagenesis, and targeted mutagenesis by genome editing. The physical and chemical mutagenesis and insertional mutagenesis, such as T-DNA insertion, are all random and uncontrollable [1]. The spontaneous mutagenesis is also random at low efficiency [2]. By contrast, genome editing can introduce mutations in a site-specific manner.

The genome editing tools include zinc-finger nucleases (ZFN), TALE nucleases (TALEN), and CRISPR nucleases [3]. The CRISPR (clustered regularly interspaced short palindromic repeats)-associated protein 9 (Cas9) system has been applied in genome editing with high efficiency in various species. This system includes Cas9 protein and single-guide RNA (sgRNA). The Cas9 protein is directed by a sgRNA for introducing precise DNA double-strand breaks. An NGG PAM is required for genome

Yiping Qi (ed.), *Plant Genome Editing with CRISPR Systems: Methods and Protocols*, Methods in Molecular Biology, vol. 1917, https://doi.org/10.1007/978-1-4939-8991-1_6,

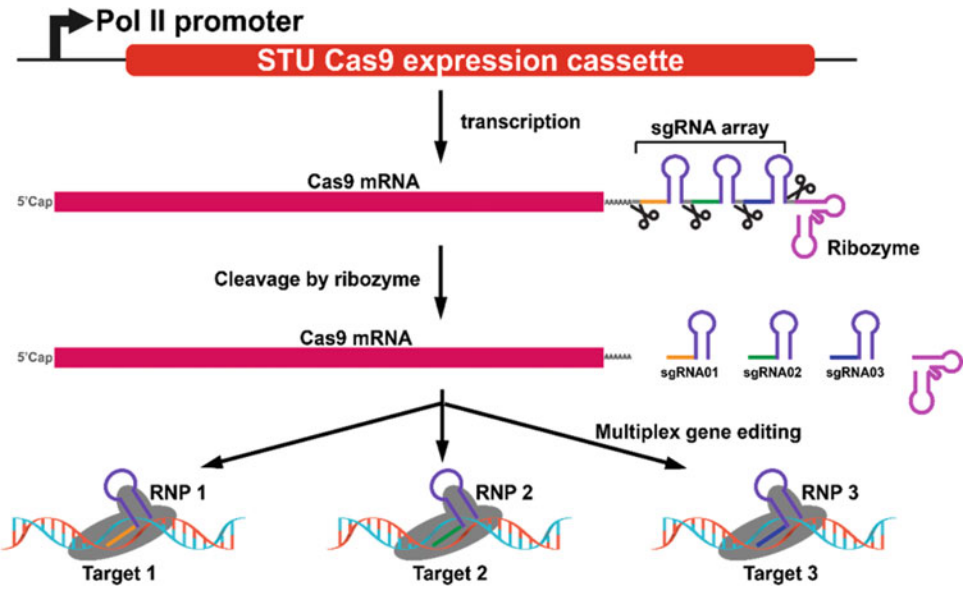

Fig. 1 Schematic illustration of multiplex genome editing using STU CRISPR-Cas9 system. RNA polymerase II catalyzes the transcription of the STU CRISPR-Cas9 primary transcripts. Once ribozyme is transcribed, it will cleave the STU CRISPR-Cas9 primary transcripts at its cleavage site. Cas9 can be targeted to different specific genomic loci by different sgRNAs. RNP: ribonucleoprotein

targeting by SpCas9, the most popular Cas9 used in genome editing [4]. The mutations created by CRISPR-Cas9 are mainly small insertions and deletions (indels) [5]. In some cases, such as noncoding sequence knockout, it may require simultaneous expression of two sgRNAs together with Cas9 to delete a chromosomal region. Such multiplex genome editing is also very useful for simultaneous editing of multiple trait genes in crop breeding, which drastically reduces the time and effort as compared to the conventional breeding. Typically, a CRISPR-Cas9 system uses Pol III promoters to transcribe sgRNAs. Hence, multiple sgRNA expression units need to be assembled into a single vector, which can be laborious and inconvenient. Here, we describe a multiplex CRISPR-Cas9 system utilizing ribozyme's self-catalyzed cleavage to process multiple sgRNAs (Fig. 1) [6]. In this system, the Cas9 and sgRNA arrays will be expressed as a single transcript unit (STU) under a single Pol II promoter. By using this method, researchers can easily achieve multiplex genome editing with high efficiency in different plant species.

2 Materials

1. DNA sequence analysis software for plasmid construction and primer design.
2. DNA oligonucleotides.

3. Taq DNA polymerase for colony PCR.
4. Q5® High-Fidelity DNA polymerase with buffer.
5. Restriction enzyme BsaI with buffer.
6. T4 DNA ligase with buffer.
7. Plasmids pTX171 and pTX172 (available from Addgene. Plasmid #89258 and Plasmid #89259, respectively).
8. LB medium: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L agar for solid medium.
9. SOC outgrowth medium: 20 g/L tryptone, 5 g/L yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose.
10. Kanamycin (filter sterilized 50 mg/mL stock; use at 50 mg/L).
11. Bacterial strains: *E. coli* ccdB resistant strain chemically competent cells, e.g., DB3.1 (Life Technologies Inc. for propagation of ccdB encoding vectors). *E. coli* DH5α chemically competent cells.
12. DNA gel extraction kit and plasmid miniprep kit.
13. NanoDrop spectrophotometer.
14. DNA gel electrophoresis equipment and supplies.
15. 50× TAE buffer: 242 g/L Tris, 57.1 mL/L acetic acid, 100 mL/L 0.5 M EDTA, pH 8.0.
16. Water bath and heat block.
17. Thermocycler.
18. 37 °C shaker and incubator.

3 Methods

3.1 Select sgRNA Targets for the Genes of Interest

Select appropriate sgRNA targets using any of the web-based sgRNA design tools such as CRISPR-P v2.0 (http://cbi.hzau.edu.cn/CRISPR2/), CRISPR RGEN tools (http://www.rgenome.net/cas-offinder/), and E-CRISP (http://www.e-crisp.org/E-CRISP/designcrispr.html). Make sure that the selected sgRNA targets are specific in the genome of interest to avoid possible off-targeting. Sequences containing a BsaI binding site should be avoided.

3.2 Generate sgRNA Array Using Two-Round PCR

Each sgRNA is flanked by a ribozyme cleavage site. The construction of sgRNA array requires two rounds of PCR amplification, as depicted in Fig. 2.

3.2.1 Generate an sgRNA Array

Design and synthesize DNA oligos (*see* **Note 1**) to generate an sgRNA array as shown below:

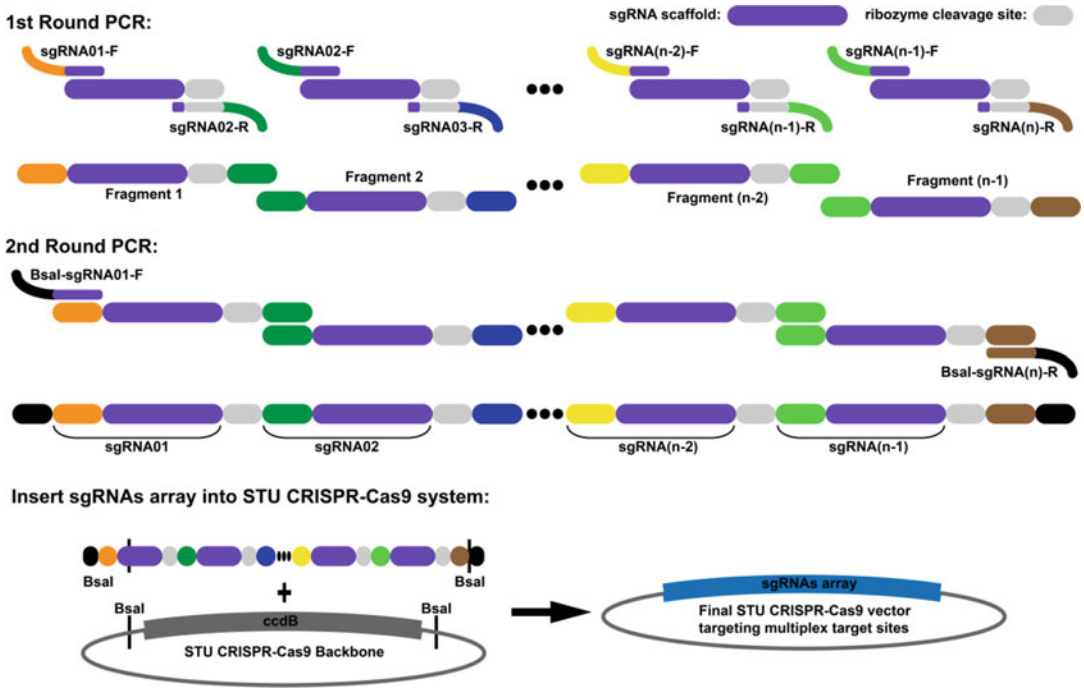

Fig. 2 Schematic illustration of the construction of STU CRISPR-Cas9 vector expressing multiplex sgRNAs

*Bsa*I-sgRNA01-F: 5′-CA**GGTCTC**ACGGA-$(N20)_1$-3′
sgRNA01-F: 5′-$(N20)_1$-gttttagagctagaaatagcaagttaa-3′
sgRNA02-F: 5′-$(N20)_2$-gttttagagctagaaatagcaagttaa-3′
sgRNA02-R: 5′-$(\underline{N20})_2$-tccggtgacaaaagcaccga-3′
. . .
sgRNA($n-1$)-F: 5′-$(N20)_{n-1}$-gttttagagctagaaatagcaagttaa-3′
sgRNA($n-1$)-R: 5′-$(\underline{N20})_{n-1}$-tccggtgacaaaagcaccga-3′
sgRNA(n)-R: 5′-$(\underline{N20})_n$-tccggtgacaaaagcaccga-3′
*Bsa*I-sgRNA(n)-R: 5′-TC**GGTCTC**CAAAC-$(\underline{N20})_n$-3′.

Here, "**GGTCTC**" is the *Bsa*I recognition sequence; "N20" is the same as the sgRNA-specific target sequence; "$\underline{N20}$" is the reverse complement of the sgRNA-specific target sequence. $(N20)_1$, $(N20)_2$ … $(N20)_{n-1}$ and $(N20)_n$ represent different sgRNA-specific target sequences. The lowercase letters are complementary to cloning sites of the STU CRISPR-Cas9 expression vector.

3.2.2 Generate the sgRNA Fragments Flanked by Ribozyme Cleavage Sites

1. Set up the first round PCR reactions as shown below for each primer pair (sgRNA01-F/sgRNA02-R, sgRNA02-F/sgRNA03-R. . .sgRNA ($n-1$)-F/sgRNA (n)-R). The number of reactions will be $n-1$ (n = number of sgRNA targets). Use pTX171 (STU CRISPR-Cas9 backbone plasmid) as template in all reactions.

5× Q5 reaction buffer	10 μL
10 mM dNTPs mixture	1 μL
10 μM forward primer	1 μL
10 μM reverse primer	1 μL
pTX171 (1 ng/μL)	1 μL
Q5 High-Fidelity DNA polymerase (2 U/μL)	0.5 μL
H_2O (to a final volume of 50 μL)	35.5 μL

2. Place the PCR reaction in a thermocycler and run the following program: 98 °C for 3 min, (98 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s) × 35 cycles, 72 °C for 3 min, 4 °C on hold.
3. Run 5 μL of each PCR product in an agarose gel to confirm successful amplification. Products of all reactions will be ~130 bp long.
4. Purify the PCR products using DNA gel extraction kit.
5. Quantify DNA concentration using a NanoDrop spectrophotometer.

3.2.3 Generate sgRNA Array

1. Set up the second round PCR reaction in 50 μL as shown below to fuse the sgRNA fragments to generate an sgRNA array:

5× Q5 reaction buffer	10 μL
10 mM dNTPs mixture	1 μL
BsaI-sgRNA01-F (10 μM)	1 μL
BsaI-sgRNA(n)-R (10 μM)	1 μL
Fragment 1 (20 ng/μL)	1 μL
Fragment 2 (20 ng/μL)	1 μL
...	...
Fragment $(n-1)$ (20 ng/μL)	1 μL
Q5 High-Fidelity DNA polymerase (2 U/μL)	0.5 μL
H_2O	Up to 50 μL

2. Place the PCR reaction in a thermocycler and run the following program: 98 °C for 3 min, (98 °C/30 s + 56 °C/30 s + 72 °C/30 s) × 35 cycles, 72 °C for 3 min, 4 °C on hold.
3. Run 5 μL of each PCR product on a gel to confirm successful amplification.
4. Purify the PCR products using DNA gel extraction kit.
5. Quantify DNA concentration using a NanoDrop spectrophotometer.

3.3 Insert the sgRNA Array into the STU CRISPR-Cas9 System

The cloning step can be performed either by conventional restriction cloning or Golden Gate reaction. Both approaches are described below.

3.3.1 Insert the sgRNA Array into the STU CRISPR-Cas9 Vector by Conventional Cloning (Option 1)

1. Digest the purified sgRNA array and STU CRISPR-Cas9 vector (pTX171 or pTX172) with BsaI as follows: Combine 1 μg of purified sgRNA array or 3 μg of STU CRISPR-Cas9 plasmid, 5 μL of 10× NEB CutSmart buffer, 1 μL of BsaI enzyme (10 U/μL), and H_2O to 50 μL. Incubate at 37 °C for 2–4 h. (*see* **Note 2**).
2. Run the digested products on 1% (w/v) agarose gel in TAE buffer.
3. Purify the digested sgRNA array and the linearized STU CRISPR-Cas9 vector using a DNA gel extraction kit. Quantify DNA concentration using a NanoDrop spectrophotometer.
4. Prepare a ligation reaction as follows: 1 μL of the digested sgRNA array (10 ng/μL), 1 μL linearized STU CRISPR-Cas9 vector (50–75 ng/μL), 2 μL T4 ligase buffer, 15 μL H_2O, and 1 μL T4 ligase. Incubate at 16 °C overnight or room temperature for 1–2 h.
5. Transform 5 μL of the reaction into 50 μL competent DH5α *E. coli* cells, and plate on LB plates supplemented with 50 mg/L kanamycin. Incubate at 37 °C overnight.
6. On the following day, check the clones by colony PCR with primers BsaI-sgRNA01-F and ZY065-RB (5′-ttctaataaacgctcttttctct-3′) (*see* **Note 3**).
7. Inoculate 1–2 correct clones into 5 mL of LB supplemented with 50 mg/L kanamycin. Incubate and shake overnight at 210 rpm at 37 °C.
8. Miniprep plasmid DNA from the LB cultures. Confirm by sequencing with primer ZY065-RB.

3.3.2 Insert the sgRNA Array into the STU CRISPR-Cas9 Vector by Golden Gate Reaction (Option 2)

1. Prepare a Golden Gate reaction as follows:

10× T4 DNA ligase buffer	2 μL
STU CRISPR-Cas9 vector (100 ng/μL)	1 μL
Purified sgRNA array (10 ng/μL)	1 μL
T4 DNA ligase (400 U/μL)	1 μL
BsaI (10 U/μL)	1 μL
H_2O	14 μL

2. Place the Golden Gate reaction in a thermocycler, and run the following program: (37 °C for 5 min, 16 °C for

10 min) × 10 cycles, 37 °C for 5 min, 80 °C for 10 min, 4 °C on hold (*see* **Note 4**).

3. Transform 5 μL of the reaction into 50 μL competent DH5α cells, and plate on LB plates supplemented with 50 mg/L kanamycin. Incubate at 37 °C overnight.
4. On the following day, check the clones by colony PCR with primers BsaI-sgRNA01-F and ZY065-RB (5′-ttctaataaacgctcttttctct-3′) (*see* **Note 3**).
5. Inoculate 1–2 correct clones into 5 mL of LB supplemented with 50 mg/L kanamycin. Incubate and shake overnight at 210 rpm at 37 °C.
6. Miniprep plasmid DNA from the LB cultures. Confirm by sequencing with primer ZY065-RB.

4 Notes

1. PAGE-purified oligos are highly recommended due to their high purity.
2. pTX171 and pTX172 use CaMV 35S promoter and maize ubiquitin 1 promoter to drive the expression of CRISPR-Cas9 single transcript unit, respectively. Both promoters are effective in rice.
3. The expected size will depend on the number of sgRNAs, which can be figured out by running an in silico cloning ahead of time.
4. The number of cycles can be increased to 15–20 times if the reactions are not efficient.

Acknowledgments

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Chapter 7

CRISPR-Act2.0: An Improved Multiplexed System for Plant Transcriptional Activation

Aimee Malzahn, Yong Zhang, and Yiping Qi

Abstract

CRISPR systems have greatly promoted research in genome editing and transcriptional regulation. CRISPR-based transcriptional repression and activation systems will be valuable for applications in engineering plant immunity, boosting metabolic production, and enhancing our knowledge of gene regulatory networks. Multiplexing of CRISPR allows multiple genes to be targeted without significant additional effort. Here, we describe our CRISPR-Act2.0 system which is an improved multiplexing transcriptional activation system in plants.

Key words CRISPR, dCas9-VP64, MCP-VP64, Plant transcriptional activation, Artificial transcription factor, Multiplex, Golden Gate assembly, Gateway cloning

1 Introduction

Genome engineering tools such as CRISPR-Cas9 have been instrumental in furthering basic and applied research. While CRISPR-Cas9 is most commonly used for creating DNA double-stranded breaks, it has also been utilized for a variety of transcriptional regulation purposes. Single or multiple genes can be repressed or activated utilizing a catalytically dead Cas9 paired with an activator, such as VP64, or a repressor. In plants, transcriptional activation has applications in plant immunity [1, 2], elucidation of regulatory networks, and increased production of plant metabolites [3, 4].

Improvements continue to be made to dCas9-based activation systems as new strategies are tested. One such strategy that we recently utilized for gene activation relies on an RNA-protein interaction from the bacteriophage MS2. The protein MCP binds to MS2 RNA stem loops. By changing the sequence of the structural guide RNA (gRNA) loops to create MS2 stem loops, the gRNA is able to recruit MCP-VP64 proteins to the binding site (Fig. 1). By creating a fusion protein of MCP and VP64, four VP64 proteins are recruited to the gRNA. This is in addition to the VP64 domain

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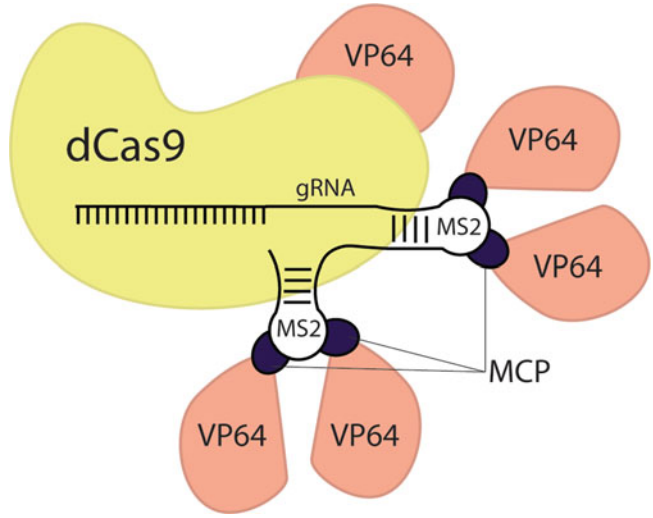

Fig. 1 Diagram of CRISPR-Act2.0 system. Four VP64 activators are recruited through MS2 stem loop and MCP RNA-protein interactions on gRNA2.0. VP64 is also fused to dCas9. In total, five activators are brought to the target site by one gRNA

already fused to dCas9 (Fig. 1) [5]. This new design is termed "CRISPR-Act2.0" and resulted in a three- to fourfold increase in activation when compared to our previous system that is solely based on dCas9-VP64 [6]. Here, the protocol for the assembly of multiplexed CRISPR-Act2.0 system is described, which utilizes Golden Gate and Gateway cloning to quickly produce a T-DNA vector without using polymerase chain reaction (PCR) (Fig. 2) [7]. The resulting T-DNA constructs can be used for transcriptional activation in monocots or dicots. As an example, we will assemble CRISPR-Act2.0 T-DNA vectors for transcriptional activation in *Arabidopsis*, a model dicot plant.

2 Materials

1. Sequences from genes of interest (e.g., *PAP1*, *FIS2*, *miR319* in *Arabidopsis*), collected from TAIR (https://www.arabidopsis.org) or IGB (http://bioviz.org/).
2. DNA editing software (e.g., Snapgene, ApE).
3. Synthetic guide RNAs (Integrated DNA Technologies).
4. Hot plate.
5. Medium to large glass beaker.
6. Vectors from Addgene (depending on your plant and overall design): pYPQ131A2.0 (99884), pYPQ131B2.0 (99885), pYPQ131C2.0 (99886), pYPQ131D2.0 (100044), pYPQ132A2.0 (99887), pYPQ132B2.0 (99888),

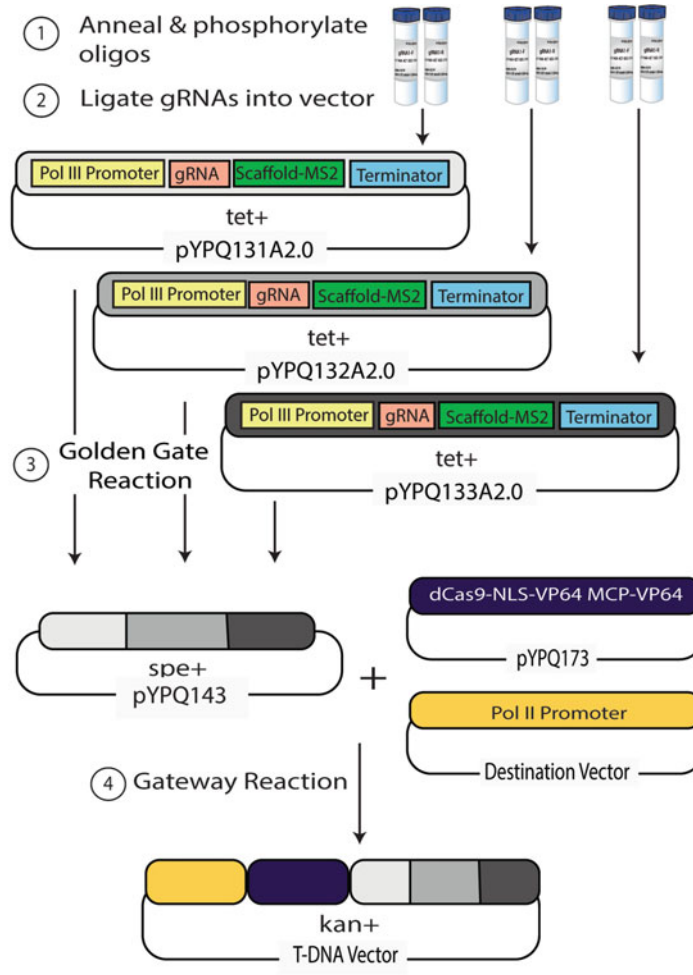

Fig. 2 Diagram of T-DNA vector construction. Step 1 depicts the annealing and phosphorylation of three gRNA pairs. In step 2, these gRNAs are each placed into a pYPQ13xA2.0 vector. Step 3 combines these gRNAs into a single gRNA unit through Golden Gate cloning. Finally, a Gateway assembly brings dCas9-VP64, gRNAs, and Pol II promoter together in a single T-DNA vector

pYPQ132C2.0 (99889), pYPQ132D2.0 (99890), pYPQ133A2.0 (99891), pYPQ133B2.0 (99892), pYPQ133C2.0 (99893), pYPQ133D2.0 (99894), pYPQ140 (99895), pYPQ142 (69294), pYPQ (69295), pYPQ141A2.0 (99896), pYPQ141B2.0 (99897), pYPQ141C2.0 (99905), pYPQ141D2.0 (99906), pYPQ173 (99907). Gateway-compatible attR1-attR2 destination vector of your choice.

7. Sequencing primers: pTC14-F2 as 5′-caagcctgattgggagaaaa-3′ (for pYPQ13x2.0 vectors), M13-F as 5′-cccagtcacgacgttg-taaaacg-3′ (for pYPQ141x2.0 vectors).
8. Restriction enzymes and buffers: BgIII, SalI, EcoRV, BsaI, NcoI, SpeI, XbaI, BamHI, EcoRI, and BsmBI (Esp31).

9. T4 polynucleotide kinase and T4 buffer.
10. Ligation reagents: T4 ligase and T4 10× buffer.
11. Invitrogen Gateway LR Clonase® II mix.
12. Sterile filtered 20 mg/mL of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in DMSO (dimethyl sulfoxide) and 0.1 M IPTG (isopropyl β-D-1-thiogalactopyranoside).
13. DH5α *E. coli* competent cells or comparable strain.
14. SOC liquid media: (2% w/v tryptone, 0.5% w/v yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulfate, 20 mM glucose).
15. LB media: (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v sodium chloride). For solid media, add 1.5% w/v agar.
16. Heat block or water bath.
17. Incubator, shaking and static.
18. Plasmid isolation kit (e.g., IBI high-speed plasmid mini kit).
19. NanoDrop spectrophotometer.
20. Centrifuge tubes: 1.7 and 2.0 mL.
21. Antibiotic stocks (sterile filtered): 10 mg/mL tetracycline in 50% ethanol; 100 mg/mL spectinomycin; 50 mg/mL kanamycin.
22. Gel electrophoresis materials: agarose, TAE or TBE buffer, gel box, casting tray, well combs, voltage source, and ethidium bromide.
23. 0.2 mL PCR strip tubes.
24. PCR purification kit (e.g., QIAquick gel extraction kit).
25. Culture tubes.
26. DTT (dithiothreitol), molecular biology grade.
27. Thermocycler.
28. Cryogenic tubes.
29. 50% glycerol.

3 Methods

3.1 T-DNA Assembly for Multiplexed Activation

1. Choose a gene of interest. Choosing what gene to target will depend on the goal of the research. Likely, the gene will come from previous interest in a specific area. If the goal is to test transcriptional activation efficiency, it is recommended to choose a gene with an easily discernable phenotype when activated or overexpressed (*see* **Note 1**). Promoter sequences, rather than coding sequencing, will be targeted for transcriptional activation (*see* **Note 2**).

Oligos	Sequence	Purpose
PAP1-gR1-top	GATTgacagctaatacataaaatg	1st gRNA targeting PAP1
PAP1-gR1-bottom	AAACcattttatgtattagctgtc	1st gRNA targeting PAP1
PAP1-gR2-top	GATTgaaaatttataaaaacacgtga	2nd gRNA targeting PAP1
PAP1-gR2-bottom	AAACtcacgtgtttttataaattttc	2nd gRNA targeting PAP1
PAP1-gR3-top	GATTggtataagtaaattggtca	3rd gRNA targeting PAP1
PAP1-gR3-bottom	AAACtgaccaatttacttatacc	3rd gRNA targeting PAP1

Fig. 3 Oligos for three sgRNAs targeting *PAP1* in *Arabidopsis*

2. Design gRNAs manually or with the help of an online tool. Guide RNAs can slightly differ in length, but a length of 20 bp is recommended. Target sites should be proximal to the transcriptional start site or near or within the promoter. To manually select gRNAs, search for the NGG PAM sequence and pick 20 bp upstream. Target sites on the reverse strand can be selected by searching for CCN PAM and picking 20 bp downstream. There are several online software programs that facilitate gRNA design, such as CRISPR-P2.0 [8]. More programs are listed by Graham and Root [9] that consider many aspects of gRNA design (*see* **Note 3**).
3. Compare gRNA to the rest of genome and look for off-target effects. In most plants, gRNAs with two or more mismatches to other sequences will not result in off-targeting. In polyploids or plants with repetitive genomes, such as maize, extra care may need to be taken to avoid off-targeting.
4. Add in overhangs for cloning and then order gRNAs as oligos. In the case of using *Arabidopsis* U6 (AtU6) promoter, 5′ 4 nt overhang sequences are (5′-GATTg-3′) for the forward gRNA oligo, and the reverse gRNA oligo is (5′-AAAC-3′) (Fig. 3). Overhang sequences for other gRNA expression promoters can be similarly designed based on the vector map.
5. Select required plasmids. Our toolkit is designed for both monocots and dicots, and users can choose between U3 and U6 promoters. Vectors with an "A" designation, such as pYPQ131A2.0, carry an AtU6 promoter. B= AtU3, C= OsU6, D= OsU3. A and B vectors are for dicots, while C and D are for monocots. If the design incorporates multiplexing, then pYPQ13x2.0 series vectors will be required. If only one gRNA is used, then this gRNA will be cloned directly into pYPQ141x2.0. These will be referred to as "gRNA expression plasmids" in the next step.
6. Digest guide RNA expression plasmids with 10 u of BgIII and SalI as follows. This step is optional, but recommended, as it will further prevent vector self-ligation. Digestion components are listed below. Incubate the reaction for 3 h to overnight at 37 °C. Purify digestion products with Qiagen QIAquick DNA

purification kit, and elute with 35 μL of molecular grade water or elution buffer (EB) (*see* **Note 4**).

First digestion		Example
gRNA plasmid (100 ng/μL)	2 μg	20 μL
10× NEB buffer 3.1	1×	4 μL
BgIII (10 u/μL·L)	10 u	1 μL
SalI-HF (10 u/μL)	10 u	1 μL
H_2O (molecular grade)	Up to 40 μL	14 μL
Total		40 μL

7. Digest the same gRNA expression plasmids with EPS31 as follows. Incubate the reaction at 37 °C overnight, and then incubate at 80 °C for 20 min to inactivate the enzymes. Purify using a PCR purification kit. DNA can be quantified with a NanoDrop spectrophotometer.

Second digestion		Example
Digested gRNA plasmid	~320 ng	32 uL
OPTIZYME 10× Buffer 4	1×	4
DTT (20 mM)	1 μL	1
EPS31 (10 u/μL)	10 u	1
H_2O (molecular grade)	Up to 40 μL	14
Total		40

8. While the plasmids are being digested, phosphorylate and anneal oligos as follows. Oligos are phosphorylated with a 30-min incubation at 37 °C. After phosphorylation, there are two options for annealing oligos. Oligos can be placed in a thermocycler with a program set to 95 °C for 5 min and then cooled down to 25 °C at 0.08 °C/s. Alternatively, the tubes can be secured to a floating rack and placed in boiling water. The water can be allowed to cool immediately after adding the oligos. We recommend the boiling water method for convenience.

Phosphorylation and annealing		Example
gRNA oligo forward (100 μM)	100 ng	1 μL
gRNA oligo reverse (100 μM)	100 ng	1 μL
10× T4 polynucleotide kinase buffer	1×	1 μL

(continued)

Phosphorylation and annealing		Example
T4 polynucleotide kinase (10 u/uL)	10 u	0.5 μL
H_2O (molecular grade)	Up to 10 uL	6.5
Total		10 μL

9. Ligate oligos into linearized gRNA expression vectors as follows. Before ligation, 1 μL of annealed oligos from the previous step should be diluted in 199 μL of molecular grade water. Incubate ligation at room temperature for 1 h to overnight. Transform 50 μL of competent *E.coli* DH5α with 10 μL of ligation reaction, and plate onto tetracycline (10 μg/μL) LB plates. Incubate plates overnight at 37 °C (*see* **Note 5**).

Ligation reaction		Example
Diluted annealed oligos (1:200)	–	1 μL
Linearized plasmid	–	1 μL
10× NEB T4 ligase buffer	1×	1 μL
T4 ligase	200 u	0.5 μL
H_2O (molecular grade)	Up to 10 uL	6.5
Total		10 μL

10. Select two or more colonies from each plate, and culture at 37 °C overnight in 5 mL of autoclaved LB broth with tetracycline (*see* **Note 6**).
11. Miniprep cultures using a plasmid isolation kit to extract the vector from 4 mL of LB broth.
12. Use Sanger sequencing to determine whether the gRNA was properly inserted. Use primer TC14-F2 for pYPQ13x2.0 vector series and primer M13-F for pYPQ141x2.0.
13. If multiple gRNAs are used, a Golden Gate reaction will place all gRNA expression cassettes from pYPQ13x2.0 vectors into a pYPQ14x vector in preparation for the Gateway reaction. If two gRNAs in pYPQ131x2.0 and pYPQ132x2.0 need to be assembled, vector pYPQ142 will be used. If three gRNAs need to be assembled, pYPQ143 will be used and so on. For the assembly of three gRNA vectors, set up a reaction as follows. Golden Gate reactions will be carried out in a thermocycler according to the procedure: 10× (37 °C for 5 min, 16 °C for 10 min), 50 °C for 5 min, 80 °C for 5 min, hold at 10 °C.

Golden Gate reaction		Example
10× T4 DNA ligase buffer	1×	1 μL
T4 DNA ligase	200 u	1 μL
BsaI	5 u	0.5 μL
pYPQ143	100 ng	0.5 μL
pYPQ131A2.0-gRNA1	100 ng	1 μL
pYPQ132A2.0-gRNA2	100 ng	1 μL
pYPQ133A2.0-gRNA3	100 ng	1 μL
H_2O (molecular grade)	Up to 10 μL	4 μL
Total		10 μL

14. Transform 50 μL of competent *E. coli* DH5α and plate onto spectinomycin (100 μg/mL) LB plates. A blue-white screen can be applied by adding 75 μL of 0.1 M IPTG and 75 μL of 20 mg/mL X-gal to the plates, spreading evenly, and allowing the chemicals to dry (1–2 h) before adding the transformed cells.
15. Select two colonies, miniprep, and digest with BamHI and EcoRV in CutSmart buffer. Run the digestion on a 0.8% agarose gel to confirm a successful Golden Gate reaction. This will be the gRNA entry vector for the following Gateway reaction.
16. Assemble Gateway reaction components for the final T-DNA vector production as follows. Dilute vectors in sterile molecular grade water as necessary. Incubate at room temperature overnight (*see* **Note 7**).

Gateway reaction		Example
Cas9 entry vector pYPQ173	50 ng	2 μL (40 ng/μL)
gRNA entry vector	50 ng	2 μL (40 ng/μL)
Destination vector	200 ng	2 μL (100 ng/μL)
LR Clonase II	1 μL	1 μL
Total		7 μL

17. Transform competent *E. coli* DH5α and plate onto kanamycin (100 μg/mL) LB plates. Incubate overnight at 37 °C and pick 2–3 colonies to culture in LB broth.
18. Miniprep cultures using a plasmid isolation kit to extract the vector from 4 mL of LB broth. Refrigerate extra culture and save for glycerol stock.

19. Digest Gateway-assembled T-DNA vectors with EcoRI. Run the digestion on a 1% agarose gel to confirm a successful assembly.
20. Make glycerol stocks of correct vectors. Inoculate 4 mL of fresh LB kan + broth with 5 μL of old culture and incubate overnight. Mix 500 μL of culture with 500 μL of 50% glycerol into a cryogenic tube for storage at −80 °C.

3.2 Higher-Order Assembly for Multiplexing

In this example, a total of nine gRNAs will be assembled through an additional cloning step. Between two and eight gRNAs make up a "unit" previously assembled into a pYPQ14x2.0 series vector through Golden Gate assembly. Units will be assembled through the compatibility of XbaI and SpeI restriction sites. This method can be used to bring together four or more gRNA cassettes more efficiently than a Golden Gate assembly.

1. Simultaneously digest 1 μg of pYPQ143-gR1,gR2,gR3 "donor" entry clone with NcoI and SpeI and 1 μg of pYPQ143-gR4,gR5,gR6 "acceptor" entry clone with NcoI and XbaI. Bring the reaction to 50 μL with sterile water and incubate overnight at 37 °C.
2. Gel purify the donor gRNA unit 1 using 0.8% agarose gel. Column purify the acceptor gRNA vector with the 13 bp sequence removed (*see* **Note 8**).
3. Mix 3 μL of digested and purified gRNA units with 1 μL of T4 DNA ligase buffer and 0.5 μL of T4 DNA ligase. Bring the reaction to 10 μL with sterile water, and leave at room temperature overnight (*see* **Note 9**).
4. Use all 10 μL of ligation reaction to transform chemically competent *E. coli*, and plate to LB spectinomycin. Incubate plates overnight at 37 °C.
5. Pick 2–4 colonies for overnight culture in LB broth with spectinomycin. Miniprep and digest, using XbaI and SpeI, to confirm. Sequence confirm with primer M13-F.
6. Repeat **steps 2–6** to insert an additional gRNA unit. The vector confirmed in the previous step will act as the donor vector, and the additional gRNA unit will act as an acceptor.
7. Once all gRNA units are in a single vector, assemble a Gateway recombination following **steps 15–19** in Subheading 3.1.

4 Notes

1. Transcriptional upregulation of some genes may result in an obvious phenotype. For example, purple leaves may be caused by upregulation of *PAP1*, which is involved in anthocyanin

production. However, posttranscriptional gene silencing (PTGS) may result in plants without a phenotype even when the transcriptional activation system works. We also hypothesize that there is an activation limit for certain genes. Beyond this limit, PTGS and small interfering RNAs may silence transcriptional activation [6].

2. Here is an example of the three gRNAs targeting *PAP1* (TAIR #AT1G56650):

   PAP1: gRNA1 is −188 bp from ATG start codon on exon 1.

   PAP1: gRNA2 is −129 bp from ATG start codon on exon 1.

   PAP1: gRNA3 is −43 bp from ATG start codon on exon 1.

3. A good gRNA will have few potential off-target sites. If the closest off-target site has at least two nucleotide mismatches close to the PAM, site off-targeting is not likely according to our recent study [10]. GC content should also be between 30 and 80% [11].
4. If problems are encountered, take note of the ingredients listed in the elution buffer. EDTA is present in TE buffers and in larger concentrations can inhibit enzymatic reactions.
5. Plates should be taken out of the incubator after overnight incubation. If left in the incubator, colonies may grow into one another, and it can be difficult to pick individual colonies. Plates can be stored at room temperature for a few days or in 4 °C for weeks to months.
6. Autoclaved LB broth can be kept in sealed containers at room temperature. LB broth with antibiotics can be stored at 4 °C for several months.
7. Note the similar sizes of the gRNA unit and the backbone. Be careful to fully separate these bands. The acceptor gRNA can also be gel purified. However, since most columns will not capture the 13 bp sequence, direct column purification will result in a higher yield than gel purification. Check the specificities for your columns of choice.
8. We often carry out ligation reactions overnight, but 2 hours is sufficient to obtain results. We also leave ligations at room temperature, but incubating at 16 °C can resolve issues with efficiency, should they arise.
9. We recommend allowing the Gateway reactions to incubate overnight, but successful reactions can be obtained with 2 h incubation.

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Part IV

CRISPR-Cas9 Editing in Monocots

Chapter 8

Generating Photoperiod-Sensitive Genic Male Sterile Rice Lines with CRISPR/Cas9

Weihang Gu, Dabing Zhang, Yiping Qi, and Zheng Yuan

Abstract

Obtaining photoperiod-sensitive genic male sterility (PGMS) lines is one of the most important steps in two-line hybrid rice breeding. Traditionally, such lines were screened and developed with a classic rice breeding system under both long-day and short-day conditions. The isolation and backcross process used for this could easily last for more than 3 years with a very low success rate. Here, we describe a straightforward method for generating *csa*-based PGMS lines by using the CRISPR-Cas9 technology in rice.

Key words Photoperiod-sensitive genic male sterility (PGMS), Two-line hybrid rice, *Carbon starved anther* (*csa*), CRISPR-Cas9, Agrobacterium-mediated transformation, Transgene-free

1 Introduction

The breakthrough of hybrid rice breeding technologies, including the three- and two-line hybrid rice systems, greatly relies on the extent of heterosis and application of the male sterile lines [1]. In the two-line hybrid rice system, photoperiod-sensitive genic male sterility (PGMS) and temperature-sensitive genic male sterility (TGMS) lines are most widely used, which can be utilized to propagate itself under short-day or low-temperature (e.g., 24 °C) conditions and to produce hybrid seeds by interplanting it with normal fertile lines under long-day or high-temperature (e.g., 29 °C) conditions. In China, the majority of *japonica* PGMS lines in the two-line system are derived from the PGMS line Nongken 58S (NK58S), which was firstly discovered in 1973 exhibiting complete male sterility under long-day (LD) conditions and restoring complete or partial fertility under short-day (SD) conditions. However, the reversibility of male fertility in the widely applied *indica* lines Peiai 64S and GD-1S, derived from NK58S, is also affected by temperature, which has largely limited their application in two-line hybrid rice production [2]. On the other hand, more than 95% male sterile lines used in two-line hybrid rice seed

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production were homozygous lines with the TGMS allele (*tms5*) [3]; all of them may suffer from unpredictable temperature changes resulting in contamination of self-hybrid seeds in the seed production process [4]. Therefore, targeted isolation of new PGMS or reverse PGMS (rPGMS) lines resistant to temperature perturbation is important in two-line hybrid rice seed production [5].

Several years ago, we isolated a rPGMS line *carbon starved anther* (*csa*) [6], which displayed male sterility under SD conditions and male fertility under LD conditions with a daily average temperature of 24–28 °C [7]. More importantly, *csa* could be used in two-line hybrid seed production by traditional rice breeding programs [7]. By the application of the newly developed CRISPR-Cas9 technology [8, 9], we recently created one rPGMS line 9522^{csa} by targeted genome editing of the *CSA* gene in *japonica* rice. Compared to traditional rice breeding, this genome editing approach for generating rPGMS rice lines is more reliable and faster, which can be finished within 1.5 years [5]. The protocol described here uses one CRISPR-Cas9 system for inducing targeted mutations at *CSA* in rice.

2 Materials

Prepare all the solutions with ultrapure water and analytical reagents. Prepare and store all reagents at room temperature unless otherwise noticed.

2.1 Vector Construction

1. CH-CRISPR-Cas9 [8] and pCAMBIA1300.
2. 96-well PCR plates.
3. Sterile water.
4. Thermocycler.
5. Oligonucleotide primers:

   For cloning of the single-guide RNA (sgRNA):

   CH-OsCSA-F: 5′-TGGCATGGCTCACGAGATGATGGG-3′ (phosphorylated)

   CH-OsCSA-R: 5′-AAACCCCATCATCTCGTGAGCCAT-3′ (phosphorylated)

   For diagnostic PCR to confirm cloning of the sgRNA:

   M13F: 5′-TGTAAAACGACGGCCAGT-3′.

   CH-OsCSA-R: 5′-AAACCCCATCATCTCGTGAGCCAT-3′.

   For Sanger sequencing to confirm cloning of the sgRNA:
   M13F: 5′-TGTAAAACGACGGCCAGT-3′.

   P2: 5′-GCGATTAAGTTGGGTAACGC-3′.

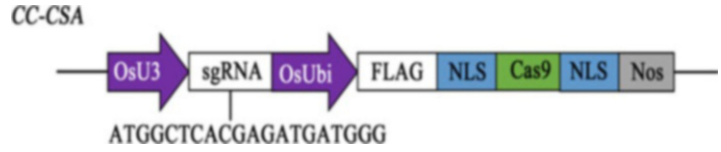

Fig. 1 Diagram of the CRISPR/Cas9 vector *CC-CSA*. Targeted mutagenesis of the *CSA* gene by the CH-CRISPR/Cas9 system. Note that the target sequence (5′-ATGGCTCACGAGATGATGGG-3′) is located at 1–20 bp in the first exon of the *CSA* gene

6. Restriction enzymes *Bbs*I, *Hind*III-HF, *Eco*RI-HF and buffers.
7. LB medium: 10 g/L tryptone, 5 g/L yeast extraction, 10 g/L NaCl.
8. Competent cells of *Escherichia coli* DH5α.
9. 42 °C water bath.
10. Selective LB agar plates supplemented with kanamycin with working concentration of 50 μg/mL, autoclaved in advance (*see* **Note 1**).
11. Centrifuge tube with a volume of 1.5 mL.
12. Glass spreading rod.
13. 96-well PCR plates.
14. 37 °C incubator.
15. Taq DNA Polymerase and buffer (*see* **Note 2**).
16. 2.5 mM dNTPs.
17. 1% agarose gel.
18. Gel purification kit.
19. Plasmid miniprep kit.
20. T4 DNA ligase and buffer (Fig. 1).

2.2 Plant Transformation and Growth

1. Seeds of rice variety: 9522.
2. Rice husker and concussion incubator.
3. 33% sodium hypochlorite.
4. Tween 20.
5. Sterile water.
6. Two-layer filter papers.
7. NBD_2 medium: 0.5 g/L casein hydrolysate, 30 g/L sucrose, 0.1 g/L inositol, 0.5 g/L L-Pro, 0.5 g/L L-GIn, 50 mL/L N6 stock solution I (56.6 g/L KNO_3, 9.26 g/L $(NH_4)_2SO_4$, 8 g/L KH_2PO_4), 25 mL/L N6 stock solution II (3.32 g/L $CaCl_2{\cdot}2H_2O$), 25 mL/L N6 stock solution III (3.7 g/L $MgSO_4{\cdot}7H_2O$), 10 mL/L B5 micronutrient I, 1 mL/L B5 micronutrient II (250 mg/L $Na_2MoO_4{\cdot}2H_2O$, 25 mg/L

$CoCl_2 \cdot 6H_2O$, 25 mg/L $CuSO_4 \cdot 5H_2O$), 10 mL/L B5 vitamin stock solution I (100 mg/L pyridoxine hydrochloride, 100 mg/L thiamine hydrochloride, 100 mg/L niacin), 10 mL/L B5 vitamin stock solution II (200 mg/L Gly), 10 mL/L 2,4-D, 50 mL/L 200× ferric salt, pH 5.8.

8. AAM-AS medium: 100 mL/L AA micronutrient stock solution, 10 mL/L AA micronutrient I stock solution, 1 mL/L AA micronutrient II stock solution, 100 mL/L AA stock solution of amino acid, 5 mL/L stock solution of ferric salt, 10 mL/L MS stock solution of vitamin, 0.5 mg/L casein hydrolysate, 2.94 g/L KCl, 36 g/L glucose, 0.1 g/L inositol, 68.5 g/L sucrose, 200 μL/L AS (acetosyringone), pH 5.2.
9. MS-H: 4.4 g/L MS powder, 500 mg/L casein hydrolysate, 2 mg/L 6-BA, 0.5 mg/L KT, 0.5 mg/L NAA, 30 g/L sucrose, 15 g/L sorbitol, 50 mg/L hygromycin, pH 5.8 (*see* **Note 2**).
10. MSNH: 2.2 g/L MS powder, 30g/L sucrose, pH 5.8.
11. YEB medium: 5 g/L beef extract, 1 g/L yeast extract, 5 g/L peptone, 5 g/L sucrose, 4 g/L $MgSO_4 \cdot 7H_2O$, 15 g/L agar, pH 7.2.
12. Competent cells of *Agrobacterium tumefaciens* EHA105.
13. Growth chamber: light intensity of 1000 μ moles/m^2/s; temperature at 18–25 °C.

2.3 Genotyping and Phenotyping

1. CTAB buffer: 20 g/L CTAB, 81.76 g/L NaCl, 12.1 g/L Tris-base, 7.44 g/L EDTA-2Na.
2. PCR and sequencing primers:

   For amplifying the target gene:

   OsCSA-F: 5′-ACCCCATTTTGCCGTTCG-3′.

   OsCSA-R: 5′-CGGCGATGAGGTTCCAGTTC-3′.

   For amplifying the CRISPR-Cas9 vector backbone:
   M13F: 5′-TGTAAAACGACGGCCAGT-3′.

   CH-OsCSA-R: 5′-AAACCCCATCATCTCGTGAGCCAT-3′.

   CAS9-CH-F: 5′-CGATAAGAACCTGCCCAACG-3′.

   CAS9-CH-R: 5′-GCTCTTTGATGCCCTCTTCG-3′.

3. Taq DNA Polymerase and buffer.
4. 96-well PCR plates.
5. Thermocycler.
6. 2.5 mM dNTPs.
7. Sterile water.

8. Potassium iodide solution (I_2-KI): 3.3 g/L I_2, 6.6 g/L KI.
9. Nikon E995 digital camera, Leica S8 APO stereomicroscope (Leica Microsystems), Leica DM2500 microscope.

3 Methods

3.1 Vector Construction (see Note 3)

1. Choose a target site of *CSA* in the first exon, which is close to the mutation position formerly verified in the rPGMS line *csa* [6]. The sequence 5′-ATGGCTCACGAGATGATGGG-3′ is chosen in this case.
2. Anneal phosphorylated oligo pair: CH-OsCSA-F and CH-OsCSA-R. Mix 1 μL of 100 μM of oligo pair in 10 μL reaction, and heat the reaction in boiling water, and let it cool to room temperature gradually.
3. Linearize 1 μg CH-CRISPR-Cas9 vector with *Bbs*I, and then run the digested reaction in 1% agarose gel, and purify the vector backbone using a gel purification kit.
4. Set up a 10 μL ligation reaction containing 1 μl 1:200 diluted annealed phosphorylated oligo pair and 1 μl gel-purified linearized vector to generate the entry vector.
5. Digest the entry vector by *Hind*III-HF and *Eco*RI-HF to generate the entry cassette, and run the digested reaction in 1% agarose gel, and purify the entry cassette using a gel purification kit.
6. Linearize 1 μg pCAMBIA1300 vector with *Hind*III-HF and *Eco*RI-HF, and then run the digested reaction in 1% agarose gel. Purify the vector backbone using a gel purification kit.
7. Set up a 10 μL ligation reaction to clone the Cas9-sgRNA cassette into linearized pCAMBIA1300 vector to generate the destination vector *CC-CSA*.
8. Thaw 60 μL of *E. coli* competent cells on ice for 20 min.
9. Dispense 30 μL of competent cells to each sterile 1.5 mL centrifuge tube.
10. Add 2 μL ligation reaction into each tube with 30 μL of competent cells, and pipette up and down to mix gently.
11. Incubate the DNA-cell mixture on ice for half an hour.
12. Heat shock the competent cells by floating the tubes at 42 °C water bath for 90 s (*see* **Note 4**).
13. Return the centrifuge tube to ice immediately and wait for 5 min.
14. Add 600 μL of LB Broth into each centrifuge tube and mix gently.

15. Recover the transformed cells by incubating at 37 °C and shaking at 220 rpm for 1 h.
16. Centrifuge at 3500 × *g* using a microcentrifuge for 1 minute, discard 500 μL LB Broth, and suspend the competent cells with the remaining 100 μL LB Broth.
17. Transfer the competent cells with a pipette to selective LB agar plates with kanamycin. Evenly distribute the cells across the entire plate with glass spreading rod.
18. Cover the lid, invert the plate, and incubate the plates overnight (12–14 h) in a 37 °C incubator to grow bacterial colonies (*see* **Note 5**).
19. Screen colonies by diagnostic PCR with primers M13F and CH-OsCSA-R.
20. Plasmid extraction using a miniprep kit.
21. Sanger sequencing using primers M13F and/or P2 to confirm successful cloning of the vector.
22. Make *E. coli* glycerol stock of correct clones for future use.

3.2 Agrobacterium Transformation

1. Thaw 60 μL of competent cells of *Agrobacterium* EHA105 on ice for 20 min. Dispense 30 μL of competent cells to each 1.5 mL volume sterilized centrifuge tube.
2. Transfer 2 μL of each of the two T-DNA binary vectors into centrifuge tubes with 30 μL of competent cells, and pipette up and down gently to mix DNA with the competent cells.
3. Incubate the DNA-cell mixture on ice and liquid nitrogen for 5 min, and perform heat shock of competent cells with the centrifuge tube floating in a 37 °C water bath for 5 min.
4. Return the centrifuge tube to ice immediately and wait for 5 min. Add 600 μL YEB into each centrifuge tube and mix gently. Recover the transformed cells by incubating at 28 °C and shaking at 220 rpm for 2 h. Centrifuge for 1 min at 6000 rpm, discard 500 μL YEB, and suspend the competent cells with the rest of YEB. Transfer the competent cells with a pipette to selective YEB agar plates, and evenly distribute the cells across the entire plate with glass spreading rod. Cover the lid, invert the plate, and incubate the inverted plates for 2–3 days in a 28 °C incubator to grow *Agrobacterium* colonies.
5. Screen colonies by diagnostic PCR, and pick successfully the transformed *Agrobacterium* for plant transformation.

3.3 Rice Transformation

1. Dehusk the seeds of rice variety (9522) and avoid any injury of the pericarp.

2. Sterilize dehusked seeds in 70% ethanol with gentle shaking for 1 minute. Pour the 70% ethanol off, and sterilize by adding 33% sodium hypochlorite containing Tween 20 for 20 min with vigorous shaking (about 220 rpm) twice. Rinse thoroughly with sterile water until they were out of extraneous odor. Dry the sterilized seeds on sterile filter paper.
3. Transfer sterilized seeds to NBD_2 medium, and incubate at 26 °C under dark condition for 3 weeks (*see* **Note 6**).
4. Excise all the roots and endosperms. Subculture for further 10 days to develop calli from mature embryos.
5. Inoculate *Agrobacterium* EHA105-*CC-CSA* in YEB liquid medium supplemented with both kanamycin (50 μg/mL) and rifampicin (20 μg/mL). Culture at 220 rpm at 28 °C until OD600 reaches 0.6 to 0.8. Collect *Agrobacterium* cells by centrifugation at 6200 × *g* at 4 °C for 10 min. Suspend bacterial pallet with AAM liquid medium supplemented with 100 μM/L acetosyringone.
6. Immerse active growing calli (yellowish white, relatively dry, and about 1–3 mm in diameter) [10] in the recombinant EHA105-*CC-CSA* suspension for 20 minutes. Transfer the calli into co-cultivation medium with two-layer filter papers (*see* **Note 7**), and co-culture the calli with agrobacterium under dark conditions at 25 °C for 3 days [11].
7. Wash the calli twice in sterile water containing 50 μg/mL Timentin to remove *Agrobacterium* after 3 days of co-cultivation. Transfer the co-cultivated calli to selective NBD_2 medium with hygromycin and Timentin. Repeat resistance selection for two times in the *Agrobacterium*-mediated rice transformation progress under dark condition at 28 °C.
8. Distinguish the calli actively growing in the medium from nontransformed calli [10], and move them to differential medium twice for differentiation with both hygromycin- and Timentin-resistant selections after selection culture in NBD_2 medium. Transfer new seedlings to regeneration medium for shoot generation, and move them to a growth chamber when they grow up (*see* **Note 8**) (Fig. 2).

3.4 Identification of Cas9-Generated Transgene-Free Mutants

1. Extract genomic DNA from wild-type (rice variety 9522) control plants and T0 transgenic plants, respectively, by a modified CTAB (cetyltrimethyl ammonium bromide) method.
2. Amplify the target sequence by PCR with primers OsCSA-F and OsCSA-R and genotype for mutagenesis at the CSA target site with Sanger sequencing with the same primers.

Fig. 2 Diagram of major steps of rice transformation. After co-cultivation, the calli are transferred to selective NBD_2 medium with hygromycin and Timentin resistance selection for two times under dark condition at 28 °C. Then, the calli actively growing in the selective medium are moved to differential medium twice for differentiation with both hygromycin- and Timentin-resistant selections. When new seedlings appeared, they are transferred to regeneration medium for inducing root generation

3. Identify heterozygous or homozygous T0 lines, and grow them to maturity for seeds of T1 generation in a growth chamber.
4. Grow T1 generation in a growth chamber. Genotype individual T1 plants to identify homozygous *9522$^{csa\text{-}1}$* mutant lines following the procedure as described in Subheading 3.4.
5. For identified homozygous lines, further genotype them with three PCR primer pairs (M13F and CH-OsCSA-R, 35s-F and 35s-R, CAS9-CH-F and CAS9-CH-R) specific to the CRISPR-Cas9 vector to identify transgene-free *9522$^{csa\text{-}1}$* homozygous T1 mutant lines.
6. Harvest T2 generation seeds from the transgene-free homozygous T1 mutant lines.

3.5 Phenotypic Characterization of csa Mutants

1. Use a previously identified *csa* mutant as a positive control [7]. Grow the transgene-free T2 *9522$^{csa\text{-}1}$* mutant lines along with the wild type and the positive control plants in a growth chamber with photoperiodic treatment as follows: the daily average temperature is 28 °C, and the day length for long-day (LD) is 14.0 h and for short-day (SD) is 10.5 h.
2. Photograph the morphology of panicles and spikelets of the transgene-free *csa* lines and controls with a Nikon E995 digital camera and a Leica S8 APO stereomicroscope (Leica Microsystems), respectively.
3. Sample anthers of the transgene-free *csa* lines and control plants carefully from three mature flowers at stage SP13 in line with the definition by Zhang and Wilson for pollen fertility

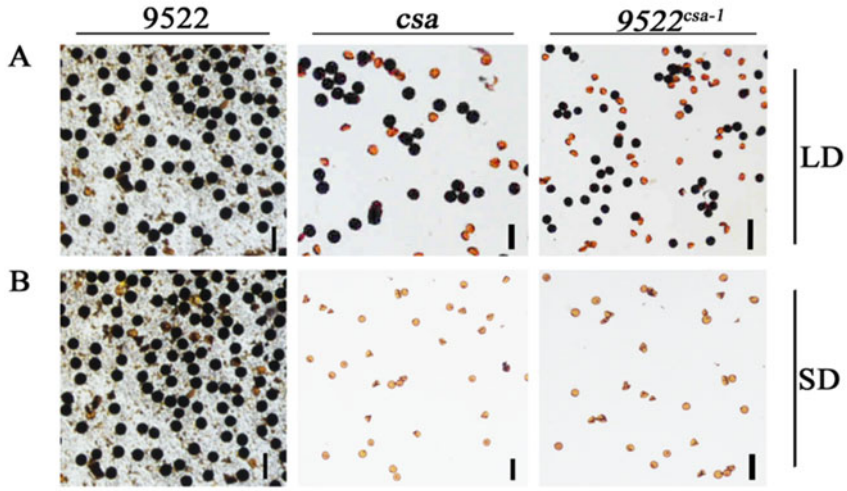

Fig. 3 Pollen fertility test of 9522, *csa*, and *9522*$^{csa-1}$. (**a**) I_2-KI staining of pollen grains of 9522, *csa* mutant, and *9522*$^{csa-1}$ lines under a LD condition. The pollens of *9522*$^{csa-1}$ lines generated by CRISPR/Cas9 are semi-sterile under a LD condition, and this phenotype is similar to the control *csa* mutant identified through random mutagenesis earlier. (**b**) I_2-KI staining of pollen grains of 9522, *csa* mutant, and *9522*$^{csa-1}$ line under a SD condition. The pollen of *9522*$^{csa-1}$ generated by CRISPR-Cas9 is sterilized under a SD condition, and this phenotype is similar to the control *csa* mutant. Note that wild type 9522 is always fertile under both LD and SD conditions. *LD* long day, *SD* short day, bar = 100 μm

detection [12], and stain them with potassium iodide solution (I_2-KI) (*see* **Note 9**). Photograph them carefully with the Leica DM2500 microscope. The example results of pollen fertility of 9522, *csa*, and *9522*$^{csa-1}$ plants under LD and SD conditions are shown in Fig. 3.

4 Notes

1. It is important to utilize all of the antibiotics right after it is thawed as a frozen stock from −20 °C. Be sure to add antibiotics to LB agar and YEB agar when the medium temperatures are lower than 60 °C.
2. Taq DNA Polymerase is preferred to be used for genotyping. In our experience, high-fidelity DNA polymerases, such as KOD-FX, Gflex, etc., didn't work well for tissue-cultured samples.
3. All operations in the transformation process should be fully accomplished in a sterile bench.
4. Efficient heat shock process requires direct contact between water and tube.
5. If there are some satellite colonies after transformation, it is better to incubate the plates overnight for additional 12 h at

30 °C and then pick the positive colonies for the next experiment.

6. Seeds are occasionally contaminated in this step. To avoid contamination, remember to check the culture plates frequently and transfer uncontaminated seeds to new plates immediately if any early signs of contamination occur in the old plates.
7. Acetosyringone will facilitate the transformation progress. Therefore, it is important to dip two-layer filter papers with 100 μM/L acetosyringone.
8. Resistance selection twice in differential medium typically lasts for 35 days in total, a little longer than the selection progress in NBD_2 medium. If there is no or little shoot regeneration, adjusting the composition of regeneration medium may be necessary. Regeneration process of rice is highly sensitive to culture conditions.
9. Freshly made potassium iodide solution (I_2-KI) is recommended to be used here for best results.

Acknowledgments

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Chapter 9

Knocking Out MicroRNA Genes in Rice with CRISPR-Cas9

Jianping Zhou, Zhaohui Zhong, Hongqiao Chen, Qian Li, Xuelian Zheng, Yiping Qi, and Yong Zhang

Abstract

Background: MicroRNAs (miRNAs) are small noncoding RNAs that play important roles in plant development and stress responses. Loss-of-function analysis of miRNA genes has been traditionally challenging due to lack of appropriate knockout tools. In this chapter, we describe a method of using CRISPR-Cas9 for knocking out microRNA genes in rice by *Agrobacterium*-mediated transformation. We also demonstrate single-strand conformation polymorphism (SSCP) as an effective genotyping method for screening CRISPR-Cas9-induced mutations.

Keywords MicroRNAs, CRISPR-Cas9, Genome editing, Rice, SSCP

1 Introduction

MicroRNAs (miRNAs) are endogenous single-stranded small noncoding RNAs (~21 nucleotides in length) that function as important posttranscriptional regulators in eukaryotes. MiRNAs play key roles in biological processes, such as growth and development and defense against stress in plants and animals [1]. Biogenesis of miRNAs starts with the processing of RNA polymerase II promoter-driven primary miRNA (pri-miRNA) transcripts which are partially self-complementary and possess the fold-back hairpin structure. The pri-miRNAs are then processed to precursor miRNAs (pre-miRNAs). And finally, the mature miRNA was formed by the RNA-induced silencing complex (RISC) [1, 2]. After processing, mature miRNAs are loaded into Argonaute (Ago) proteins forming miRNA/Ago complexes which will search for cognate messenger RNAs (mRNA) and target them for degradation and/or translational silencing by highly specific complementary base-pairing mechanisms [3, 4].

In plants, posttranscriptional silencing technologies that inhibit miRNAs [5–7] have been used for loss-of-function analysis. However, these methods produced variable results in miRNA inhibition

Yiping Qi (ed.), *Plant Genome Editing with CRISPR Systems: Methods and Protocols*, Methods in Molecular Biology, vol. 1917, https://doi.org/10.1007/978-1-4939-8991-1_9,

[8]. In recent years, genome editing technologies have emerged as useful tools for targeted mutagenesis [9–11]. Clustered regularly interspaced short palindromic repeats and associated protein (CRISPR-Cas) has become a powerful and robust genome editing tool in diverse organisms [12–18]. CRISPR-Cas9 is highly facile as it targets DNA using a single-guide RNA (sgRNA) through RNA-DNA base pairing. This nucleotide-based targeting mechanism has made CRISPR-Cas9 the preferred sequence-specific nuclease (SSN) for genome editing across organisms. Since its first demonstration in editing plant genomes in 2013 [14, 19, 20], CRISPR-Cas9 has been constantly improved and widely used for editing genomes of many plant species [21]. It is now feasible to use CRISPR-Cas9 to directly generate miRNA knockout mutants, which are superior for carrying out reverse genetic research [18]. Here, we describe the use of a CRISPR-Cas9 T-DNA binary vector, pZHY988 (*see* Fig. 1), to generate knockout mutants of miRNA genes in rice. Our results suggest CRISPR-Cas9 is a powerful tool for functional study of miRNA genes in plants.

2 Materials

All chemicals used should be of reagent grade. All solutions should be prepared with ultrapure water.

2.1 Plant

1. Seeds of *Oryza sativa* L. japonica cv. Nipponbare.

2.2 Bacterial Strain and Growth Medium

1. Chemically competent *Escherichia coli* DH5a and *Agrobacterium tumefaciens* strain EHA105.
2. LB solid medium: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 15 g/L agar.

2.3 Plasmids

1. pZHY988: CRISPR-Cas9 expression T-DNA binary vector (*see* Fig. 1).

2.4 Chemicals, Buffers and Solutions

1. dNTPs.
2. Taq polymerase.
3. Plasmid mini extraction kit.
4. DNA gel extraction kit.
5. Isopropanol.
6. 70 and 100% ethanol.
7. Gelrite.
8. Carbenicillin.
9. 2× CTAB buffer (1 L):100 mM/L Tris-HCl, 1400 mM/L NaCl, 20 mM/L EDTA, 20 g CTAB.

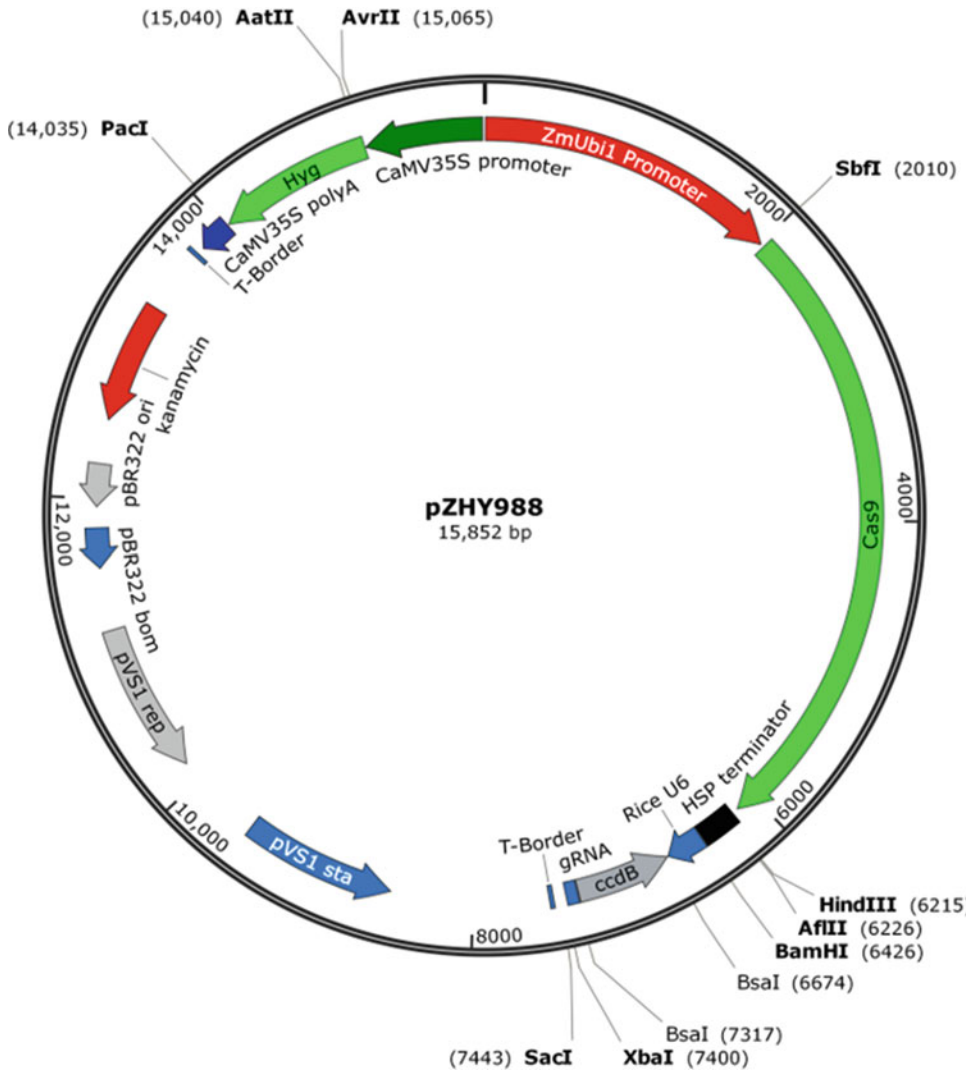

Fig. 1 Map of the pZHY988 T-DNA binary vector

10. 0.1× TE buffer: 1 mM Tris-HCl (pH 8.0), 0.1 mM EDTA (pH 8.0).
11. 15% Acr/Bis (29:1) buffer: 145 g/L acrylamide, 5 g/L bisacrylamide in 1 TBE buffer.
12. 1× TBE buffer: 10.8 g/L Tris base, 5.5 g/L boric acid, 2 mM, 2 mM/L Na_2EDTA (PH = 8.0).
13. Denatured buffer: 95% formamide deionized, 10 mmol/L EDTA, 1 mg/mL bromophenol blue, 1 mg/mL xylene cyanide, 10 mM Na_2EDTA.
14. 10% aps: 100 g/L ammonium persulfate.
15. 0.1% $AgNO_3$: 1 g/L $AgNO_3$.

16. 2.5% NaOH: 25 g/L NaOH.
17. 4% Na_2CO_3: 40 g/L Na_2CO_3.
18. TEMED.
19. 37% formaldehyde.
20. 2.5% sodium hypochlorite.
21. N6 media (working concentration) (pH 5.8): 463 mg/L $(NH_4)_2SO_4$, 2.83 g/L KNO_3, 166 mg/L $CaCl_2{\cdot}2H_2O$, 185 mg/L $MgSO_4{\cdot}7H_2O_4$, 400 mg/L KH_2PO_4, 0.37.3 mg/L Na_2EDTA, 27.8 mg/L $FeSO_4{\cdot}7H_2O$, 4.4 mg/L $MnSO_4{\cdot}4H_2O$, 1.5 mg/L $ZnSO_4{\cdot}7H_2O$, 0.8 mg/L KI, 1.6 mg/L H_3BO_3, 300 mg/L casamino acid, 2 mg/L glycine, 2.878 g/LL proline, 100 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 1.0 mg/L thiamine HCl, 2 mg/L 2, 4-D, 30 g/L sucrose.
22. 2N6-AS media (working concentration) (pH 5.2): 463 mg/L $(NH_4)_2SO_4$, 2.83 g/L KNO_3, 166 mg/L $CaCl_2{\cdot}2H_2O$, 185 mg/L $MgSO_4{\cdot}7H_2O_4$, 400 mg/L KH_2PO_4, 37.3 mg/L Na_2EDTA, 27.8 mg/L$FeSO_4{\cdot}7H_2O$, 4.4 mg/L$MnSO_4{\cdot}4H_2O$, 1.5 mg/L $ZnSO_4{\cdot}7H_2O$, 0.8 mg/L KI, 1.6 mg/L H_3BO_3, 300 mg/L casamino acid, 2 mg/L glycine, 100 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 1 mg/L thiamine HCl, 2 mg/L 2, 4-D, 10 mg/L acetosyringone, 30.0 g/L sucrose, 10.0 g/L glucose.
23. AAM media (working concentration) (pH 5.2): 250 mg/L $MgSO_4{\cdot}7H_2O$, 150 mg/L $CaCl_2{\cdot}2H_2O$, 150 mg/L $NaH_2PO_4{\cdot}2H_2O$, 3 g/L KCl, 40 mg/L Fe-EDTA, 10 mg/L $MnSO_4{\cdot}4H_2O$, 2 mg/L $ZnSO_4{\cdot}7H_2O$, 0.025 mg/L $CuSO_4{\cdot}5H_2O$, 0.025 mg/L $CoCl_2{\cdot}6H_2O$, 0.75 mg/L KI, 3 mg/L H_3BO_3, 0.25 mg/L $Na_2MoO_4{\cdot}2H_2O$, 500 mg/L casamino acid, 7.5 mg/L glycine, 176.7 mg/L L-arginine, 900 mg/L L-glutamine, 300 mg/L L-aspartic acid, 100 mg/L myo-inositol, 1 mg/L nicotinic acid, 1 mg/L pyridoxine HCl, 1 mg/L thiamine HCl, 2 mg/L acetosyringone, 68.5 g/L sucrose, 36 g/L glucose.
24. RE-III media (working concentration) (pH 5.8): 1.9 g/L KNO_3, 1.65 g/L $(NH)_4NO_3$, 370 mg/L $MgSO_4{\cdot}7H_2O$, 440 mg/L $CaCl_2{\cdot}2H_2O$, 170 mg/L KH_2PO_4, 37.3 mg/LNa_2EDTA, 27.8 mg/L $FeSO_4{\cdot}7H_2O$, 22.3 mg/L$MnSO_4{\cdot}4H_2O$, 8.6 mg/L $ZnSO_4{\cdot}7H_2O$, 0.025 mg/L $CuSO_4{\cdot}5H_2O$, 0.025 mg/L $CoCl_2{\cdot}6H_2O$, 083 mg/L KI, 6.2 mg/L H_3BO_3, 0.25 mg/L $Na_2MoO_4{\cdot}2H_2O$, 2 g/L casamino acid, 2 mg/L glycine, 100 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 0.1 mg/L

thiamine HCl, 0.02 mg/L NAA, 2.0 mg/L kinetin, 30 g/L sucrose, 30 g/L sorbitol.

25. HF media (working concentration) (pH 5.8): 1.9 g/L KNO_3, 1.65 g/L$(NH)_4NO_3$, 370 mg/L $MgSO_4{\cdot}7H_2O$, 440 mg/L $CaCl_2{\cdot}2H_2O$, 170 mg/L KH_2PO_4, 37.3 mg/L Na_2EDTA, 27.8 mg/L $FeSO_4{\cdot}7H_2O$, 22.3 mg/L $MnSO_4{\cdot}4H_2O$, 8.6 mg/L $ZnSO_4{\cdot}7H_2O$, 0.025 mg/L $CuSO_4{\cdot}5H_2O$, 0.025 mg/L $CoCl_2{\cdot}6H_2O$, 0.83 mg/L KI, 6.2 mg/L H_3BO_3, 0.25 mg/L $Na_2MoO_4{\cdot}2H_2O$, 2 mg/L glycine, 100 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 0.1 mg/L thiamine HCl, 30 g/L sucrose.

3 Methods

3.1 Vector Construction

1. Select a 20-bp sequence (sgRNA) of the targeted region (around the region of mature miRNA or miRNA*; Fig. 2) with an NGG PAM site (*see* **note 1**). Add the adapters to the oligonucleotide sense and antisense sequences used for cloning into the backbone vector pZHY988 (*see* **note 2**).
2. Synthesize both oligonucleotides with a commercial vendor and dissolve the oligonucleotides in ddH_2O to 100 μM (*see* **note 3**).
3. Mix the two dissolved oligonucleotides. Heat in boiling water for 5 min, and slowly cool down to room temperature to anneal them (*see* **note 4**).
4. Dilute the annealed oligonucleotides tenfold (*see* **note 5**).
5. Set up a Golden Gate reaction as follows for cloning sgRNAs into the Cas9 expression vector.

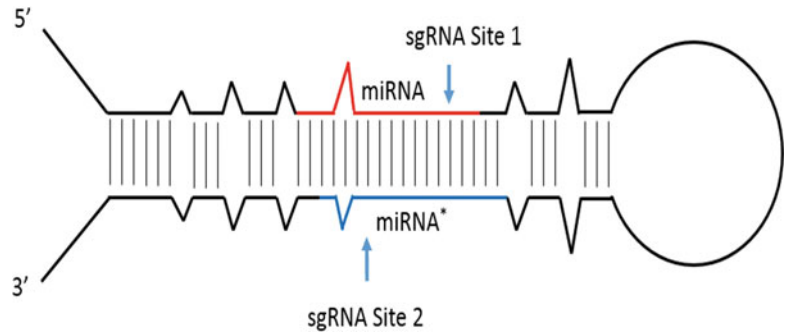

Fig. 2 The primary microRNA (pri-miRNA) transcript. The mature miRNA and miRNA* are color-coded in red and blue, respectively. The mature miRNA and miRNA* sequences can be used to design sgRNA site 1 and sgRNA site 2, respectively

pZHY988 (100 ng/μL)	1 μL
Diluted annealed oligos	2 μL
10× T4 DNA ligase buffer	2 μL
T4 DNA ligase(400 U/μL)	1 μL
Bsa I (10 U/μL)	1 μL
H_2O	13 μL

6. Incubate Golden Gate reactions in a thermal cycler using the following program: 10 cycles of 5 min at 37 °C and 10 min at 16 °C, and then heat to 37 °C for another 5 min and 80 °C for 10 min.
7. Transform 5 μL of the ligation mixture into *E. coli* DH5a competent cells. Spread the transformed cells on LB plates supplemented with 50 mg/L kanamycin, and then incubate overnight at 37 °C.
8. Select about five to ten resistant clones to identify correct clones using colony PCR with the primers of the oligonucleotide (sgRNA) sense sequence and ZY065RB (*see* **note 6**).
9. Verify the insertion of the oligonucleotide by sanger sequencing (*see* **note 7**).
10. Transform the sequencing-confirmed recombinant vector into competent *Agrobacterium tumefaciens* strain EHA105 for rice plant transformation (*see* **note 8**).

3.2 Rice Stable Transformation (see Note 9)

1. Dehull rice seeds and sterilize them with 70% ethanol for 1 min prior to washing in sterile water.
2. Further sterilize the seeds with 2.5% sodium hypochlorite containing one drop of Tween-20 per 50 mL for 15 min, and then wash five times in sterile water.
3. Further sterilize the seeds with 2.5% sodium hypochlorite for 15 min, and then wash five times in sterile water.
4. Culture the sterilized seeds on N6D solid medium with 0.4% Gelrite at 32 °C under continuous light in the growth chamber for 5–7 days.
5. Suspend the *Agrobacterium* strain EHA105 carrying the recombinant vector in AAM medium, and infect precultured seeds/calli for 1.5 min (*see* **note 10**).
6. Blot dry seeds/calli with a sterilized filter paper to remove excess bacteria.
7. Transfer the infected seeds/calli onto a sterilized filter paper that had been moistened with AAM medium, and place onto

2N6-AS solid medium with 0.4% Gelrite. Incubate at 25 °C in the dark for 3 days.

8. Wash the seeds/calli five times in sterilized water, and then wash once in sterilized water containing 500 mg/L carbenicillin.
9. Blot dry the seeds/calli with a sterilized filter paper, and culture them on N6D medium containing 50 mg/L hygromycin and 400 mg/L carbenicillin under continuous light at 32 °C for 2 weeks (*see* **note 11**).
10. Transfer the proliferating calli arising from the scutellum to RE-III medium at 32 °C under illumination with a light intensity of 6000 lx and a photoperiod of 16 h day/8 h night.
11. Transfer the plantlets arising from the calli to HF medium to induce roots at 32 °C for at least 3 weeks.
12. Transfer the transgenic seedlings into the soil (*see* **note 12**).

3.3 Mutation Identification by SSCP

1. Take about 20 mg of leaf tissue from each transgenic line, and put into a 2 mL centrifuge tube with grinding beads.
2. Freeze the samples in liquid nitrogen and homogenize the tissue on a vortex.
3. Add 600–700 μL of 2× CTAB and mix gently. Incubate at 65 °C for 30–60 min.
4. Let the samples cool to room temperature. Extract with 500 μL chloroform ($CHCl_3$)/isoamyl alcohol (24:1). Vortex and spin at 12,000 × *g* with a microcentrifuge for 10 min.
5. Transfer the supernatant to a new tube, and precipitate the genomic DNA with an equal volume of isopropanol alcohol. The genomic DNA is precipitated at −20 °C for 2 h in isopropanol and then centrifuged at 12,000 × *g* for 15 min at 4 °C.
6. Wash with 70% cold ethanol twice and air-dry on benchtop or in a SpeedVac for 20–30 min.
7. Dissolve each sample DNA with 50 μL of 0.1× TE buffer or ddH_2O at 4 °C overnight or 1 h at room temperature.
8. Analyze the genomic DNA on an agarose gel (0.7–1.0%) or with a NanoDrop machine. Use 2 μL as template DNA for PCR.
9. Design the primer pairs for the PCR-SSCP analysis according to the target genome sequence (*see* **note 13**).
10. Set up a typical 25 μL PCR reaction containing 1 U Taq DNA polymerase, 0.5 mM each of forward and reverse primer pairs, 1.5 mM $MgCl_2$, 200 mM dNTP mix, and 4 μL genomic DNA template (~50 ng).
11. Set up a standard PCR condition as follows: 94 °C for 5 min; 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s for 32 cycles; 72 °C for 5 min; hold at 10 °C.

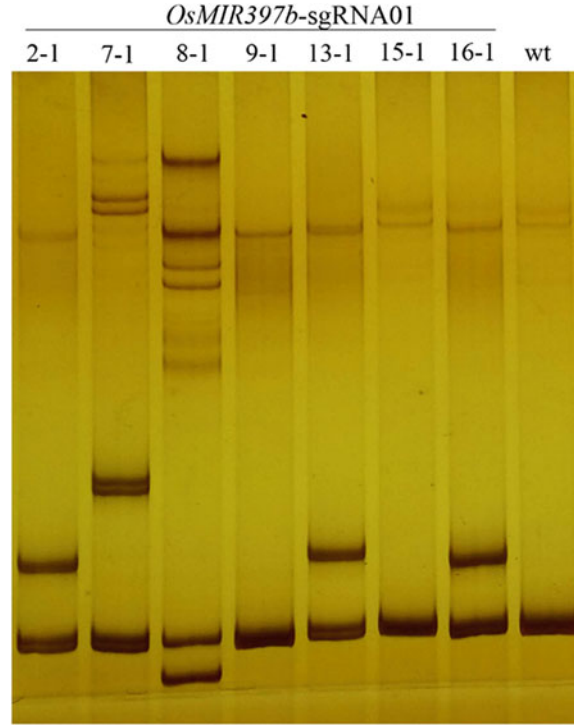

Fig. 3 SSCP screening for genome-edited T0 lines targeted with *OsMIR397b*-sgRNA01. Compared to the wild type (wt), the extra bands in the lower panel are heteroduplex DNA, and the bands in the upper panel are single-strand DNA. All samples except 9-1 and 15-1 contain targeted mutations based on this SSCP analysis

12. Denature PCR products for 5 min at 95 °C in a PCR machine (*see* **note 14**) and then immediately put into an ice box afterward to minimize self-annealing.
13. Prepare 15% non-denaturing polyacrylamide gels (*see* **note 15**).
14. Load 5 μL denatured sample and electrophoresis at 45 mA for 4.5–6 h with 1× TBE buffer (*see* **note 16**).
15. After electrophoresis, put polyacrylamide gels in a container and wash twice using water.
16. Add into the container with the silver-staining solution which contains 0.1% $AgNO_3$ (100 mL volume with 0.1 g $AgNO_3$ and 200 μL 37% CH_2O). Shake gently in the dark for 10 min.
17. Transfer the gels to a container with the developing solution which contains 2.5% NaOH (100 mL volume with 2.5 g NaOH, 400 μL 37% CH_2O, and 1 mL 4% Na_2CO_3). Shake gently for about 10 min under illumination.
18. Stop the developing process with water until the target bands have clearly appeared. Take picture of the gel using a camera (see an example in Fig. 3).

19. Reveal mutation identified by sanger sequencing of the PCR products from SSCP-positive lines (*see* **note 17**).

4 Notes

1. The suitable PAM site should be found in a mature miRNA sequence.
2. The structure of the oligonucleotide pairs is as follows:

   Oligo I: 5′-gtgtgNNNNNNNNNNNNNNNNNNN-3′.

   Oligo II: 3′-cNNNNNNNNNNNNNNNNNNNcaaa-5′.

   NN... sequence in Oligo I represents the 20-nt sequence of the target (sense oligo). NN... sequence in Oligo II represents the complementary sequence of the target (antisense oligo). Note the adaptor nucleotides for cloning are added and specified.
3. The synthetic oligonucleotides are dissolved in ddH_2O for more than 30 min at room temperature. Before adding water, the tube containing the oligonucleotides should be centrifuged for 5 min.
4. Alternatively, it can be annealed with the initial denaturation at 95 °C for 5 min using a PCR thermocycler. After that, gradually cool down to room temperature.
5. Make sure annealed sgRNA oligos don't contain *Bsa* I site.
6. The sequence of ZY065RB is 5′-ttctaataaacgctcttttctct-3′. The PCR system is as follows: 25 mix contains 1 U Taq DNA polymerase, 0.5 mM forward and reverse primer pairs, 1.5 mM $MgCl_2$, 200 mM dNTP mix, and 2 μL clone cell solution template. Use a standard PCR condition as follows: 94 °C for 5 min; 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s for 32 cycles; 72 °C for 5 min; hold at 10 °C.
7. The insertion of the oligonucleotide could be sanger-sequenced using the primer ZY065RB.
8. One protocol of transforming the vector into competent *Agrobacterium tumefaciens* was described previously [22].
9. *Agrobacterium*-mediated rice transformation is based on a method described previously [23].
10. The agrobacteria density at OD600 is approximately 0.1.
11. The calli are checked every 5 days. Any contaminated calli should be removed.
12. Before planting the seedlings, the medium should be washed away with water.

13. The design of a primer pair should follow this rule: The length of the PCR product should be 200–350 bp in which the target site should be near the center.
14. 5 μL PCR products is added to 5 μL denatured buffer.
15. The two gels are set up as follows: 42 mL 15% Acr/Bis, 300 ul 10% aps, 20 μL TEMED. More details are described in a previous protocol [24].
16. Adjust the electrophoresis time according to the length of PCR products.
17. If edited lines are biallelic or heterozygous, the chromatograms of sanger sequencing will contain mixed peaks which can be decoded by computer programs such as DsDecode. To identify chimeric or mosaic mutations from some edited lines, it is better to do a TA cloning of the PCR products first, followed by sanger sequencing of individual clones.

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Chapter 10

An *Agrobacterium*-Mediated CRISPR/Cas9 Platform for Genome Editing in Maize

Keunsub Lee, Huilan Zhu, Bing Yang, and Kan Wang

Abstract

Precise genome engineering can be efficiently made using the revolutionary tool named CRISPR/Cas (clustered regularly interspaced short palindromic repeat/CRISPR-associated protein) systems. Adapted from the bacterial immune system, CRISPR/Cas systems can generate highly specific double-strand breaks (DSBs) at the target site, and desired sequence modifications can be introduced during the DSB repair process, such as nonhomologous end-joining (NHEJ) or homology-directed repair (HDR) pathways. CRISPR/Cas9 is the most widely used genome editing tool for targeted mutagenesis, precise sequence modification, transcriptional reprogramming, epigenome editing, disease treatment, and many more. The ease of use and high specificity make CRISPR/Cas9 a great tool not only for basic researches but also for crop trait improvements, such as higher grain yield, better tolerance to abiotic stresses, enhanced disease resistance, and better nutritional contents. In this protocol, we present a step-by-step guide to the CRISPR/Cas9-mediated targeted mutagenesis in maize Hi II genotype. Detailed procedures will guide through the essential steps including gRNA design, CRISPR/Cas9 vector construction, *Agrobacterium*-mediated maize immature embryo transformation, and molecular analysis of the transgenic plants to identify desired mutant lines.

Key words *Agrobacterium*-mediated transformation, CRISPR/Cas9, Genome editing, Maize, Targeted mutagenesis

1 Introduction

The recently developed genome editing technology using the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins showed a great potential for crop improvements through highly efficient and specific genome modifications [1–3]. Among currently available CRISPR/Cas systems, Cas9 endonuclease from the *Streptococcus pyogenes* (SpCas9) and its engineered derivatives have been most commonly used for a wide range of applications including targeted mutagenesis [4–8], transcriptional reprogramming [9–13], precise gene replacement [14–16], epigenome editing [17, 18], base editing [19–22], CRISPR imaging [23–25], and disease treatment [26–28].

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CRISPR/Cas9 belongs to the class 2 CRISPR/Cas system and consists of the Cas9 effector protein that makes double-strand DNA breaks (DSBs), the crRNA array that specifies target sequences, and the *trans*-activating CRISPR RNA (tracrRNA) that facilitates crRNA-guided DNA cleavage as well as crRNA processing [4]. The fusion of the crRNA with the tracrRNA into the single-guide RNA (sgRNA) further simplified the CRISPR/Cas9 system [4], making it a versatile tool for genome engineering. Typically, a 20-bp DNA target immediately upstream of 5′-NGG protospacer adjacent motifs (PAMs) within a desired gene is cloned into the sgRNA scaffold, which is then transcribed as a ~96 nt sgRNA. It is important to select a unique 20-bp target within a given genome to avoid potential off-target mutations [29]. The Cas9/sgRNA complex generates blunt-end DSBs at the 3-bp upstream of the PAM [4]. Desired gene modifications can be made during the DSB repair procedures. Highly efficient targeted mutations can be generated via nonhomologous end-joining (NHEJ) repair, which can introduce short indels at the DSBs [30]. Alternatively, specific gene modifications can be introduced via homology-directed recombination (HDR) pathway by providing a repair template with desired sequences [5].

Recently, the CRISPR/Cas9 system has been successfully adapted to generate targeted mutations in maize [31–36]. In this protocol, we provide a step-by-step guide to generate targeted mutations in maize Hi II line using the CRISPR/Cas9 system. The overall procedure involves the following steps: (1) target selection and gRNA design preferentially within an exon, (2) vector construction using either multiple cloning sites (MCS) or Gateway cloning, (3) introduction of the constructs into *Agrobacterium* strain, (4) transformation of maize immature embryos, and (5) molecular analysis of the transgenic plants to screen desired mutants. Detailed procedures are provided with additional notes. This protocol has been successfully used in our labs to generate targeted mutations in maize Hi II and B104 lines [34].

2 Materials

2.1 A. tumefaciens Strains, Binary Vectors, and DNA Constructs

1. EHA101 [37] and EHA105 [38] (*see* **Note 1**): EHA101 is a disarmed, nopaline-type *Agrobacterium tumefaciens* strain derived from A281, a hyper-virulent *A. tumefaciens* strain causing crown gall on plants. EHA101 has a kanamycin resistance gene cassette on Ti plasmid. EHA105 was generated from EHA101 through site-directed deletion of the kanamycin resistance gene cassette from the Ti plasmid; thus they are identical other than the kanamycin resistance.
2. pENTR-gRNA1 [34; Fig. 1a]: This is an entry vector that can accept two gRNAs under two independent rice U6 promoters,

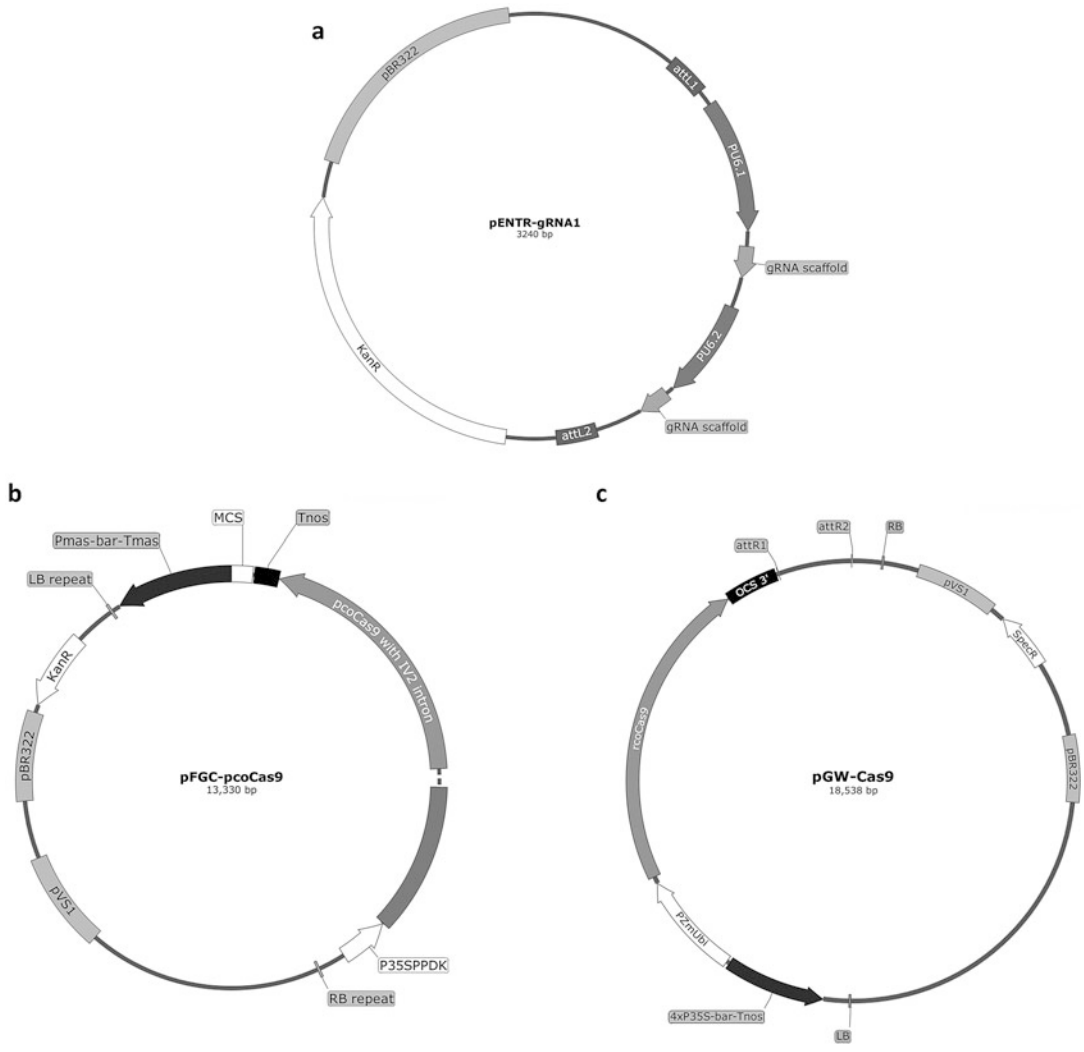

Fig. 1 Constructs for CRISPR-/Cas9-mediated targeted mutagenesis in maize. Schematic representations of the plasmid constructs used for *Agrobacterium*-mediated maize transformation: (**a**) pENTR-gRNA1, (**b**) pFGC-pcoCas9, and (**c**) pGW-Cas9. attL1, attL2, attR1, and attR2: Gateway recombination sequences; PU6.1 and PU6.2, *Oryza sativa* U6 small RNA promoters; gRNA, single-guide RNA scaffold; Kan^R, kanamycin resistance gene; RB, right border; LB, left border; P35SPPDK, hybrid promoter consisting of the cauliflower mosaic virus 35S enhancer fused to the maize C4PPDK basal promoter; pcoCas9, plant codon-optimized Cas9; MCS, multiple cloning sites; Pmas, mannopine synthase promoter; Tmas, mannopine synthase terminator; *bar*, bialaphos resistance gene; OCS3′, octopine synthase 3′ untranslated region; rcoCas9, rice codon-optimized Cas9; PZmUbi, *Zea mays* ubiquitin promoter; Tnos, nopaline synthase terminator; P35S, cauliflower mosaic virus 35S RNA gene promoter; T35S, cauliflower mosaic virus 35S terminator; TrbcS, *Pisum sativum* rbcS E9 terminator; pVS1, replication origin from *Pseudomonas aeruginosa*; pBR322, replication origin from pMB1; and $Spec^R$, spectinomycin resistance gene (*aad*A)

PU6.1 and PU6.2. Each guide sequence can be inserted into the two gRNA cassettes using restriction endonucleases *Btg*ZI and *Bsa*I, respectively. This vector has pBR322 replication origin and kanamycin resistance gene (*aphA3*) for bacterial selection.

3. pFGC-pcoCas9 (*see* **Note 2**; Fig. 1b): pFGC-pcoCas9 was a gift from Jen Sheen (Addgene plasmid #52256). This is a binary vector that expresses a plant codon-optimized Cas9 (pcoCas9) by a hybrid constitutive 35SPPDK promoter [39]. It has multiple cloning sites (MCS) to accept gRNA cassettes. This vector contains two replication origins, pVS1 and pBR322, for *Agrobacterium* and *E. coli*, respectively. Kanamycin-resistant marker gene (*aphA3*) is for bacterial selection. An herbicide (bialaphos)-resistant gene, phosphinothricin acetyltransferase gene from *Streptomyces hygroscopicus*, is used as a selectable marker gene for maize transformation [40].
4. pGW-Cas9 [34; Fig. 1c]: This is a binary vector that expresses a rice codon-optimized Cas9 under maize ubiquitin1 promoter [PZmUbi; 34]. The vector contains a broad-host-range pVS1 replication origin for *Agrobacterium* and pBR322 replication origin for *E. coli*. Spectinomycin resistance gene (*aadA*) is used for bacterial selection. Bialaphos-resistant gene driven by 4x 35S promoter (cauliflower mosaic virus 35S promoter) is used as a selectable marker for maize transformation. gRNA cassettes can be introduced through Gateway recombination.

2.2 Reagents for Molecular Cloning

1. Restriction enzymes: *Bsa*I, *BtgZ*I, *Eco*RI, *Xba*I, and *Hin*dIII.
2. Gateway™ LR Clonase™ II enzyme mix (ThermoFisher Scientific).
3. Proteinase K solution (2 μg/μL).
4. T4 DNA ligase (NEB).
5. Phusion high-fidelity DNA polymerase (ThermoFisher Scientific).
6. 5× Phusion HF buffer (ThermoFisher Scientific).
7. dNTP mix: 2.5 mM each (ThermoFisher Scientific).
8. Annealing buffer: 10 mM Tris-Cl, 1 mM ethylenediaminetetraacetic acid (EDTA), 50 mM sodium chloride (NaCl).
9. TE buffer (pH 8.0): 10 mM Tris-Cl, 1 mM EDTA.
10. ExoSAP-IT PCR cleanup kit (ThermoFisher Scientific).
11. PCR purification kit (e.g., QIAquick PCR purification kit).
12. Gel extraction kit (e.g., QIAquick gel extraction kit).
13. PCR cloning kit (e.g., CloneJET PCR cloning kit).
14. Plasmid DNA miniprep kit (e.g., QIAprep spin miniprep kit).

15. RNase A (ThermoFisher Scientific).
16. *E. coli* competent cells (e.g., DH5α).
17. Thermocycler.
18. Tabletop centrifuge.
19. Gel electrophoresis apparatus.

2.3 Plant Material

1. Maize seeds: Hybrid Hi II parent A and parent B seed can be requested from the Maize Genetics Coop (https://maizecoop.cropsci.uiuc.edu). Greenhouse-grown immature embryos of 1.2–1.8 mm long are used for infection experiments (*see* **Note 3**).

2.4 Stock Solutions

1. Modified MS vitamin stock (1000×): 0.05 g/L nicotinic acid, 0.5 g/L thiamine HCl, 0.5 g/L pyridoxine HCl, and 2.0 g/L glycine (*see* **Note 4**).
2. N6 vitamin stock (1000×): 0.5 g/L nicotinic acid, 1.0 g/L thiamine HCl, 0.5 g/L, pyridoxine HCl, and 2.0 g/L glycine (*see* **Note 4**).
3. 2,4-dichlorophenoxyacetic acid (2,4-D): 2 mg/mL (*see* **Note 5**).
4. Silver nitrate: 50 mM (*see* **Note 6**).
5. Acetosyringone (AS): 100 mM (*see* **Note 7**).
6. L-cysteine (100 mg/mL) (*see* **Note 8**).
7. Bialaphos (Gold Biotechnology): 1 mg/mL (*see* **Note 9**).
8. Glufosinate (Sigma-Aldrich): 1 mg/mL (*see* **Note 9**).
9. Carbenicillin: 250 mg/mL (see **Note 9**).
10. Cefotaxime: 200 mg/mL (see **Note 9**).
11. Vancomycin: 200 mg/mL (see **Note 9**).
12. Kanamycin sulfate: 50 mg/mL (see **Note 9**).
13. Spectinomycin sulfate: 100 mg/mL (see **Note 9**).
14. DNA extraction buffer: 2% CTAB (cetyltrimethyl ammonium bromide), 1.5 M NaCl, 0.1 M Tris-Cl (pH 8.0), 20 mM EDTA, 1% β-mercaptoethanol (add freshly before use).
15. Phenol:chloroform:isoamyl alcohol mixture [25:24:1 (v/v)].
16. Isopropanol.
17. 70% (v/v) ethanol.

2.5 Culture Media

1. LB medium [41]: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl. For solid medium, add 15 g/L Bacto agar and autoclave.
2. SOC medium [42]: 20 g/L peptone, 5 g/L yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose.

3. YEP medium [43]: 10 g/L yeast extract, 10 g/L peptone, 5 g/L NaCl, pH 6.8. Add 15 g/L Bacto agar (for solid medium) and autoclave. Antibiotics are added to the cooled medium before the medium is poured to 100 × 15 mm petri dishes.
4. All media and compositions needed for maize transformation are listed in Table 1 (*see* **Note 10**). pH of the media should be adjusted before the gelling agents are added. Autoclave condition is typically 121 °C for 30 min for a 4 L media load. Heat-sensitive chemicals should be first filter sterilized and added after the media are cooled to approximately 60 °C. Media are poured to 100 × 25 mm petri plates in a laminar flow bench and are stored at room temperature (22–25 °C) in the dark.

3 Methods

3.1 Design gRNAs for Targeted Mutagenesis (Glossy2 Gene as an Example)

It is highly recommended to sequence the gene of interest in the target genotype to be transformed, because a mismatch between the guide and target sequence may significantly impair targeting efficiency. Specific gRNAs can be designed using various web tools such as CGAT [http://cbc.gdcb.iastate.edu/cgat/; 44], CRISPR-P [http://crispr.hzau.edu.cn/CRISPR2/; 45], and Cas-Designer [http://rgenome.net/cas-designer/; 46]. Here, we demonstrate how to use CGAT to design gRNAs for targeted mutagenesis in maize *glossy2* (*gl2*), which is involved in epicuticular wax biosynthesis and deposition in young maize tissues (*see* **Note 11**).

1. Obtain B73 *gl2* sequence from the MaizeGDB, the maize genetics, and genomics database [www.maizegdb.org; 47], by searching the database using "glossy2" as a keyword.
2. Obtain the gene ID for *gl2* (GRMZM2G098239) from the B73 reference genome data [RefGen_v3]. *gl2* is located on chromosome 2.
3. On CGAT, gRNAs can be designed by (a) selecting a gene from the database or by (b) providing gene sequence. (a) Select "Maize" from the genome list, and then select chromosome 2. Copy and paste the gene ID (GRMZM2G098239) into the "Enter Gene ID" window, and select "GRMZM2G098239_T01." Alternatively, (b) copy the *gl2* gene full sequence, and paste into the "PASTE INPUT SEQUENCE" window.
4. Select "23" for "Target Length" to design 20 nt guides.
5. Set "Max Allowed Nucleotide Repeats" to 2 and GC contents to 40–60% (*see* **Note 12**).

Table 1
Media compositions for *Agrobacterium*-mediated Hi II transformation

Hi II media(per liter)	Major salts[a]	L-proline	Myo-inositol	2,4-D	MES	Sucrose	Glucose	$CuSO_4$	pH	Gelling agents[b]		Vit (1000×)[c]	AS	$AgNO_3$	L-cysteine	Cefotaxime	Vancomycin	Selective agents[d]
Infection	4 g N6	0.7 g	–	1.5 mg	–	68.4 g	36 g	–	5.2	–	Autoclave and add reagents at right	1 mL N6	100 μM	–	–	–	–	–
Co-cultivation	4 g N6	0.7 g	–	1.5 mg	–	30 g	–	–	5.8	3 g G		1 mL N6	100 μM	5 μM	300 mg	–	–	–
Resting	4 g N6	0.7 g	–	1.5 mg	0.5 g	30 g	–	–	5.8	8 g A		1 mL N6	–	5 μM	–	100 mg	100 mg	–
Selection I	4 g N6	0.7 g	–	1.5 mg	0.5 g	30 g	–	–	5.8	8 g A		1 mL N6	–	5 μM	–	100 mg	100 mg	1.5 mg bar
Selection II	4 g N6	0.7 g	–	1.5 mg	0.5 g	30 g	–	–	5.8	8 g A		1 mL N6	–	20 μM	–	100 mg	100 mg	3 mg bar
Regeneration I	4.3 g MS	–	100 mg	0.25 mg	–	30 g	–	–	5.8	3 g G		1 mL MS	–	–	–	100 mg	–	2 mg bar
Regeneration II	4.3 g MS	–	100 mg	–	–	60 g	–	1 mg	5.8	3 g G		1 mL MS	–	–	–	100 mg	–	6 mg glu
Regeneration III	4.3 g MS	–	100 mg	–	–	30 g	–	–	5.8	3 g G		1 mL MS	–	–	–	–	–	–

[a]Major Salts: N6 = N6 salts, MS = MS salts
[b]Gelling agents: G = Gelrite, A = purified agar
[c]Vit (1000×): N6 = N6 vitamins, MS = modified MS vitamins, *see* **Note 4**
[d]Selective agents: bar = bialaphos, glu = glufosinate, *see* **Note 9**

6. Select "Maize: *Zea mays* ssp. mays B73 refGen_v3" for optional off-target analysis. Click the "ANALYZE" button to design gRNAs.
7. Refer to *gl2* gene model at the MaizeGDB, and choose proper gRNAs which target exons.
8. Check potential off-targets, and select gRNAs without highly similar off-targets (fewer than two mismatches).
9. Here, a 23 nt target within the exon1 (chr:8113841-8114309) is selected (5′-GCAGTCGTTGCACTTGATGTAGG-3′; PAM is underlined and should not be included in the spacer sequence of the guide RNA cassette) for targeted mutagenesis in Hi II *gl2*.

3.2 CRISPR Reagent Assembly into T-DNA Binary Vector

The CRISPR/Cas9 constructs with desired gRNA can be made using various cloning methods. In this protocol, we show two different methods utilizing (1) multiple cloning sites and (2) Gateway cloning.

3.2.1 Using a Multiple Cloning Site Vector (pFGC-pcoCas9: Addgene #52256)

1. New gRNA cassettes can be rapidly assembled by overlapping PCR as described in Li et al. [48].
2. Use your favorite DNA synthesis service to synthesize the following oligos:

   PrimerF1 (5′-NNN + **restriction site** + AAGAACGAACTAAGCCGGAC-3′): 5′-CCG**GAATTC**AAGAACGAACTAAGCCGGAC-3′.

   PrimerR1 (5′-*reverse complement of 20 nt guide* + AACACAAGCGGCAGCGCG-3′): 5′-*ACATCAAGTGCAACGACTGC*AACACAAGCGGCAGCGCG-3′.

   PrimerF2 (5′-*20 nt guide* + GTTTTAGAGCTAGAAATAGC-3′): 5′-*GCAGTCGTTGCACTTGATGT*GTTTTAGAGCTAGAAATAGC-3′.

   PrimerR2 (5′-NNN + **restriction site** + TAATGCCAACTTTGTACA-3′): 5′-TGC**TCTAGA**TAATGCCAACTTTGTACA-3′.
3. In the first round of PCR, PrimerF1 and PrimerR1 amplify the PU6.1 + 20 nt guide (~350 bp), while PrimerF2 and PrimerR2 [48] amplify the 20 nt guide + gRNA scaffold (~250 bp).
4. Determine optimal annealing temperature for each primer pair using the ThermoFisher Tm calculator (https://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/tm-calculator.html).

5. Set up first round PCR reactions as follows:

Component	Volume (μL)
pENTR-gRNA1 (template: 5–10 ng)	X
5X Phusion HF buffer	10
dNTP mix (2.5 mM each)	4
Forward primer: PrimerF1 or PrimerF2	2.5
Forward primer: PrimerR1 or PrimerR2	2.5
Phusion high-fidelity DNA polymerase (2 U/μL)	0.5
Nuclease-free water	Add to 50
Total volume	50

6. Program thermocycler conditions as follows:

Step	Temperature (°C)	Time	Cycles
Initial denaturation	98	30 s	1
Denaturation	98	10 s	30
Annealing	50	20 s	
Extension	72	20 s	
Final extension	72	5 min	1
Storage	4	hold	1

7. Apply 2–5 μL of reaction mix to a 2% agarose gel electrophoresis to verify PCR amplification.
8. Purify the PCR products (~350 bp and ~250 bp) using QIAquick PCR purification kit or other methods.
9. For QIAquick PCR purification kit, add 5 volumes of Buffer PB to 1 volume of the PCR product and mix.
10. Transfer the sample to a QIAquick column, and centrifuge for 1 min.
11. Discard flow-through, and wash the column once with 750 μL Buffer PE.
12. Centrifuge for 1 min and discard the flow-through.
13. Centrifuge for 1 min to dry the column.
14. Elute DNA with 30 μL of nuclease-free water.
15. Quantify DNA concentration using a NanoDrop.
16. In the second round of PCR, the overlapping sequence of the PrimerR1 and PrimerF2, i.e., the 20 nt guide sequence, works as primers to amplify the full-length PU6.1-gRNA1 cassette.

17. Set up second round PCR as follows:

Component	Volume (μL)
5× Phusion HF buffer	10
Reaction 1 product from **step 14**	0.5
Reaction 2 product from **step 14**	0.5
dNTP mix (2.5 mM each)	4
Phusion high-fidelity DNA polymerase (2 U/μL)	0.5
Nuclease-free water	Add to 50
Total volume	50

18. Program the thermocycler conditions as follows:

Step	Temperature (°C)	Time	Cycles
Initial denaturation	98	30 s	1
Denaturation	98	10 s	30
Annealing	63	20 s	
Extension	72	20 s	
Final extension	72	5 min	1
Storage	4	hold	1

19. Repeat **step 7** to verify full-length PU6.1-gRNA1 cassette amplification (~580 bp).
20. Repeat **steps 8–15** to purify the PCR products.
21. Digest the PU6.1-gRNA1 cassette and pFGC-pcoCas9 with *Eco*RI and *Xba*I overnight (*see* **Note 13**).
22. Purify the digested PU6.1-gRNA1 cassette and pFGC-pcoCas9, and quantify the concentrations.
23. Set up a ligation reaction as follows:

Component	Volume (μL)
10× T4 DNA ligase buffer	1
pFGC-pcoCas9 DNA from **step 22** (~50 ng)	X
PU6.1-gRNA cassette from **step 22** (~10 ng)	Y
T4 DNA ligase	1
Nuclease-free water	Add to 10
Total volume	10

24. Incubate at room temperature for 10 min.
25. Chill on ice, and transform 1–5 μL of the reaction mix into 50 μL *E. coli* (DH5α) competent cells.
26. Incubate the competent cells on ice for 30 min.
27. Heat shock by incubating at 42 °C for 45 s, and chill on ice for at least 1 min before adding 500 μL of SOC medium and shaking; incubate at 37 °C for 1 h.
28. Spread 50–100 μL of the culture on a LB agar plate amended with 50 μg/mL of kanamycin.
29. Pick multiple single colonies for plasmid DNA miniprep, and verify the insertion of the gRNA cassette by sequencing using the oligonucleotide MCSseq 5′-AATAAAAACTGACTCGGA-3′ [48].

3.2.2 Using a Gateway Cloning Vector

1. Use your favorite DNA synthesis service to synthesize complementary oligonucleotides with desired overhangs (5′-gtgt for sense-strand oligo and 5′-aaac for antisense-strand oligo) for the selected target:

   Oligonucleotide 1: 5′-gtgtGCAGTCGTTGCACTTGATGT-3′.

   Oligonucleotide 2: 5′-aaacACATCAAGTGCAACGACTGC-3′.
2. Digest 1–2 μg of pENTR-gRNA1 with *Bsa*I (*see* **Note 13**) and agarose gel purify the cut DNA using QIAquick gel extraction kit.
3. Dissolve the oligonucleotides in nuclease-free water to a 100 μM concentration. For example, if an oligonucleotide is 72.2 nmol, then add 722 μL of nuclease-free water.
4. Anneal oligonucleotides: Add 0.5 μL (50 pmole) of each of the two oligos into a PCR tube containing 49 μL of the annealing buffer to bring the final concentration to 1 pmole/μL.
5. Mix well by vortexing and briefly spin down.
6. Incubate the tube in a thermal cycler with the following cycling profile: 5 min at 95 °C followed by 70 cycles of 1 min incubation at 94–25 °C by decreasing the temperature 1 °C per cycle (*see* **Note 14**).
7. Finally, add 3 μL of the annealed oligonucleotides to a 1.5 mL microcentrifuge tube containing 27 μL of nuclease-free water.
8. Set up gRNA ligation reaction as follows:

Component	Volume (μL)
10× T4 DNA ligase buffer	1
*Bsa*I-digested pENTR-gRNA1 DNA from **step 8** (50 ng)	X
Annealed oligonucleotides from **step 7**	1
T4 DNA ligase	1
Nuclease-free water	Add to 10
Total volume	10

9. Incubate at room temperature for 10 min.
10. Chill on ice, and transform 1–5 μL of the reaction mix into 50 μL *E. coli* (DH5α) competent cells.
11. Incubate the competent cells on ice for 30 min.
12. Heat shock by incubating at 42 °C for 45 s, and chill on ice for at least 1 min before adding 500 μL of SOC medium and shaking at 37 °C for 1 h.
13. Spread 50–100 μL of the culture on a LB agar plate amended with 50 μg/mL of kanamycin.
14. Pick multiple colonies for plasmid miniprep, and verify the insertion by sequencing using the oligo U6P-F1b 5-′-CGTTGAGGGGAGACAGGTTTAG-3′.
15. Prepare LR reaction by adding 50–150 ng of the entry clone, 150 ng of pGW-Cas9, and TE buffer to 8 μL into a 1.5 mL microcentrifuge tube.
16. Thaw the LR Clonase™ II enzyme mix on ice, and briefly mix twice by vortexing for 2 s.
17. Add 2 μL of the LR Clonase™ II enzyme mix to the reaction, and mix well by vortexing briefly twice. Spin down briefly.
18. Incubate reactions at 25 °C for 1 h.
19. To terminate the reaction, add 1 μL of the Proteinase K solution, and vortex briefly.
20. Incubate the sample at 37 °C for 10 min.
21. Transform 1 μL of each LR reaction into 50 μL of *E. coli* (DH5α) competent cells as described above.
22. Spread 50–100 μL of the culture on a LB agar plate with 100 μg/mL of spectinomycin.
23. Pick multiple colonies for plasmid miniprep, and verify the gRNA cassette insertion and vector integrity by sequencing using the oligo U6P-F1b 5′-CGTTGAGGGGAGACAGGTTTAG-3′ or restriction enzyme digestion using HindIII. Digestion with HindIII cuts the vector into six fragments: 13 kb, 2.6 kb, 1.4 kb, 0.8 kb, 0.6 kb, and 126 bp (*see* **Note 15**).

3.3 Introduce Binary Vectors to Agrobacterium Strain

pFGC-pcoCas9 construct needs to be introduced into EHA105 due to the kanamycin selection in bacteria, whereas pGW-Cas9 construct can be introduced into EHA101. The binary vectors can be introduced into *Agrobacterium* strains by the freeze-thaw transformation protocol [49] which is described as follows:

1. Thaw *Agrobacterium* competent cells (use 250 μL per sample) on ice, and add 1 μL of plasmid DNA (0.1–1 μg).
2. Incubate the mixture for 5 min on ice, 5 min in liquid nitrogen, and 5 min at 37 °C.
3. Transfer the mixture to a 15 mL tube, and add 1 mL of YEP.
4. Shake the cells at 28 °C for 2 h.
5. Collect the cells by centrifugation for 2 min at 5180 × *g*, and resuspend cells in 200 μL of YEP medium.
6. Spread the cells on a YEP agar plate containing appropriate antibiotics, and incubate the cells for 2 days at 28 °C.
7. After 2 days, pick multiple single colonies for plasmid analysis to confirm successful transformation.

3.4 Agrobacterium-Mediated Hi II Transformation

The brief protocol for maize Hi II transformation described below is based on previous publication [50].

3.4.1 Infection and Co-cultivation

1. *Agrobacterium* cultures are initiated from a glycerol stock and grown on YEP plates (with 100 mg/L spectinomycin and 50 mg/L kanamycin) for 2 days at 28 °C.
2. On the day of embryo infection, one full loop of bacterial culture was suspended in 5 mL infection medium in a 50 mL Falcon tube.
3. Place the *Agrobacterium* suspension on a shaker or a Vortex Genie platform for incubation for 2 h at room temperature (22–25 °C).
4. Use the infection medium to adjust *Agrobacterium* cell density to 0.3–0.4 of OD_{550} before infection.
5. For infection, maize Hi II immature embryos (IEs, ~1.5 mm) are dissected, and up to 70 IEs are put into an Eppendorf tube containing 1 mL of infection liquid medium.
6. Wash the IEs twice using the infection medium, and remove the liquid.
7. Add 1 mL of *Agrobacterium* suspension from the **step 4**. Gently invert the tube 20 times to ensure thorough mixing of IEs with the bacteria.
8. After the 5 min infection, IEs are transferred to a co-cultivation plate. Use pipet tips to remove bacterial suspension as much as possible.

9. Orientate the IEs to ensure that the embryo-axis side is in contact with the medium (round scutellum side up, *see* **Note 16**).
10. Wrap the plates with vent tape, and incubate at 20 °C in the dark for 2–3 days.

3.4.2 Selection and Regeneration of Transgenic Plants

1. Three days after the co-cultivation, transfer the IEs to resting medium. The plates are incubated at 28 °C in the dark for 1 week (*see* **Note 17**).
2. After 1 week on the resting medium, the IEs are transferred to Selection I medium and incubated at 28 °C in the dark for 2 weeks.
3. The IEs are then transferred to Selection II medium and incubated in the dark for 3 weeks.
4. Majority of the embryos may turn brown and stop growing, but a few rapidly growing embryogenic type II calli start to emerge from some embryos. Subculture each proliferating callus to fresh Selection II medium, and incubate at 28 °C in the dark for 3 weeks.
5. Each growing callus is considered as a putative independent transgenic event. They are transferred to Regeneration I medium (*see* **Note 18**) and incubated at 28 °C in the dark for 10–14 days.
6. Using a dissecting microscope, transfer 15 to 20 bialaphos-resistant, embryogenic type II callus pieces (~4 mm) from each event to Regeneration II medium (*see* **Note 19**). Incubate the plates in the dark (25 °C) for 2–3 weeks.
7. Using a dissecting microscope, transfer 12 to 15 mature somatic embryos (opaque, white, and some with coleoptile) to Regeneration III medium. Plates are incubated at 25 °C, in the light (80–100 $\mu E/m^2/s$, 16,8 photoperiod).
8. Sprouting leaves and roots can be seen within 1 week. Rooted plantlets can be transferred to soil in another 10 days. Molecular analysis can be done on individual plants at this stage.

3.5 Targeted Mutagenesis Analysis

3.5.1 Analysis of T0 Transgenic Plants and Seed Production

1. Extract total genomic DNA from transgenic T0 plants using the modified CTAB method [51].
2. Pool 1–2 cm leaf segments from 2 to 3 young leaves for each T0 plant (*see* **Note 20**).
3. PCR amplify the *gl2* exon1 region including the target site using the oligonucleotides Zm-gl2-F1 5′-GCGCCGAGTACAATACAAGG-3′ and Zm-gl2-R1 5′-GAATTGATTGCAAGGCTGTG-3′. This primer pair will amplify a 1045 bp PCR product.

4. Set up PCR reactions as follows:

Component	Volume (μL)
Genomic DNA from transgenic plants (50–100 ng)	X
5× Phusion HF buffer	4
dNTP mix (2.5 mM each)	1.6
Forward primer: Zm-gl2-F1 (10 μM)	1
Reverse primer: Zm-gl2-R1 (10 μM)	1
Phusion high-fidelity DNA polymerase (2 U/μL)	0.2
Nuclease-free water	Add to 20
Total volume	20

5. The thermocycler conditions are as follows:

Step	Temperature (°C)	Time	Cycles
Initial denaturation	98	30 s	1
Denaturation	98	10 s	30
Annealing	63	20 s	
Extension	72	20 s	
Final extension	72	5 min	1
Storage	4	hold	1

6. Use 2–5 μL of PCR product for 1% agarose gel electrophoresis to verify single-band amplifications.
7. Cleanup PCR products using the ExoSAP-IT treatment. Mix 5 μL PCR product with 2 μL ExoSAP-IT reagent, and incubate the mixture at 37 °C for 30 min, and then inactivate the enzymes at 80 °C for 10 min.
8. Sequence the PCR products using the oligonucleotide Zm-gl2-seq 5′-GTTCGAGCAGCATGAGGAG-3′.
9. Short indel mutations can be detected by analyzing the trace files using the Tracking of Indels by Decomposition [TIDE; 52] or DSDecode [53] with default parameters.
10. Alternatively, PCR products can be cloned into a cloning vector such as pJET1.2 (ThermoFisher Scientific) and sequenced using the sequencing primers from the cloning vector (forward sequencing primer, 5-′-CGACTCACTATAGGGAGAGCGGC-3′; reverse sequencing primer, 5′-AAGAACATCGATTTTCCATGGCAG-3′). Make sure to sequence multiple clones to verify indel mutations.

11. Typical outcomes of TIDE analysis are shown in Fig. 2 (*see* **Note 21**). A wild-type plant carries no indel mutations (Fig. 2a), whereas a monoallelic mutant contains one mutated and a wild-type alleles (Fig. 2b). A biallelic mutant (Fig. 2c) carries two different mutated alleles, and a homozygous mutant (Fig. 2d) has the same mutation at both alleles. A mosaic mutant carries more than two different mutated alleles with or without a wild-type allele (*see* **Note 22**).
12. Transgenic T0 lines carrying desired mutations on the target site (both mono- and biallelic candidates) are brought to growth facility for maturation (*see* **Note 23**).
13. Use wild-type B73 pollen to pollinate transgenic T0 maize plants (*see* **Note 24**).
14. Seeds are harvested and recorded.

3.5.2 Phenotypical and Genotypical Analysis of T1 Transgenic Plants

1. For progeny analysis of the *gl2* gene mutation, germinate 20 seeds per line in soil.
2. Seven days post-germination, the *gl2* loss-of-function mutants can be identified by spraying the seedling leaf surface with water mist.
3. Homozygous or biallelic *gl2* mutants lack epicuticular wax layer and retain water droplets when misted (Fig. 3a), whereas the wild-type or heterozygous plants will display a glossy surface that repels water adhesion (Fig. 3b).
4. Perform mutation analysis on both *gl2* and *GL2* plants using the steps described in Subheading 3.5.1 (*see* **Note 25**).
5. To obtain transgene-free loss-of-function mutants, perform transgene analysis to identify T1 lines with desired mutation without transgenes (*see* **Note 26**).
6. Grow transgene-free T1 lines to maturity, and pollinate with its own pollens (selfing) or pollens from other transgene-free mutant lines (sib crossing).
7. Perform phenotypical and genotypical analysis of T2 lines if necessary as described in **steps 1–4** to identify transgene-free loss-of-function mutants.

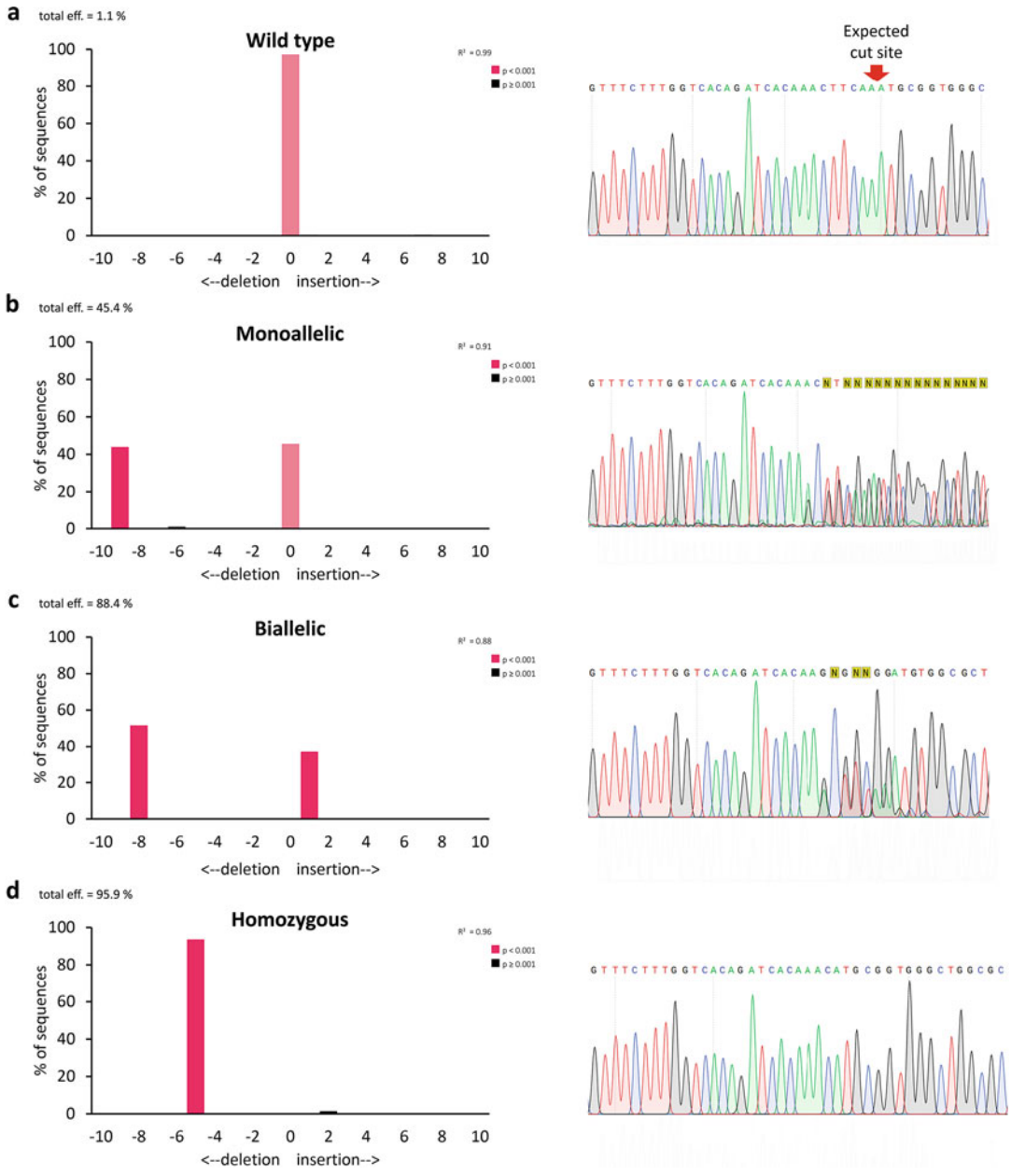

Fig. 2 Tracking of Indels by Decomposition (TIDE) analysis for genotyping transgenic maize plants. Sanger sequencing trace files were analyzed using the TIDE analysis, and typical outcomes are shown for (**a**) wild-type, (**b**) monoallelic (9 bp deletion), (**c**) biallelic (8 bp deletion/1 bp insertion), and (**d**) homozygous mutants (5 bp deletion). Chromogram of each trace file was shown next to the TIDE results (*see* **Note 21**)

Fig. 3 Phenotypes of *gl2* mutant and wild-type maize plants. (**a**) The *gl2* loss-of-function mutant exhibits dull leaf surface on which water droplets adhere when misted, whereas (**b**) the wild-type plant (*GL2*) has a glossy surface which repels water adhesion

4 Notes

1. Other *Agrobacterium tumefaciens* strains such as AGL-1, GV3101, and LBA4404 can be used for transformation [54]. EHA101 has been used extensively and successfully in our laboratory but has kanamycin resistance gene thus cannot be used for a binary vector that has kanamycin resistance gene for bacterial selection.
2. Mannopine synthase promoter [55] driving bialaphos-resistant gene may not be strong enough for maize transformation. We have had limited success with constructs using Pmas driving bialaphos resistance gene. Thus, pFGC-pcoCas9 is not optimal for maize transformation, but the same vector construction procedure using the multiple cloning sites can be applied to similar vectors.
3. Both F_1 and F_2 Hi II immature embryos can be used for transformation. To produce F_1 Hi II seeds, Hi II parent A (pA) are used as female to be pollinated by Hi II parent B (pB). Because of the poor synchronization of pA and pB, F_1 immature embryo production can be challenging. Therefore, we typically use F_2 embryos (produced from sib-pollinated F1 plants) for all Hi II transformation. Immature ears can be stored at 4 °C for 1–4 days.
4. The vitamin solution stocks are filter sterilized, aliquoted, and stored in 50 mL Falcon tubes at −20 °C.

5. 200 mg of 2,4-D powder is dissolved in 1 mL 1 N KOH on a magnetic stirrer with low heat until completely dissolved. Deionized water is added up to a final volume of 100 mL. The solution is stored at 4 °C.
6. 0.85 g of silver nitrate is dissolved in 100 mL of deionized water. The stock solution is filter sterilized, aliquoted, and stored in the dark at 4 °C for up to 1 year.
7. 0.392 g of AS is dissolved in 10 mL of dimethyl sulfoxide (DMSO). This solution is diluted 1:1 with deionized water and filter sterilized. Aliquots (0.5 mL) of stock solution are stored at −20 °C for up to 6 months. Media with AS should be made freshly and used within 1–2 days.
8. L-cysteine solution is made fresh each time when co-cultivation medium is prepared. The stock solution is filter sterilized and added to cooled, autoclaved co-cultivation medium for a final concentration of 300 mg/L. Any unused cysteine stock solution is discarded.
9. Selective agents and antibiotics are dissolved in sterilized deionized water. The stock solution is filter sterilized, aliquoted, and stored at 4 °C for up to 6 months.
10. N6 and MS major salts are purchased from PhytoTechnology Laboratories. Gelling agents such as Gelrite and purified agar are purchased from PhytoTechnology Laboratories and ThermoFisher Scientific, respectively.
11. Hi II parents were derived from A188 × B73 cross [56], and sequencing analysis confirmed that they indeed have *gl2* sequence identical to that of B73. Therefore, we use B73 *gl2* sequence to design gRNAs.
12. Adjust "Max Allowed Nucleotide Repeats" or "GC content" if desired gRNAs are not available with default parameters.
13. Complete digestion of the empty vector is critical to minimize the background. Overnight digestion of 2 μg of plasmid DNA in a 50 μL reaction with 10–20 U of enzymes is recommended.
14. Alternatively, incubate the oligonucleotides in a heating block at 95 °C for 5 min, and gradually reduce the heat until the oligonucleotides have reached room temperature.
15. Digest empty pGW-Cas9 DNA with *Hin*dIII as a control. pGW-Cas9 should have five bands: 13 kb, 2.6 kb, 2.2 kb, 0.6 kb, and 126 bp.
16. Dissecting microscope may be used to ensure the embryo orientation.
17. Resting step can be omitted to shorten the in vitro process.
18. Each event can be cultured on one plate. However, multiple events can be placed on one plate (to save resources) as long as

enough spaces are made available between each callus event to ensure proper separation.

19. We use glufosinate ammonium instead of bialaphos as selective agent for Regeneration II medium (Table 1). This is because glufosinate is readily obtained and less expensive compared to bialaphos. Both selective agents contain the same active ingredient phosphinothricin. It is important to continue the selection during the regeneration period to ensure only the transgenic callus will form mature somatic embryos on this medium. On the other hand, using glufosinate during the early selection stages of transformation appeared to be less effective than bialaphos in our hands.
20. Clonal transgenic plants derived from the same callus can bear different mutations at the target site.
21. The sequence examples shown in Fig. 2 are only for demonstration purposes. The transgenic maize lines were not generated with the gRNA designed in this protocol.
22. If resource permits, perform transgene copy number analysis on T0 plants to identify single- or low-copy transgene insertion candidates for seed production and progeny analysis.
23. Detailed greenhouse care and seed production can be found in previous publication [50].
24. We typically backcross transgenic plants with wild-type maize pollen for two reasons: (a) transgenic plants are detasseled to prevent transgenic pollen dispersal, and (b) transgenic plants often have unsynchronized flowering time due to in vitro culture stresses.
25. Because the transgenic plants are pollinated by wild-type pollens, it is common that the resulting T1 seeds contain newly generated mutations in addition to the inherited mutations. It is highly recommended to screen multiple T1 progenies to identify desired mutant lines.
26. Monoallelic mutant plants with no transgene insertions can be identified in T1 generation. If the desired combination is not found, continue another generation of backcross with the B73. The transgene-free mutant lines can be self- or sib-crossed to generate loss-of-function mutant lines carrying mutations at both alleles of the target gene.

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Part V

CRISPR-Cas9 Editing in Dicots

Chapter 11

Fluorescence Marker-Assisted Isolation of Cas9-Free and CRISPR-Edited *Arabidopsis* Plants

Hanchuanzhi Yu and Yunde Zhao

Abstract

CRISPR/Cas9 gene editing technology has successfully introduced modifications at target DNA sequences in many plant species including *Arabidopsis*. After the target gene is edited, the CRISPR/Cas9 construct needs to be removed to ensure genetic stability and to gain any regulatory approval for commercial applications. However, removal of the transgenes by genetic segregation, backcross, and genotyping is very laborious and time-consuming. The methods we report here allow fast and effective isolation of transgene-free T2 *Arabidopsis* plants with the desired modifications at the target genes. We express a fluorescence protein mCherry under the control of a seed-specific promoter *At2S3* and placed the cassette into the *CRISPR/Cas9* vector. Therefore, we can use mCherry as a proxy for the presence of *Cas9*, and we are able to visually isolate the Cas9-free *Arabidopsis* plants with heritable mutations at the T2 generation. We targeted two sites in the *ABP1* gene to demonstrate the effectiveness of our approach.

Key words CRISPR/Cas9, mCherry, *Agrobacterium* transformation, Floral dipping, RGR, ABP1

1 Introduction

CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated) system has been widely used in genome engineering in zebrafish, mouse, human cell lines, plants, and many other organisms [1–10]. Gene editing by CRISPR requires the expression of the Cas9 protein, the production of a guide RNA, and the presence of the NGG PAM (protospacer adjacent motif) site adjacent to the 3′ end of the target sequence. The guide RNA is complementary to the target sequence and guides Cas9 protein to bind the target sequence. Cas9 protein generates specific double-strand breaks (DSBs) in the target sequence. Subsequently, the DSBs are repaired by the error-prone nonhomologous end-joining (NHEJ) pathway, often causing small deletions or insertions [6]. Because of its simplicity and effectiveness, CRISPR/Cas9 has become the choice of gene editing in essentially any transformable organisms.

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CRISPR/Cas9 gene editing technology has improved significantly over the past few years in terms of efficiency and applicability. One area still needs dramatic improvement is the removal of the transgenes after CRISPR/Cas9 fulfilled its gene editing function. In the presence of Cas9, it is difficult to determine whether the observed mutations are transmitted from the previous generation or newly created by Cas9/gRNA at the current generation. It is almost impossible to conduct phenotypic characterization of an edited plant if Cas9 is still in the plant because Cas9 may generate off-target mutations and may make the genome unstable. Moreover, the presence of *Cas9* transgene almost guarantees that government regulatory agencies will not approve any commercial applications of the edited plants. Therefore, it is essential to remove the CRISPR/Cas9 construct after the target genes have been edited.

Traditionally, Cas9-free and transgene-free plants are identified by PCR-based methods using Cas9-specific primers. The methods are not efficient and are very time-consuming. Extensive labor is needed to grow the plants, extract DNA, conduct PCR, and perform gel electrophoreses. Moreover, at most 25% T2 *Arabidopsis* plants are transgene-free. Here we present an effective strategy to isolate Cas9-free *Arabidopsis* plants with heritable mutations generated by CRISPR/Cas9 [11]. We inserted a mCherry-expressing cassette into the CRISPR/Cas9 vector so that we were able to visually select Cas9-free plants at T2 generation under a fluorescent microscope (Fig. 1) [11]. We targeted two sites, CRP2 and CRP3 sites, in the *ABP1* (auxin binding protein 1) gene as a proof of concept (Fig. 1) [11]. We also generated a large deletion by simultaneously expressing two gRNAs to simplify the screening process for mutants (Fig. 1).

2 Materials

2.1 Growth of Arabidopsis

1. *Arabidopsis thaliana* ecotype Columbia (Col-0) is used in this study.
2. MS media: Dissolve 4.33 g Murashige and Skoog (MS) basal salt into 2 L DI water, then add 230 μL 1 N NaOH. Prepare five 500 mL PYREX bottles. Add 2.6 g agar-agar to each bottle, and then add 400 mL MS mixture to each bottle. Keep the lid loose. Autoclave at 121 °C for 30 min. Add about 30 mL media to each Petri plate, and allow the agar to solidify in the hood.

2.2 Vector Construction

1. Phusion mixture (for 10 reactions): 2.5 μL Phusion enzyme from NEB, 20 μL 5× HF Phusion buffer, 10 μL dNTP, 2.5 μL forward primer, 2.5 μL reverse primer, 61.5 μL H_2O.

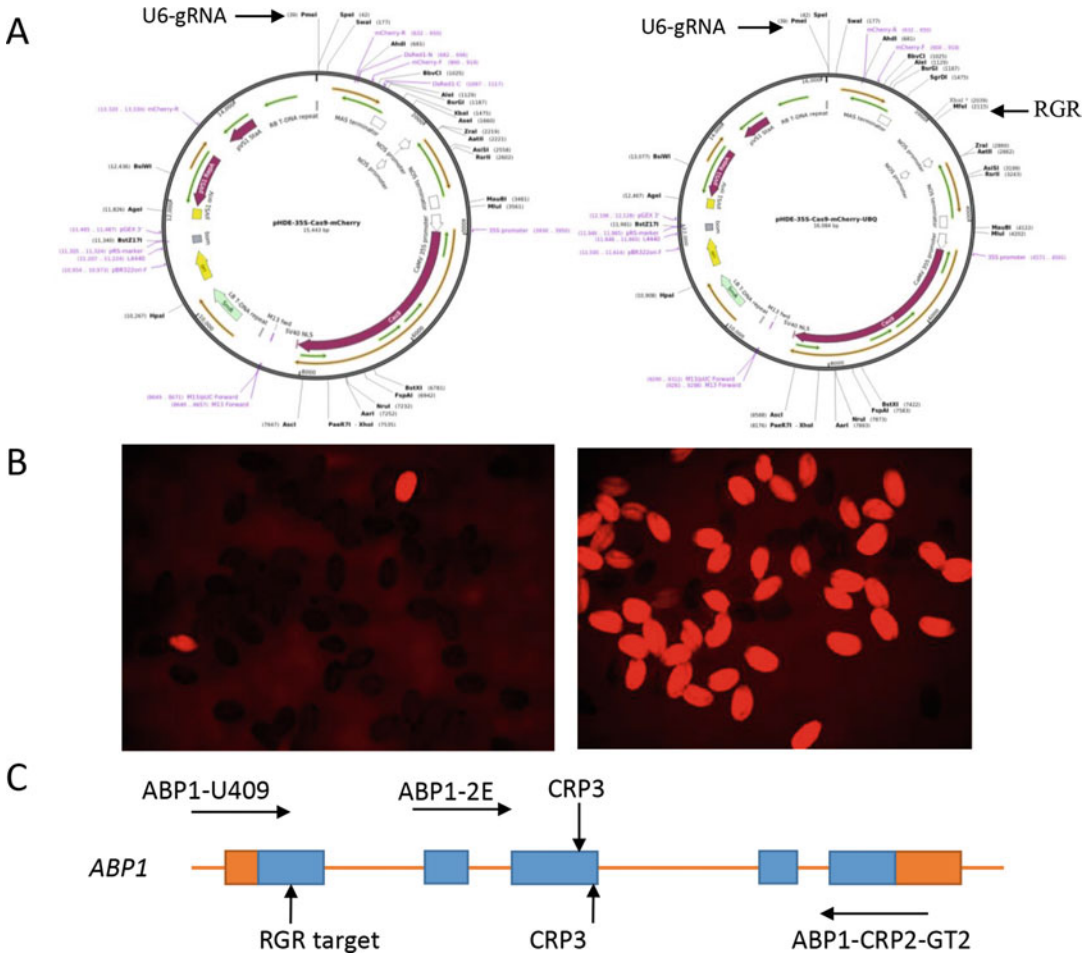

Fig. 1 Fluorescence marker-assisted isolation of Cas9-free and CRISPR-edited *Arabidopsis* plants. (**a**) Vector maps of pHDE-35S-Cas9-mCherry and pHDE-35S-Cas9-UBQ10-mCherry showing the insertion sites of U6-gRNA and RGR. U6-gRNA unit is cloned into the PmeI site in both vectors. RGR unit is cloned into the MfeI site in pHDE-35SCas9-UBQ10-mCherry. (**b**) Identification of seeds with the CRISPR/Cas9 transgenes. Left panel shows that transgenic seeds can be identified as red at T1 generation. Note that the majority of seeds are black, because *Arabidopsis* transformation efficiency is usually less than 3%. The right panel shows the seeds harvested from a T1 plant. T-DNA insertion in T1 plants is usually heterozygous, yielding less than 25% of black seeds, which are presumed Cas9-free. Black seeds without Cas9 are selected at T2 generation. (**c**) *ABP1* gene is used as an example for targeted mutagenesis by CRISPR/Cas9. The *ABP1* gene structures along with the Cas9 targets are shown. Locations of the genotyping primers are shown

2. 1% agarose gel: 1 g agarose, 100 mL 1× TAE buffer.
3. LB media (1 L): 10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl, pH to 7.5 with NaOH, DI water to 1 L.
4. *E. coli* DH5αcompetent cell stored at −80 °C.
5. P1 buffer 500 mL: RNase A added (100 μg/mL), 25 mL Tris-Cl pH 8.0, 10 mL 0.5 M EDTA, 465 mL DI H_2O. Store at 4 °C.

6. P2 buffer 500 mL: 4 g NaOH (200 mM final), 5 g SDS (1% final), 500 mL H_2O. Store at room temperature.
7. P3 buffer 500 mL: 147.21 g KAC, 57.5 mL HAC, H_2O to 500 mL. Store at 4 °C.

2.3 Agrobacterium-Mediated Transformation Using Floral Dipping

1. *Agrobacterium tumefaciens* strain GV 3001.
2. 3% sucrose solution: 0.22 g Murashige and Skoog (MS) basal salt, 3 g sucrose, 32 μL Silwet L-77, 100 mL DI H_2O.
3. Electroporator.

2.4 Selection of Mutant Seeds

1. Fluorescence microscope equipped with a mCherry filter.
2. Genotyping primers.

3 Methods

3.1 Growth of Arabidopsis

1. Put the seeds in a microfuge tube. Add 800 μL 75% ethanol, and shake for 10 min. Discard the ethanol, add 1 mL 100% ethanol, and shake for 10 min. Transfer the seeds onto a sterile filter paper, and dry in the hood.
2. Sow seeds on MS plates by tapping the filter papers. Seal the plates with tape, and stratify the seeds for 2 days at 4 °C. Grow under long-day conditions (16 h light/8 h dark) at 22 °C for 5 days.
3. Transplant the seedlings to soil. Grow in the growth room under long-day conditions (16 h light/8 h dark) at 22 °C until flowering.

3.2 Vector Construction

U6-gRNA unit is cloned into the PmeI site in both vectors pHDE-35S-Cas9-mCherry and pHDE-35S-Cas9-UBQ10-mCherry by Gibson assembly [12]. Ribozyme-gRNA-Ribozyme (RGR) unit [13] is cloned into the MfeI site in pHDE-35SCas9-UBQ10-mCherry by Gibson assembly [14, 15]. A simplified protocol is described as follows:

1. Add 1 μL DNA (5 ng) template to 99 μL Phusion mixture. Aliquot 20 μL mixture to five PCR tubes. PCR condition (Phusion program) was 10 cycles of 98 °C for 10 s, 57 °C for 15 s, and 72 °C for 20 s, followed by 35 cycles of 98°C for 10 s and 72 °C for 20 s (*see* **Note 1**).
2. DNA purification: Run 1% agarose gel, cut out the desired band, freeze the gel fragment at −20 °C for 5 min, and centrifuge for 5 min at 15,000 × *g* (*see* **Note 2**).
3. Ligation: Mix 0.3 μL linearized plasmid, 0.7 μL of the PCR product, and 3 μL 1.33× Gibson assembly mixture. Incubate at 50 °C in a PCR machine for 1 h (*see* **Note 3**).

4. Heat shock: Add the Gibson assembly product to 50 μL *E. coli* DH5αcompetent cells. Put on ice for 20 min, transfer to 42 °C water bath for 1.5 min, and put on ice for another 3 min. Add the product to 200 μL LB media, and place it in 37 °C incubator for 30 min. Spread on LB plate containing 50 μg/mL spectinomycin, and incubate at 37°C overnight (~15 h).
5. Colony culture: Pick ten colonies, and put them into individual PCR tubes. Add 10 μL Phusion mixture to each tube. Run the PCR Phusion program. Run 1% agarose gel to check positive colonies. For each positive colony, add 3 mL LB and 3 μL 50 mg/mL spectinomycin to a test tube, and dip the colonies into the test tube. Place the test tubes on a 37 °C shaker overnight (*see* **Note 4**).
6. Purification of plasmid: For each colony, transfer the overnight culture to two centrifuge tubes, and centrifuge 2 min at 15,000 × *g*. Use pipette to remove all the supernatant. Add 200 μL P1 buffer to resuspend the cells, and then add 200 μL P2 buffer, inverse gently for 6 times, and sit on ice for 2–5 min. Add 200 μL P3 buffer, and inverse gently for 5 times. Place on ice for 10 min, and then centrifuge for 10 min at 15,000 × *g*. Discard the precipitate, and transfer 550 μL supernatant to a new microfuge tube. Add the same volume (550 μL) of isopropanol and mix. Put on ice for 10 min, and centrifuge for 10 min at 15,000 × *g*. Discard the supernatant. Add 500 μL 75% ethanol, and centrifuge for 2 min. Discard the supernatant, and dry. Add 30 μL H_2O to dissolve DNA by pipetting up and down. Take 2 μL of the product, and run 1% agarose gel to check if the size of plasmid is correct.

3.3 Agrobacterium-Mediated Transformation Using Floral Dipping [16]

1. Chill the electroporation cuvette on ice for 3–5 min.
2. Mix 0.5 μL plasmid DNA with 50 μL *Agrobacterium* on ice in the cuvette. Add to the side, and then tap down to eliminate the air bubbles. Place on ice for 20–30 min.
3. Set the electroporator to 1640 V. Dry outside of the cuvette with tissue paper. Insert the cuvette into the cuvette chamber with notch facing toward you, and close cuvette chamber lid. Push start button until beep.
4. Add 200 μL LB to the cuvette, pipette up and down to mix, and transfer to a 1 mL microfuge tube. Place the tube at room temperature for 1 hour. Spread the solution on LB-spectinomycin plate, and place in 28 °C incubator for 2–3 days.
5. Pick colonies. For each colony, add 3 mL LB, and place on 28 °C shaker overnight.
6. Take 100 μL *Agrobacterium* solution and 100 mL LB to a flask, and place on 28 °C shaker overnight.

7. Spin down the *Agrobacterium* solution, discard the supernatant, and resuspend in 100 mL 3% sucrose MS solution. Add 30 μL Silwet L-77 and mix.
8. Transfer the solution to a bottle, and use a sprayer to spray the solution on the plants [16]. Place the plants in a dark environment, and cover the plants for 1 day. Clean up the bottle with 10% bleach (*see* **Note 5**).

3.4 Screen for Editing Events in Arabidopsis

1. Harvest T1 seeds, and select the red seeds under a fluorescence microscope equipped with a mCherry filter (*see* **Note 6**).
2. Grow T1 plants, and extract DNA from leaf tissues of 2-week-old T1 plants.
3. To screen mutations at the CRP2 target, perform PCR using primers ABP1-U409 and ABP1-CRP2-GT2. Use restriction enzyme BsaJI to digest the PCR product.
4. To screen mutations at the CRP3 site, perform PCR using primers ABP1-2E and ABP1-CRP2-GT2. Use restriction enzyme TaqI to digest the PCR product.
5. To screen large deletions, perform PCR using primers ABP1-U409 and ABP1-CRP2-GT2.

3.5 Screen for Cas9-Free and CRISPR-Edited Arabidopsis *T2 Plants*

1. Harvest seeds from individual plants that showed positive editing events at the target site.
2. Select the black seeds (non-fluorescence, Cas9-free) under a fluorescence microscope equipped with a mCherry filter (*see* **Note 7**). At least 96 non-fluorescence seeds are selected from each T1 plant.
3. Germinate the non-fluorescence seeds, and grow the T2 plants in the green house.
4. Extract genomic DNA from each plant, and genotype the plants using PCR and restriction digestion (*see* **Note 8**). Primers are listed in Table 1.
5. Sequence the target genes to reveal the identity of mutations.

4 Notes

1. Other high-fidelity DNA polymerases such as Pfu can also be used. The PCR program needs to be adjusted according to the size of fragment and primer lengths. Phusion-based PCR can be conducted following manufacturer's recommendation.
2. The desired DNA fragment can be purified from the gel slice using commercially available gel extract kit and spin columns. We found that it is not necessary to purify the DNA fragment.

Table 1
List of genotyping primers

Primer	Sequence
ABP1-U409	CCTCATCACACAACAAAGTCACTC
ABP1-CRP2-GT2	CATGAGGACCTGCAGGTGTTG
ABP1-2E	TTGCCAATCGTGAGGAATATTAG

Freeze and thaw followed by centrifugation is sufficient to yield the needed DNA fragment.

3. Commercially available Gibson assembly kits usually recommend 20 μL reaction. A total of 4 μL reaction is sufficient.
4. Colony PCR may not be necessary. The Gibson assembly reaction usually is very efficient and produces very few false positive colonies.
5. *Arabidopsis* is usually transformed by floral dipping. We found that using a spray bottle, which can be purchased from the Home Depot or other stores, is faster and uses less *Agrobacterium* culture. Transformation efficiency is usually more than 2%.
6. Seeds with the mCherry marker should be very obvious. T1 plants can be screened using either antibiotics or mCherry. The former is more efficient and requires very little labor, but antibiotics do affect plant growth even if they are resistant. Selection of T1 seeds using the fluorescence marker requires hands on time, but this method saves space.
7. The number of T-DNA insertions can be estimated based on the ratio of red seeds over black seeds. Usually, multiple insertions lead to higher expression of Cas9 and gRNA, thus higher editing efficiency.
8. The mCherry marker is an excellent proxy for the presence of the CRISPR/Cas9 construct. The final edited plants still should be genotyped using Cas9-specific primers to confirm that the plants are indeed *Cas9*-free.

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Chapter 12

Creating Targeted Gene Knockouts in *Brassica oleracea* Using CRISPR/Cas9

Tom Lawrenson, Penny Hundleby, and Wendy Harwood

Abstract

While public and political views on genetic modification (*inserting* "foreign" *genes* to elicit *new traits*) have resulted in limited exploitation of the technology in some parts of the world, the new era of genome editing (to *edit existing genes* to gain *new traits/genetic variation*) has the potential to change the biotech landscape. Genome editing offers a faster and simpler approach to gene knockout in both single and multiple genetic locations, within a single or small number of generations, in a way that has not been possible through alternative breeding methods. Here we describe an *Agrobacterium*-mediated delivery approach to deliver Cas9 and dual sgRNAs into 4-day-old cotyledonary petioles of *Brassica oleracea*. Mutations are detected in approximately 10% of primary transgenic plants and go on in subsequent T1 and T2 generations to segregate away from the T-DNA. This enables the recovery of non-transgenic, genome-edited plants carrying a variety of mutations at the target locus.

Key words *Brassica oleracea*, Vector, CRISPR/Cas9, Knockout, Mutant, Gene edit, Transgene free, Genome editing

1 Introduction

The clear leader in the genome editing tool box is CRISPR technology. Since the first application of CRISPR-/Cas9-mediated genome editing in plants [1–4], it has proven to be functional in almost all species where it has been tested. In the most common application of the technology, it has been used to introduce double-strand breaks (DSBs) at a particular locus which are then repaired by the host cells' own repair machinery, the error-prone process of non-homologous end joining (NHEJ). This inaccurate repair typically results in the loss or gain of one or a few nucleotides, and this is sufficient, in most cases, to result in a loss of function of the target gene [5].

Cas9 is an RNA-guided endonuclease from the bacterium *Streptococcus pyogenes* and is associated with the CRISPR (clustered regularly interspersed short palindromic repeats) locus in the bacterium, where together they form part of a type II adaptive immune

Yiping Qi (ed.), *Plant Genome Editing with CRISPR Systems: Methods and Protocols*, Methods in Molecular Biology, vol. 1917, https://doi.org/10.1007/978-1-4939-8991-1_12,

system [6]. When encountering foreign DNA, the bacterium uses the Cas9-guide RNA complex to interrogate the foreign DNA, and if it is recognized, due to a previous encounter with the same invading sequence, then cleavage occurs resulting in the destruction of the invading bacteriophage or virus. The native guide RNA is composed of two separate RNA molecules which hybridize before complexing with Cas9. For the purpose of genome editing, the two RNA molecules have been successfully combined so that they can be provided as a single-guide RNA (sgRNA) [7]. Briefly by designing the 5′ end (protospacer) of an sgRNA such that it is complementary in sequence to a specific target locus, this sgRNA can complex with nuclear-localized Cas9 to introduce DSBs at specific loci in plant genomes.

The target gene-specific 5′ protospacer region is just 20 nucleotides in length, while the remaining sgRNA is identical regardless of the intended target (Fig. 1). The Cas9/sgRNA complex is able to search the target genome sequence, and when a suitable match is found, a DSB is introduced. A requirement for sgRNA functionality is the presence of homologous genomic sequence that is directly followed by NGG which is referred to as the PAM (protospacer adjacent motif). If this entire 23-base sequence is found in the genome (20 from the sgRNA plus PAM), then the Cas9 endonuclease will cut at a point 3 bp from the PAM motif. In higher eukaryotic plants, the principal DNA repair mechanism is NHEJ. This repair mechanism is error prone resulting in indels in some cases, although many DSBs will be perfectly repaired so they are never detected.

The length of the sgRNA responsible for targeting is relatively short at only 20 nucleotides so it is sometimes difficult, especially in larger crop genomes, to ensure that this sequence is not present in other "off-target" locations. Even if the match between the sgRNA and the genomic target is not perfect, there is a chance of off-target mutations [5]. BLAST searches against the *Brassica* genome using the full 23 nucleotide sequence (protospacer plus PAM) as a query together with other CRISPR/Cas9 online design tools can allow the selection of guides with no, or a minimal number of, predicted off targets. For plant breeders the occurrence of off-target mutagenesis is not a major constraint, as any undesirable mutations will be predictable, testable, and therefore avoidable by sensible guide selection and identification of lines where off-target activity did not occur. Off-target activity is a fraction of on-target editing, and so precision can be obtained by screening enough lines [5]. However, off-target activity can also be an advantage; for example, co-targeting functionally redundant multiple gene copies which have divergent nucleotide sequence was successfully reported in *B. oleracea* [5], *B. napus* [8–10], and *B. carinata* [11].

To date most CRISPR genome editing approaches still rely on producing stable transgenic lines to introduce the guide RNA and

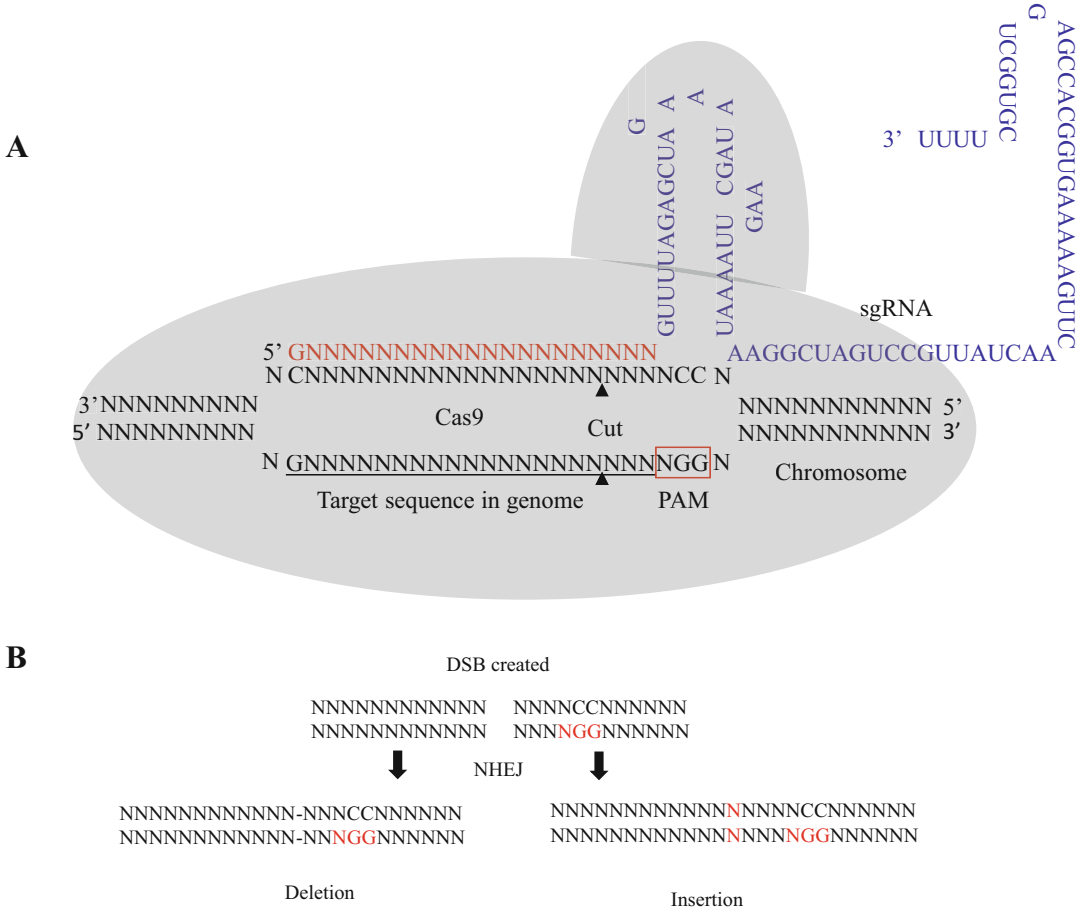

Fig. 1 CRISPR-/Cas9-targeted mutagenesis system. (**a**) Schematic of Cas9/sgRNA complex targeting a sequence in chromosomal DNA. The 5′ 20 nucleotides of the sgRNA (protospacer) are complementary to the top strand of the chromosome and are directly followed by a PAM which allows Cas9 to make a DSB. The non-variable section of sgRNA remains identical regardless of the intended target. (**b**) DSBs are repaired by error-prone NHEJ resulting in small insertions and deletions (indels)

Cas9. Protocols for the routine transformation of *Brassica* using T-DNA delivery via *Agrobacterium tumefaciens* were previously reported [12]. In the current chapter, we detail an approach to introduce CRISPR-/Cas9-targeted mutations using four transcriptional cassettes (a plant selectable marker, Cas9, and two sgRNAs) within a single T-DNA binary vector. Figure 2 shows the cloning of individual gRNAs. Figure 3 shows the structure of a dual guide vector we use for such mutagenesis in *B. oleracea*. Adjacent to the left border is the first cassette which provides resistance to kanamycin and allows regeneration of *B. oleracea* plants which contain the T-DNA integrated into the genome. The second cassette is for expression of nuclear-localized Cas9, while the third and fourth

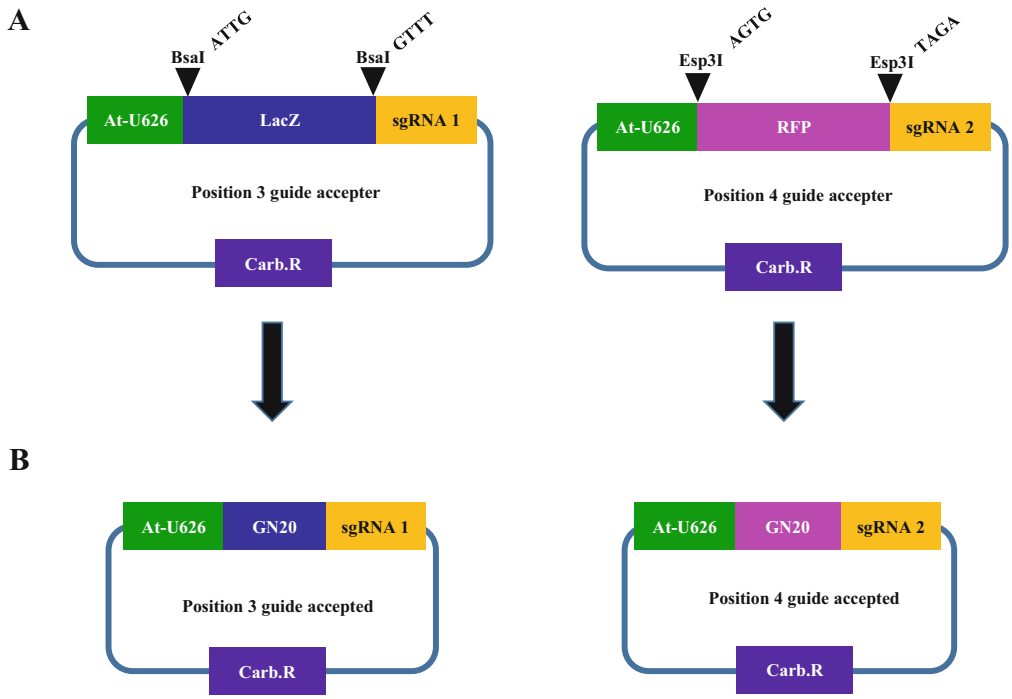

Fig. 2 Cloning of sgRNAs. (**a**) Cloning of sgRNA oligos into position 3 and 4 level 1 guide accepters at *BsaI* and *Esp3I* sites, respectively. Four base Golden Gate overhangs are shown which are compatible to the hybridized oligo pair sticky ends (*see* Table 1). (**b**) Successful cloning allows replacement of lacZ and RFP color markers by the sgRNA transcriptional cassettes in position 3 and 4 level 1 vectors, respectively. Each single-guide RNA (sgRNA1 and sgRNA2) is driven by *Arabidopsis thaliana* U626 promoter *(P-AtU626)*

cassettes utilize *Arabidopsis* U626 promoters to drive transcription of sgRNAs specific to the target locus. We have found that some sgRNAs work and some do not, with efficiency varying greatly depending on the specific sgRNAs used. A great deal of work has been undertaken to enable selection of efficient sgRNAs [13, 14]. However, this largely relates to mammalian genomes, and we have found the best approach is to test by actual transformation into *B. oleracea*.

Our standard protocol involves assembly of two dual sgRNA vectors, each with a pair of unique sgRNAs, giving a total of four per target gene (Fig. 4a). This strategy has enabled us to target a total of ten genes to date, and even in the least efficient case, one sgRNA out of four was functional. In some cases, two, three, or four sgRNAs were active. A potential benefit of using a dual sgRNA vector is that if both sgRNAs are active, then simultaneous DSBs at the two target genomic loci may occur resulting in the deletion of the entire region between the pair of sgRNAs [15]. This approach may have value, allowing, for example, the removal of an entire exon.

For each binary construct, we routinely produce 50 independent transgenic lines (Fig. 4b). These T0 lines are then screened for

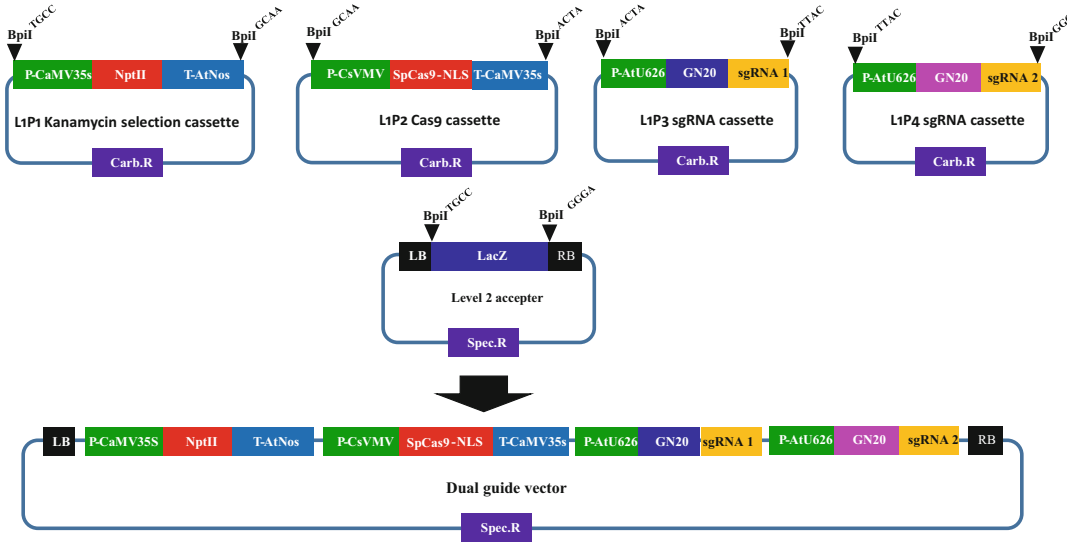

Fig. 3 Golden Gate assembly of the T-DNA expression vector. Level 1 cassettes are assembled into the level 2 accepter via *BpiI* cloning. Level 1 position 1 (L1P1) kanamycin resistance cassette consists of the *neoomycin phosphotransferase* coding sequence (*nptII*) driven by the 35 s promoter (P-CaMV35s) and terminated by the nopaline synthase terminator from *Agrobacterium tumefaciens* (*T-AtNos*). Level 1 position 2 (L1P2) Cas9 expression cassette consists of sequence encoding Cas9 from *Streptococcus pyogenes* with a carboxy-terminal nuclear localization signal from simian vacuolating virus 40 (SpCas9:NLS) driven by the cassava vein mosaic virus promoter (P-CsVMV) and terminated by the 35 s terminator sequence (T-CaMV35s). Level 1 position 3 and 4 (L1P3/L1P4) single-guide RNAs (sgRNA1 and sgRNA2) are each driven by the *Arabidopsis thaliana* U626 promoter (*P-AtU626*). Selection of these plasmids in bacteria is carbenicillin (level 1) and spectinomycin (level 2). Left border (LB) and right border (RB) T-DNA sequences are indicated

the presence of targeted mutations, and lines with mutations are moved forward into the T1 generation. After sowing T1 seeds and screening seedlings, we expect to find progenies which have lost their T-DNA via segregation but retain the targeted mutations. It is likely that the T1 progenies will also have segregated for the various mutations seen in the T0 parent, and so there is a good chance of obtaining homozygous mutants which are transgene-free in the T1 generation.

Screening for the presence of mutations can be carried out using a number of methodologies including restriction enzyme/PCR-based methods [16]. However, we have found that direct sequencing of PCR amplicons which cover the target sites is relatively quick and simple and gives detailed information on the events occurring at the target locus (Fig. 4c). Cas9 cuts within the 20-base target sequence between bases 3 and 4 counting from directly 5′ to the PAM (Fig. 1). Therefore, where indels are present, sequencing chromatograms typically become double or triple peaked from precisely this cut point, whereas the preceding sequence consists of clean single peaks (Fig. 4c). This result indicates that there is a mixture of alleles, many of which will be potentially useful for

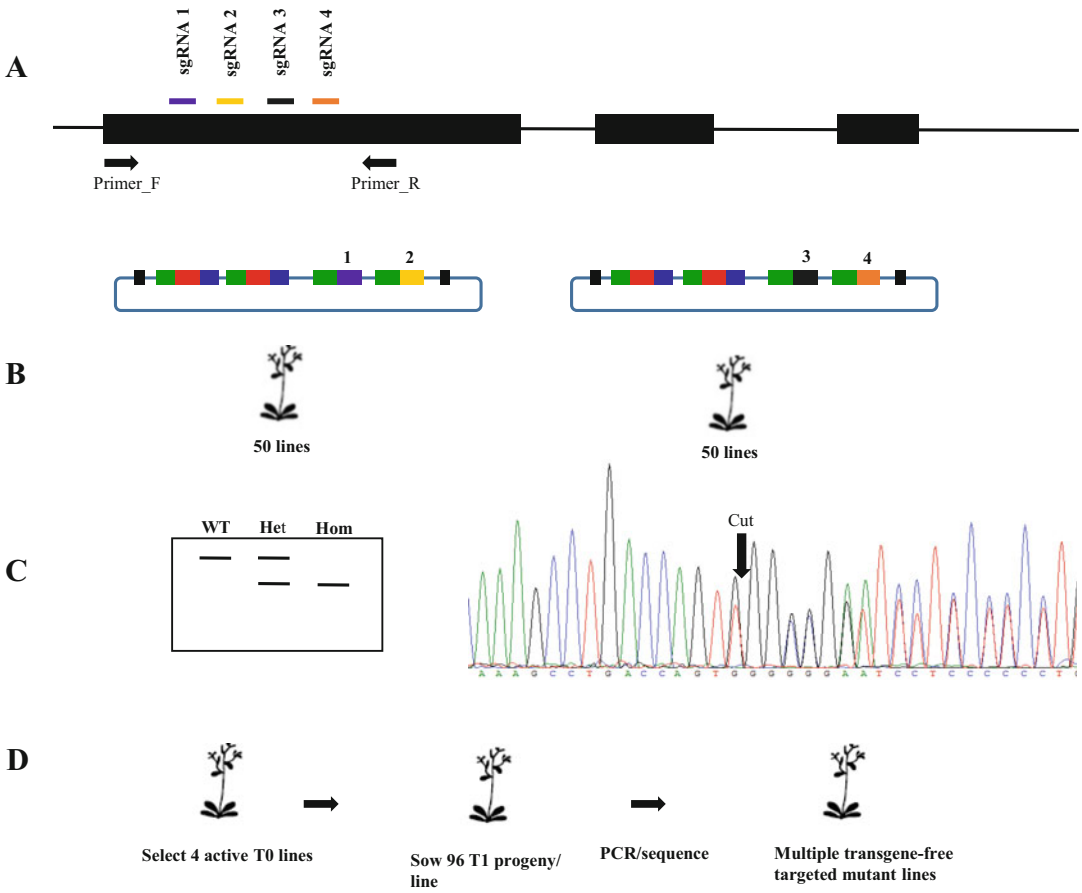

Fig. 4 An experimental flow diagram. (**a**) Four sgRNAs are designed to target the first exon of a gene. (**b**) Two dual guided binary constructs are assembled, each containing a pair of sgRNA transcriptional cassettes. Fifty independent transgenic *B. oleracea* lines are made for each of the constructs. (**c**) Screen transgenic plants via PCR/sequencing using forward and reverse primers. Some lines may display significant deletions leading to band shift on agarose gels which can be heterozygous or homozygous in appearance. Mutations can be revealed by sequencing. Double peaks arising from the Cas9 cut site are indicative of mutations. (**d**) By screening T1 progeny from active T0 lines, transgene-free targeted mutants can be recovered

causing a loss-of-function phenotype. A specific benefit of using a dual sgRNA strategy is that when both sgRNAs are active, simultaneous cutting can allow the entire fragment between two target sites to be deleted, resulting in PCR amplicons that are considerably shorter. This significant size difference relative to wild type is easy to detect on agarose gels as a band shift (Fig. 4c).

Once T0 mutant plants, with either indels or large deletions, have been identified, they are grown to seed. Mutations should segregate from the T-DNA in the T1 generation allowing T-DNA-free mutants to be identified (Fig. 4d). T1 plants which contain no T-DNA can be identified by PCR for the selectable marker or Cas9 genes, and mutations can be screened for using the same PCR/sequencing procedure as used in the T0. The aim is to identify lines

containing no T-DNA and ideally homozygous mutations. While the T-DNA should segregate in a Mendelian fashion in T1, often the targeted mutations do not. Where editing occurred early in the T0, for example, in the founder cell of regenerated plants, T1 mutagenesis may reach 100% (only mutant alleles in siblings). However, where editing has occurred later during T0 plant regeneration, the plants may be chimeric, and fewer T1 siblings are likely to be mutated meaning that a greater number of T1 plants will need to be screened.

This method is modified from our protocol published for barley in the *Methods in Molecular Biology* volume entitled *Barley Methods and Protocols*, Chapter 14 (in press).

2 Materials

2.1 Selection of Target Sequences

1. Online tools are constantly evolving for this purpose, but currently one such facility which links to the *B. oleracea* genome can be found at http://crispr.hzau.edu.cn/cgi-bin/CRISPR/CRISPR.
2. BLAST searches against the *B. oleracea* genome to check off targets can also be done at Ensembl Plants http://plants.ensembl.org/index.html.

2.2 Construct Assembly

1. All oligos and primers can be ordered as PCR grade from suppliers such as Sigma.
2. Level 1 and 2 plasmids are available from Addgene (*see* **Note 1**).
3. *Bsa1* may be purchased from New England Biolabs and stored at −20 °C.
4. T4 ligase is best purchased from New England Biolabs and stored at −20 °C.
5. Water used should be deionized and sterile.
6. A thermocycler.
7. Electrocompetent *E. coli* cells (available from Thermo Fisher).
8. 2 mm electroporation cuvettes (available from Geneflow Ltd).
9. Bio-Rad Gene Pulser 2 or equivalent.
10. Carbenicillin 1000×: 10 mL water, 1 g carbenicillin disodium. Mix to dissolve, then filter sterilize, and store in 1 mL aliquots at −20 °C.
11. IPTG 1000×: 238 mg IPTG, 10 mL water. Mix to dissolve, then filter sterilize, and store in 1 mL aliquots at −20 °C.
12. X-gal 100×: 200 mg X-gal, 10 mL of DMSO. Mix to dissolve, and store in 1 mL aliquots at −20 °C for up to 6 months.
13. Plasmid Miniprep Kit (available from Qiagen).

14. BigDye® cycle sequencing kit (Terminator v3.1 Ready Reaction Mix plus 5X buffer).
15. *Esp3I*, *BsaI*, and *BpiI* (best obtained from Thermo Fisher).
16. 0.2 mL PCR tubes.
17. Spectinomycin 1000×: 1 g spectinomycin dihydrochloride pentahydrate, 10 mL water. Mix to dissolve, then filter sterilize, and store in 1 mL aliquots at −20 °C.
18. LB Medium: 5 g/L yeast extract, 10 g/L NaCl, 10 g/L tryptone, 15 g/l Bacto Agar. Autoclave at 120 °C for 20 min.
19. Laboratory heat block with metal insert to fit Eppendorf tubes.
20. Hybridization buffer: 10 mM Tris–HCl pH 7.5, 50 mM NaCl, 1 mM EDTA.

2.3 Genomic DNA Extraction from B. oleracea

1. 1.5 mL Eppendorf tubes.
2. Buffer1: 200 mM Tris–HCl, pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS.
3. Micropestles to fit 1.5 mL Eppendorf tubes.
4. Propan-2-ol.
5. 70% ethanol.
6. Microfuge.
7. TE: Tris-EDTA buffer at pH 8.

2.4 PCR and Sequencing of Target Loci

1. Commercially available 2× PCR master mix.
2. A thermocycler.
3. PCR grade primers.
4. Molecular biology grade agarose.
5. 10× TBE gel running buffer.
6. Deionized water.
7. Ethidium bromide stock: 10 mg ethidium bromide, 1 mL water. Mix to dissolve and store at 4 °C.
8. Gel loading buffer (commercially available).
9. DNA size marker appropriate to expected band sizes.
10. Alkaline phosphatase (1 unit/μL) which is heat inactivated at 65 °C.
11. Exonuclease 1 (10 units/μL) which is heat inactivated at 80 °C.
12. BigDye® Cycle Sequencing Kit (Terminator v3.1 Ready Reaction Mix plus 5X buffer).

2.5 Identification of Transgene-Free Mutant Lines

1. 9 cm diameter Whatman paper disks.
2. 9 cm petri dishes.
3. Micropore tape.

4. Levington's F1 compost or peat and sand to sow seeds.
5. Levington's F2 compost for pricking out and growing on.
6. 4 cm wide × 6 cm deep pots in 8 × 12 (96) cell format.

3 Methods

3.1 Selection of Target Sequences in Brassica *Genome*

1. Identify 23 nucleotide sequences which are unique in the first exon of the target gene(s). The sequence should conform to the template GN20GG (Fig. 1) and can be present on either the sense or antisense strand. Potential off-target sites should be checked to ensure that mutagenesis is as specific as possible (*see* **Note 2**).
2. Verify target sequences by sequencing: When four target loci have been identified for the target gene, a PCR amplicon (s) covering the target sites should be designed and tested using template genomic DNA extracted from *B. oleracea* DH1012 (*see* **Note 3**). The PCR should be capable of amplifying clean single bands reliably which can be directly sequenced after a SAPX cleanup to yield chromatograms with single peaks covering the target regions. *See* Subheading 3.3 for genomic DNA extraction and SAPX cleanup. *See* Subheading 3.4 for PCR/sequencing of target loci. This sequence can also be used to check whether polymorphisms exist between the database accession used in target selection and the *B. oleracea* DH1012 to be used here in the transformation.
3. If **step 2** is achieved, construct assembly can commence. If **step 2** has not been achieved, then different target sequences should be selected, in a different region or exon where PCR may be more achievable (*see* **Note 4**).

3.2 Construct Assembly

3.2.1 Clone a sgRNA into the Position 3 Guide Acceptor Vector

1. Design appropriate protospacers with each as two complementary oligos. The oligos should include the overhanging ends shown in Table 1 which allow insertion into the *Bsa1* linearized accepter vector (Fig. 2). Table 1 also shows an example target sequence and how this is represented in the complementary oligo pair. Note that the PAM is *not* included in the oligo sequence.
2. Hybridize complementary oligos by preparing the pair at 2 μM in the hybridization buffer within an Eppendorf tube. Heat to 95 °C for 3 min using a metal heat block. Switch the block off, and allow it to slowly return to room temperature, which should take about 45 min.
3. Add the following to a 0.2 mL PCR tube: 100 ng position 3 guide accepter, 1 μL of hybridized oligo pair, 1 μL of 10 × T4 ligase buffer, water to 8.5 μL, 0.5 μL (10 units) *Bsa1*, 1 μL

Table 1
Template oligos for making sgRNA sequences

Generic template	5'ATTGNNNNNNNNNNNNNNNNNN 3' 3' NNNNNNNNNNNNNNNNNNCAAA5'	5' AGTGCTTGNNNNNNNNNNNNNNNNNNNGTTT 3' 3' GAACNNNNNNNNNNNNNNNNNNCAAAATCT 5'
Example target	GTGACCATGGAGGACGTGGTCGG	GACGGCGGCCACGACCTCCATGG
Example incorporated	5' ATTGTGACCATGGAGGACGTGGT 3' 3' ACTGGTACCTCCTGCACCACAAA 5'	5' AGTGCTTGACGGCGGCCACGACCTCCAGTTT 3' 3' GAACTGCCGCCGGTGCTGGAGGTCAAAATCT 5'

Compatible restriction enzyme site overhangs are shown in red. The PAMs in example target sequences are underlined

(400 units) T4 ligase. Start the reaction immediately in a thermocycler as follows: 1 × 20 s at 37 °C, 26 × (37 °C for 3 min, 16 °C for 4 min), 1 × 50 °C for 5 min, 1 × 80 °C for 5 min.

4. Transform 1 μL of the reaction into electrocompetent *E. coli* cells using 2 mm electroporation cuvettes, and conduct electroporation with conditions specified by the manufacturer. Plate the cells onto LB agar containing 100 mg/L carbenicillin, 0.1 mM IPTG, and 200 μg/mL X-gal. Incubate overnight at 37 °C.
5. The day after, inoculate three white colonies into separate 10 mL liquid LB vessels containing 100 mg/L carbenicillin. Grow the cells overnight at 37 °C with vigorous shaking (*see* **Note 5**). Extract plasmid DNA with a miniprep kit.
6. Sequence each plasmid by Sanger sequencing using the primer 5′TAGGAGGGAATCGAACTAGGAATATTG3′. Reactions are set as follows: 200 ng plasmid, water to 6.5 μL, 1.5 μL BigDye 3.1 buffer, 1 μL of 10 μM primer, 1 μL of BigDye version 3.1 (added last). Run the reaction as follows: 1 × 96 °C for 1 min, 25 × (96 °C for 10 s/50 °C for 5 s/60 °C for 4 min). Send complete reactions to one of the specialist companies dealing with capillary electrophoresis of such materials. Alternatively, plasmids and the primer can be provided to a commercial vendor specialized on Sanger sequencing.
   Validate successful cloning of sgRNA into the position 3 guide accepter vector. The correct sequence around the incorporation site is shown below: 5' TAGAGTCGAAGTAGTGATT G(NX19)GTTTTAGAG 3′. The sense strand is depicted, and the first base of transcription is the G directly 5′ to the NX19. Ns indicate protospacer sequence, and the G directly 3′ to this is the beginning of the non-variable section of sgRNA. The entire sequence of the position 3 guide accepter vector is available via Addgene (*see* **Note 1**).

3.2.2 Clone an sgRNA into the Position 4 Guide Acceptor Vector (Concurrent with Subheading 3.2.1)

1. Design appropriate protospacers with each as two complementary oligos. The oligos should include the overhanging ends shown in Table 1 which allow insertion into the *Esp3I* linearized accepter vector (Fig. 2). Table 1 also shows an example target sequence and how this is represented in the complementary oligo pair. Note that the PAM is *not* included in the oligo sequence.
2. Hybridize the pair as in **step 2** of Subheading 3.2.1 above.
3. Add the following to a 0.2 mL PCR tube: 100 ng position 4 guide accepter, 1 μL of hybridized oligo pair, 1 μL of 10 × T4 ligase buffer, water to 8.5 μL, 0.5 μL (10 units) *Esp3I*, 1 μL (400 units) T4 ligase. Start the reaction immediately in a thermocycler as follows: 1 × 20 s at 37 °C, 26 × (37 °C for 3 min, 16 °C for 4 min), 1 × 50 °C for 5 min, 1 × 80 °C for 5 min.
4. Transform the reaction into electrocompetent *E. coli* cells (refer to **step 4** of Subheading 3.2.1 for details).
5. Culture cells and miniprep plasmids (refer to **step 5** of Subheading 3.2.1 for details).
6. Sequence the plasmids by Sanger sequencing (refer to **step 6** of Subheading 3.2.1 for details).
7. Validate successful cloning of sgRNA into the position 4 guide accepter vector (refer to **step** 7 of Subheading 3.2.1 for details).

3.2.3 Golden Gate Assembly of a Final T-DNA Vector

1. Set up a Golden Gate assembly with four level 1 components (L1P1 *Brassica* kanamycin selection, L1P2 Cas9, L1P3 sgRNA cassette 1, and L1P4 sgRNA cassette 2) and a level 2 accepter (*see* **Note 1**) (Fig. 3) in a 0.2 mL PCR tube as follows: 100 ng level 2 accepter plasmid, 300 ng L1P1 kanamycin selection cassette, 300 ng L1P2 Cas9 cassette, 300 ng L1P3 sgRNA cassette 1, 300 ng sgRNA cassette 2, 1 μL of 10 × T4 ligase buffer, water to 8.5 μL, 0.5 μL (10 units) *BpiI*, and 1 μL (400 units) T4 ligase. Run the reaction immediately in a thermocycler as follows: 1 × 20 s at 37 °C, 26 × (37 °C for 3 min, 16 °C for 4 min), 1 × 50 °C for 5 min, 1 × 80 °C for 5 min.
2. Transform 1 μL of the reaction into electrocompetent *E. coli* cells by electroporation. Plate the cells onto LB agar containing 100 mg/L spectinomycin, 0.1 mM IPTG, and 200 μg/mL X-gal. Incubate overnight at 37 °C.
3. The day after, inoculate three white colonies into separate 10 mL liquid LB vessels containing 100 mg/L carbenicillin. Grow the cells overnight at 37 °C with vigorous shaking. Extract plasmid DNA with a miniprep kit.

4. Verify the plasmid clones by restriction digestion and sequencing. The full sequence for all level 1 and 2 component plasmids is available via Addgene (*see* **Note 1**).

3.3 Brassica Transformation and Genomic DNA Extraction

1. Transform *Agrobacterium tumefaciens* strain AGL1 with the assembled dual sgRNA T-DNA vector, and then transform *B. oleracea* DH1012 by *Agrobacterium*-mediated transformation (*see* **Note 6**).
2. Collect leaf samples from T0 *B. oleracea* plants for DNA extraction. Take two pieces of leaf around 1 cm^2 from different parts of T0 plants, and pool to make a single DNA prep. Collect samples in 1.5 mL Eppendorf tubes, and keep cool on ice during leaf collection.
3. Add 600 μL buffer 1 to each tube, and grind the leaves using micropestles until all large particles are fragmented and the liquid becomes dark green.
4. Spin the tubes in a benchtop microfuge at the full speed for 10 min.
5. Transfer 500 μL of the supernatant to a fresh 1.5 mL Eppendorf tube.
6. Add 500 μL propan-2-ol (equal volume). Vortex and spin it at the full speed in a microfuge for 20 min. Discard the liquid carefully, and wash the pellet with 0.5 mL of 70% ethanol.
7. Spin the tubes again at the full speed for 10 min. Carefully remove all liquid, and allow the pellet to air-dry for 20 min. Resuspend the pellets each in 100 μL of 1 × TE.

3.4 PCR and Sequencing of Target Loci for Mutations

1. Design primers for amplifying target loci using primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/), and test these primers using wild-type DNA extracted using the protocol described in Subheading 3.3.
2. Carry out PCR in 20 μL volumes using commercially available 2× master mixes. Add primers to a final concentration of 150 nM, and use 1 μL of extracted genomic DNA as template.
3. Run 5 μL of each reaction in 1% agarose gel made with 1× TBE supplemented with ethidium bromide to a concentration of 0.5 μg/mL (5 μL of 10 mg/mL stock per 100 mL gel). Verify if the amplicons are of expected sizes. Large deletions resulting from targeted mutagenesis may be visible by a band shift to a lower position relative to a wild-type control (Fig. 4c).
4. Prepare the remaining 15 μL of PCR for sequencing by adding 1 unit of alkaline phosphatase (heat inactivatable) and 10 units of exonuclease 1 (heat inactivatable). Incubate at 37 °C for 30 min and then 80 °C for 20 min.

5. Sequence PCR amplicons by Sanger sequencing as in **step 6** of Subheading 3.2.1 (*see* **Note 7**).
6. Decode the sequencing results (Fig. 4c) by computer programs such as DeDecode (*see* Chapter 3 of this book).
7. Grow T0 lines where mutagenesis has been detected to T1 generation for mutation inheritance as well as segregation of the T-DNA.

3.5 Identification of Transgene-Free Mutant Lines

1. Select 4 active T0 mutant lines and from each collect 40 harvested seeds. Place them on four sheets of wet Whatman paper within 9 cm petri dishes, and seal with micropore tape. Store the plates at 4 °C for 2 days before moving to 23 °C under 16-h day length of 70 μmol/m^2/s. Seeds should germinate uniformly in about 4 days.
2. Transplant 24 T1 seedlings per T0 line in soil in 4 cm pots which are available in 96-cell formats for ease of organization. Grow plants in a glasshouse with day/night temperatures of 18 °C/12 °C, 16-h day length, with supplementary lighting (high-pressure sodium lamps with an average bench reading of 200 μmol/m^2/s^1). Fertilize plants weekly with a 2:1:1 NPK fertilizer. After around 3–4 weeks, the plants should be well established, and leaf material may be sampled and used for DNA extraction as described previously in Subheading 3.3.
3. Genotype individual T1 lines for targeted mutations with the protocol described in Subheading 3.4. This time due to chromosome segregation, there is a much higher likelihood of identifying homozygous mutants.
4. To test for the presence or absence of the T-DNA, conduct PCR with primers specific to the *nptII* coding sequence. Set up each PCR reaction as follows: 10 μL of 2 × PCR master mix, 7 μL water, 1 μL of template DNA, and 1 μL each of the following primer pair at 10 μM stock concentration—F_ATGAACAAGATGGATTGCAC, R_ TGAGATGACAGGAGATCCTG. Run PCR reactions as follows: 1 × 94 °C for 3 min, 35 × (94 °C for 30 s, 58 °C for 45 s, 72 °C for 30 s). Include no template negative control and genomic DNA sample known to contain *nptII* as a positive control (*see* **Note 8**).
5. Run 5 μL of each reaction in 2% agarose gel. A single band of 313 bp indicates the presence of the T-DNA transgene. Otherwise, the T1 lines are most likely transgene-free. T1 plants which contain no T-DNA but have been observed to contain targeted mutations can now be classified as transgene-free mutants. Homozygous mutants which are transgene-free may be found at this stage; however it may be necessary to go into T2 generation to identify such lines.

4 Notes

1. Plasmids used here are available at Addgene along with the corresponding sequences and maps: L1P1 kanamycin selection cassette (pICSL11055; Addgene #68252), L1P2 Cas9 expression cassette (pICSL11060; Addgene #68264), L1P3 guide accepter (pBoL1P3GA; Addgene #112909), L1P4 guide accepter (pBoL1P4GA; Addgene #112910), L2 accepter (pAGM8031; Addgene #48037).
2. Various online tools are available to aid in guide selection and also give a score for predicted off-target activity. Many are linked to various genomes, and one which currently does this for *B. oleracea* can be found at http://crispr.hzau.edu.cn/cgi-bin/CRISPR/CRISPR. Mismatches to off-target sites at the PAM distal end of the sgRNA will be tolerated more so than at the proximal end and will still potentially allow off-target mutagenesis to occur. If mismatches to off-target sites occur in the eight nucleotides directly adjacent to the PAM within the protospacer, these are less tolerated and much more likely to prevent off-target activity.
3. Shorter amplicons generally work better than longer ones. We prefer to amplify between 300 and 900 base pair fragments where possible. Primers should be designed no closer than 100 nucleotides from the closest target sequence to accommodate Sanger sequencing.
4. Where target sequences conforming to GN20GG are limiting, then it is possible to simplify this to N21GG. Because the preferred start base of transcription from the *Arabidopsis* U626 promoter is G, a 5′ G should still be included in the cloned oligo sequence. The only impact this has to the oligo design (Table 1) is that it now contains NX20 instead of NX19. The PAM is still *not* included in the oligo sequence.
5. The L1P3 guide accepter contains a lacZ cassette between the two *Bsa1* sites. Successful oligo insertion is indicated by a loss of blue coloration when grown on X-gal/IPTG. The L1P4 accepter contains a cassette giving purple coloration between two *Esp3I* sites. Where oligo insertion has not occurred, colonies will develop a faint purple color. Where the oligos have inserted, colonies will be white.
6. A detailed method for the transformation of *B. oleracea* genotype DH 1012 can be found at https://www.jic.ac.uk/media/cms_page_media/638/Brassica%20oleracea%20transformation%20protocol.pdf.
7. We have found the amount of SAPX clean PCR product used as Sanger sequencing template quite flexible. Generally, 1 μL amplicons up to 1 kb in length is sufficient when the 5 μL

tested on agarose gave a bright band. However, the BigDye 3.1 manufacturer recommendations are to use 3–10 ng from 200 to 500 bp, 5–20 ng from 500 to 1000 bp, and 10–40 ng from 1000 to 2000 bp.

8. When genotyping T1 material for the presence/absence of T-DNA, it is of utmost importance that false positives or negatives are not allowed to occur. Assuming the suggested positive control results in the 313 bp band as it should, the greatest risk is of lines which actually contain no T-DNA giving a spurious 313 bp band. To avoid this problem, keep *NptII* plasmids well away, use fresh solutions, and always use filter tips.

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Chapter 13

Application of CRISPR/Cas9-Mediated Gene Editing in Tomato

Nathan T. Reem and Joyce Van Eck

Abstract

CRISPR-/Cas9-mediated gene editing has been demonstrated in a number of food crops including tomato. Tomato (*Solanum lycopersicum*) is both an important food crop and a model plant species that has been used extensively for studying gene function, especially as it relates to fruit biology. This duality in purpose combined with readily available resources (mutant populations, genome sequences, transformation methodology) makes tomato an ideal candidate for gene editing. The CRISPR/Cas9 system routinely used in our laboratory has been applied to various tomato genotypes and the wild species, *Solanum pimpinellifolium*. The vector system is based on Golden Gate cloning techniques. Cassettes that contain the neomycin phosphotransferase II (*NPTII*) selectable marker gene that confers resistance to kanamycin, a human codon-optimized Cas9 driven by the CaMV 35S promoter, and guide RNA (gRNA) under control of the *Arabidopsis* U6 polymerase promoter are assembled into a T-DNA vector. Generally, we design CRISPR/Cas9 constructs that contain two gRNAs per gene target. However, we have been successful with inclusion of up to eight gRNAs to simultaneously target multiple genes and regions. Introduction of CRISPR-/Cas9-designed constructs into tomato is accomplished by transformation methodology based on *Agrobacterium tumefaciens* infection of young cotyledon sections and selection on kanamycin-containing medium based on the presence of the *NPTII* gene. The approaches for the development of CRISPR/Cas9 constructs and genotypic analyses (PCR-based amplicon sequencing and T7 endonuclease) are detailed in this chapter.

Key words Genome editing, CRISPR/Cas9, PCR amplicon sequencing, Solanaceae, *Solanum lycopersicum*, *Solanum pimpinellifolium*, T7 endonuclease 1 (T7E1) assay

1 Introduction

Soon after the first reports of CRISPR/Cas9-mediated gene editing, there was a rapid succession of reports that demonstrated successful editing in a multitude of organisms including plants. The long-awaited ability to precisely and straightforwardly edit genes of interest sparked what could be viewed as a revolution for investigation of gene function across all organisms [1]. Tomato presented an ideal test model to demonstrate the feasibility in an important food crop because of all the resources available that

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include genomic sequence for more than 500 genotypes as well as wild species, transcriptome data, mutant populations, and *Agrobacterium*-mediated transformation methodology that has been applied to many genotypes [2–4]. All of these resources provide a comprehensive platform that aids CRISPR/Cas9 gene editing in tomato.

Tomato (*Solanum lycopersicum*), native to South America, is a member of the Solanaceae family, which is comprised of nearly 3000 species. There are two market types of tomatoes, fresh market and processing. According to the Agricultural Marketing Resource Center, in 2015 the US dollar value for fresh market tomatoes was 1.22 billion and 1.39 billion for processing types, which are used to make products such as juice, sauces, and ketchup [5]. Tomato is a perennial plant that has two different growth habits. The most common form is indeterminate that represents a sprawling type of plant architecture, whereas determinate is a compact form of growth.

From the first proof-of-concept experiments to demonstrate effective editing to experiments where genes were edited to alter plant architecture, study characteristics of fruit biology, and investigate metabolic engineering approaches, CRISPR/Cas9 has proven to be a powerful tool for advancing gene function studies and trait modification in tomato [6–8]. In addition to recovery of transgenic lines that contained the intended insertion/deletion mutations (indels), indels were shown to be transmitted into the next generations, and through segregation, lines were found that did not contain T-DNA but retained the indels [7, 9]. The ability to precisely target a gene to affect its expression has value beyond studies of gene function. As is the case with an important food crop like tomato, gene editing will play a key role in facilitating improvement programs through breeding efforts in some instances, such as where tight linkages of genes might preclude modification of a trait.

The methods reported in this chapter for design of CRISPR/Cas9 constructs for gene editing in tomato are based on a vector system previously described and is available through Addgene (www.addgene.org) [7, 10, 11]. Briefly, the method follows a Golden Gate cloning system that contains cassettes for the neomycin phosphotransferase II (*NPTII*) selectable marker gene that confers resistance to kanamycin, a human codon-optimized Cas9, and a guide RNA driven by the U6 promoter from *Arabidopsis*. We routinely include two gRNAs to target a gene of interest but have gone as high as eight gRNAs within one construct to target multiple genes. CRISPR/Cas9 constructs are delivered by an *Agrobacterium*-mediated transformation method into tomato cotyledons [4]. Putative edited lines are genotyped by either a PCR-based amplicon sequencing method or T7 Endonuclease 1 (T7E1) digestion assay [7, 12]. Edited lines are transferred to soil and evaluated for phenotype modifications as compared to non-edited control plants (wild type).

2 Materials

2.1 CRISPR Construct Design

1. Plasmid vectors—obtained from Addgene (www.addgene.org): pICH86966, pICSL01009, pAGM4723, pICH47751, pICH47761, pICH47732_NPTII, pICH47742_35S:Cas9, and pICH41780.
2. Phusion DNA polymerase (or other high-fidelity polymerase) along with all needed components for PCR (dNTPs, research-grade water, $MgCl_2$, etc.).
3. 2% agarose gels.
4. Restriction endonucleases: ApoI, BsaI, BbsI/BpiI (*see* **Note 1**), HindIII, and PmeI.
5. Primers:
   (a) pICH51F: 5′-aggatatattggcgggtaaac
   (b) pICH51R: 5′-ctgcatccaccccagtacat
   (c) pAGM4723F: 5′-ataagcccatcagggagcag
   (d) pAGM4723R: 5′-cggataaacctttcacgcc
   (e) 35S-Cas9-F: 5′-ctgacgtaagggatgacgcac
   (f) Cas9-R: 5′-catctcattactaaagatctcc
6. Forward primers for gRNA synthesis: see below for primer design.
7. Reverse primer for gRNA synthesis:
   (a) CR-gRNA-R: tgtggtctcaAGCGTAATGCCAACTTTGTAC
8. T4 DNA Ligase with buffer and 10× bovine serum albumin (BSA).
9. Ultracompetent *E. coli* (DH5α, DH10β, or others).
10. LB plates with X-gal (20 μg/mL) and carbenicillin (50 μg/mL).
11. LB plates with kanamycin (50 μg/mL).

2.2 Genotyping of Transgenic Plants

1. CTAB extraction buffer: 100 mM Tris, pH 8.0, 20 mM EDTA, pH 8.0, 0.35 mM NaCl, 0.137 mM cetyltrimethyl ammonium bromide (CTAB), 1.25 mM polyvinylpyrrolidone, 2% β-mercaptoethanol.
2. Taq DNA polymerase with 3′ A-overhangs.
3. T7 Endonuclease I.

3 Methods

3.1 Selection and Design of Guide RNAs

Guide RNA (gRNA) design and selection are critical steps in successful genome editing with CRISPR/Cas9. The major constraint for Cas9 is its recognition of the protospacer adjacent motif (PAM) site, 5'-NGG-3′. Thus, the gRNAs must contain the sequence directly upstream (5′) of, but not including, the PAM. If the overall goal is to knock out gene expression, it is important to target a region of the coding sequence near the transcriptional start site. If the goal is to modify expression levels, the promoter region can also be targeted with multiple gRNAs.

The best option for gRNA design is to use a webtool to optimize gRNA design: CRISPR-P and CRISPR-Direct are two design webtools that are freely available [13, 14]. The advantage of using a CRISPR design webtool is the reduced likelihood of undesirable off-target DNA recognition, since most tools scan the genome for highly matched sequences. gRNAs can also be designed manually, but it is important to BLAST the gRNA (including the PAM) sequences back to the genome. Fortunately, the *Solanum lycopersicum* genome is sequenced, and thus likelihood of off-targeting can be determined; this is impossible to do in species without a draft or complete genome.

After selecting the gRNA, primers should be synthesized using the following sequence:

5′-tgtggtctcaATTGNNNNNNNNNNNNNNNNNNgttttagagctagaaatagcaag-3′,

where N = ~19 bp directly upstream (to the 5′ of) the target PAM. Do not include the PAM sequence in the gRNA primer.

For each gRNA or pair of gRNAs, design a second set of PCR primers flanking the region for later use. These genotyping primers will be used for sequencing and should be at least 50 bp outside of the target region for good sequence quality. This primer set will be used later, when screening plants for insertion/deletions (*see* **Note 2**).

3.2 Synthesis of Guide RNAs

Our lab uses a vector system described by Nekrasov et al. (2013) because of its simplicity and low cost per sample. This protocol describes cloning with vectors from this system. However, there are many other useful vector systems for preparing and cloning gRNAs, all of which should be suitable for genome editing. For the readers' convenience, we have included a flowchart depicting an overview of CRISPR design (Fig. 1).

1. PCR amplify each guide RNA separately using the plasmid pICH86966 as a template in a 30–50 μL reaction.
2. To confirm amplification, run 3 μL of sample on a 2% agarose gel, and observe a single band 150 bp in length. Purify the

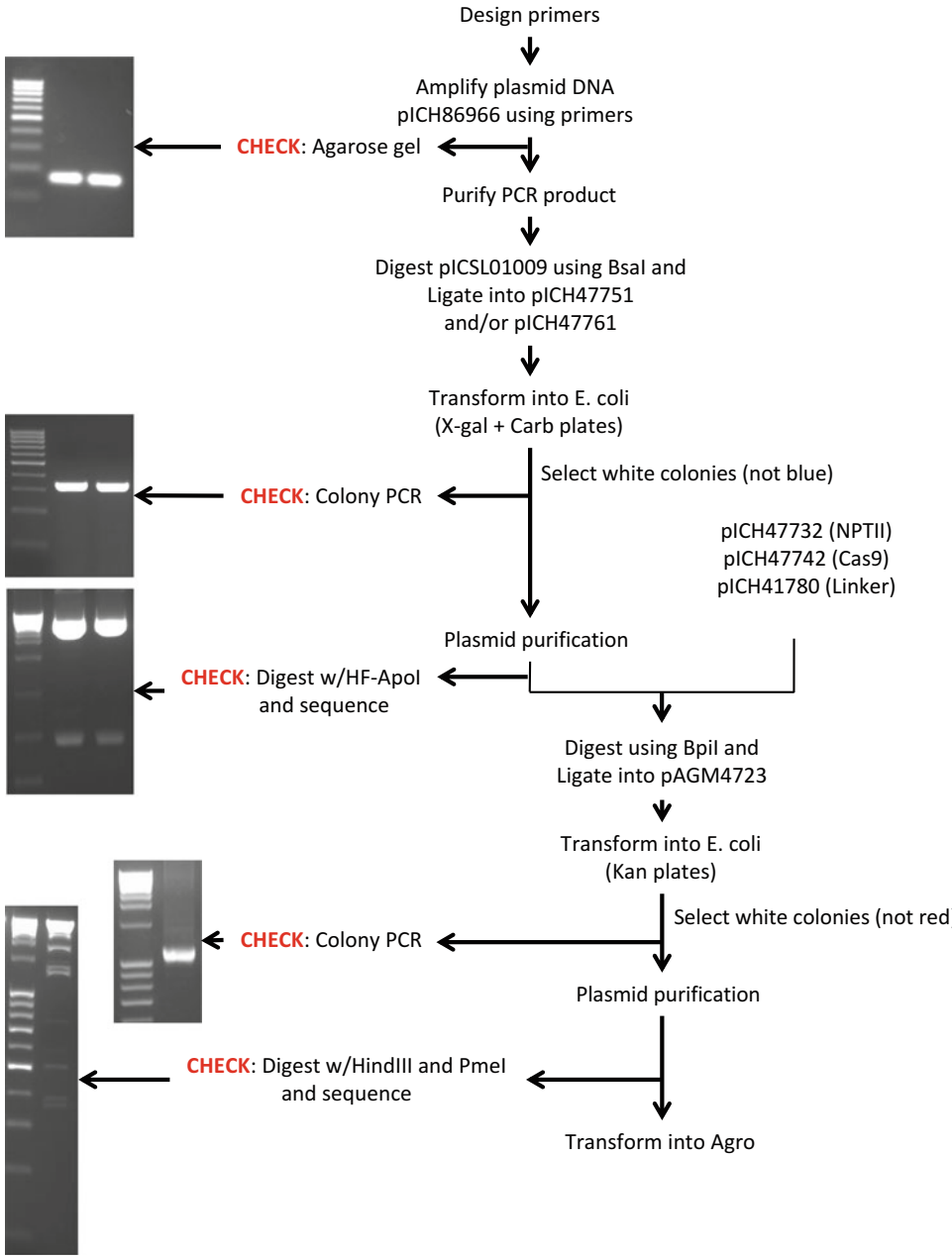

Fig. 1 CRISPR design flowchart. Abbreviated steps for synthesis of CRISPR/Cas9 constructs are shown. Gel images show expected fragment sizes at each PCR and restriction digest step

remaining PCR reaction, and measure the concentration. Proceed immediately to Level 1 cloning.

3.3 Level 1 Cloning

1. Prepare digestion-ligation reactions separately for each gRNA, adding 100 ng of each DNA component in separate PCR tubes (Fig. 2a). Mix the reactions thoroughly by pipetting, and

a

Guide 1	Guide 2	Volume
10X T4 Ligase Buffer	10X T4 Ligase Buffer	1.5 µL
10X BSA	10X BSA	1.5 µL
PCR Guide 1 (100 ng)	PCR Guide 2 (100 ng)	X µL
pICH47751 (100 ng)	pICH47761 (100 ng)	X µL
pICSL01009_u6pro (100 ng)	pICSL01009_u6pro (100 ng)	X µL
BsaI	BsaI	1.0 µL
T4 DNA Ligase	T4 DNA Ligase	1.0 µL
Water	Water	To 15 µL

b

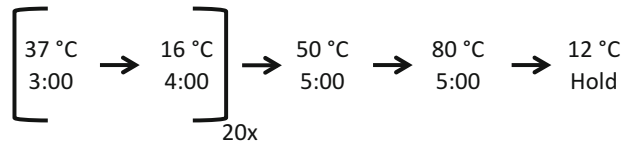

Fig. 2 Reaction conditions for Level 1 digestion-ligation. (**a**) Master mix for Level 1 cloning, which incorporates the gRNA and U6 promoter sequences into the pICH47751/61 vector backbone. (**b**) Thermocycler protocol for quick digestion-ligation of Level 1 vectors

perform digestion-ligation in a thermocycler using the reaction conditions shown in Fig. 2b.

2. Heat shock 50 µL of chemically competent *E. coli* with 3 µL of digestion-ligation reaction. Plate cells on LB/X-Gal/carbenicillin, and incubate overnight at 37 °C (*see* **Note 3**).
3. The next day, blue and white colonies should be present. Pick four white colonies for colony PCR using pICH51F and pICH51R primers. Positive clones produce 350 bp amplicons; negative clones produce 800 bp amplicons.
4. Grow positive colonies in liquid LB with carbenicillin overnight, and purify plasmid DNA.
5. Run confirmatory restriction digestion of 250–1000 ng of plasmid DNA with ApoI. Positive clones should produce bands at 3.9 kb and 690 bp (*see* **Note 4**).
6. Sequence the plasmids using the pICH51-F primer to confirm no mutations are present in the gRNA sequence.

3.4 Level 2 Cloning

1. Prepare the Level 2 digestion-ligation reaction by adding each component according to Fig. 3. Run the same thermocycler protocol as Level 1 digestion-ligation (Fig. 2b).
2. Transform *E. coli* with 3 µL of reaction, and then plate 50 µL on LB/kanamycin plates.
3. After overnight incubation, orange and white colonies should both be present; select four white colonies for colony PCR with

Reagent	Volume (μL)
10X T4 Ligase buffer	1.5 μL
pICH47732_NPTII (100 ng)	X μL
pICH47742_35S:Cas9 (100 ng)	X μL
pICH47751-Guide 1 (100 ng)	X μL
pICH47761-Guide 2 (100 ng)	X μL
pICH41780 (100 ng)	X μL
pAGM4723 (100 ng)	X μL
BpiI	1 μL
T4 Ligase	1 μL
Water	to 15 μL

Fig. 3 Master mix for Level 2 cloning. This step incorporates the Level 1 gRNA +U6 promoter with 35s:Cas9 and NPTII selection medium into the pAGM4723 vector backbone

primers CR-pAGM-F and CR-pAGM-R. Positive samples will contain a band 1136 bp in length.

4. Purify plasmid DNA from a positive colony. Elute plasmid DNA in 40 μL of distilled or deionized water, not elution buffer (*see* **Note 5**).
5. Run a confirmatory restriction digest of 250–1000 ng of plasmid using the restriction enzymes HindIII and PmeI. Bands present should be 73, 366, 387, 509, 1300, 1800, 2500, and 5500 bp.
6. Sequence the positive plasmid using CR-pAGM-F and CR-pAGM-R primers for further validation.

3.5 Agrobacterium-Mediated Transformation

Agrobacterium-mediated transformation of tomato is a lengthy, time-intensive process that requires specialized equipment, lab setup, and expertise. Many labs are not well equipped to conduct plant transformations. Thus, many researchers choose to utilize services offered through plant transformation facilities to ensure efficient transformation of their constructs. While some researchers will prefer to undertake transformation themselves, it involves many steps that cannot be described succinctly in this chapter; it deserves its own separate protocol, and we refer the reader to a detailed protocol described by Van Eck et al. [4].

3.6 Confirmation of Successful Edits

For the readers' convenience, we have included a flowchart showing the general steps for confirmation of CRISPR edits (Fig. 4).

1. Carefully excise a ~1 cm^2 piece of leaf tissue from recovered transgenic lines, transfer to a 1.5 mL microcentrifuge tube, and freeze for DNA extraction. Our lab extracts DNA from in vitro grown plants using a modified CTAB DNA extraction [15].

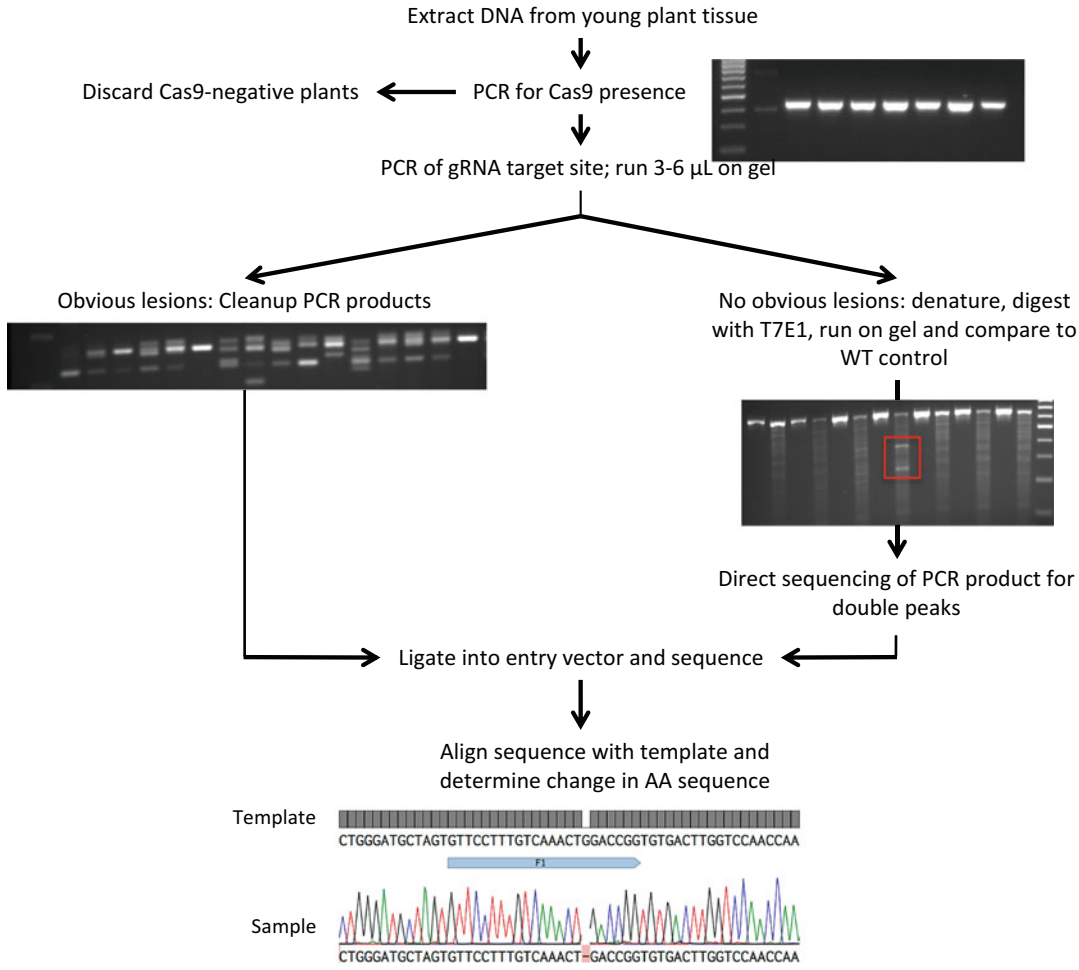

Fig. 4 Flowchart for analysis of CRISPR edits in transformed plants. After DNA extraction, plants are PCR assayed for large deletions and, if necessary, T7E1 assayed for small indels. PCR fragments are sequenced and aligned to the template sequence to confirm sequence changes

2. After DNA extraction, determine concentration, and dilute samples to a uniform DNA concentration (e.g., 10 ng/µL).
3. Perform a PCR for presence of Cas9 using primers 35S-Cas9-F and Cas9-R. Discard lines that are Cas9 negative.
4. Perform PCR to amplify the gRNA target site, using a Taq DNA polymerase that leaves 3′ A-overhangs for downstream TA cloning.
5. Run a small amount (3–6 µL) of each PCR reaction on a 2–3% agarose gel, and image it to determine the presence of large deletions. Put the remaining volume of PCR reactions on ice for later (*see* **Note 6**).

6. Samples with multiple bands, or a single band smaller than the control, have most likely undergone nonhomologous end joining (NHEJ)-mediated deletions from both gRNAs. Clean up the PCR products of these samples, and sequence directly, or subclone them into an entry TA cloning vector, followed by sequencing of individual clones (*see* **Note 7**).
7. If only one band is present in a sample, it is possible that only one of the gRNAs have caused NHEJ-mediated deletions of ~1–4 bp. To discern whether a single cut has been made, we use a T7 endonuclease I (T7E1)-based assay derived from [16]. T7E1 is a simple assay that cleaves mismatched DNA base pairs, resulting in unique fragmentation patterns after gel electrophoresis. The brief protocol is as follows (*see* **Note 8**):
   (a) From the remaining PCR solution, aliquot 8.5 μL into a new PCR tube.
   (b) In a thermocycler, heat the solution to 95 °C for 5 min to completely denature the amplicon, and then slowly decrease the temperature to 16 °C at the slowest ramp speed available (our lab uses −1 °C/s).
   (c) After denaturation, add 1 μL 10× NEBuffer 2 and 0.5 μL T7E1 enzyme to a total volume of 10 μL, and incubate at 37 °C for at least 30 min.
   (d) After incubation, run the T7E1 samples on a gel alongside 8.5 μL of original PCR product.
8. The resulting gel should contain lanes of smears with subtle bands of differing sizes (Fig. 4). Carefully observe fragment sizes in the control sample, and compare them with fragments in Cas9-positive samples. Samples with faint bands that are not present in control samples are candidates for successful edits (Fig. 4, red box). If the primers are at different distances from the gRNAs, it is possible to determine which gRNA is cutting based on the length of fragments present (*see* **Note 2**).
9. For the samples displaying unique bands after PCR or T7E1, clean up and purify the undigested PCR fragments, and sequence directly. Purified PCR fragments can also be ligated into an entry TA cloning vector, transformed into *E. coli,* and sequenced to determine specific targeted mutations.

3.7 Selection and Breeding of Plants with Genome Edits

1. After sequencing, align sequence file with reference gene, and determine the size of edit and the resulting change in amino acid sequence.
2. Transplant well-rooted T_0 plants with unique edits from in vitro conditions to soil, and grow to maturity in a greenhouse. These plants are selfed to produce T_1 progeny. Fruits are

picked when ripened, and seeds are collected from individual fruits.

3. Plant a population of 50 or more T_1 seeds in soil and PCR screen for the same deletions seen in the parental T_0 lines using the same methods described above. Screen plants by PCR for the presence of Cas9. Keep Cas9-negative edited plants and discard Cas9-positive plants.
4. Let Cas9-negative edited plants grow until maturity. During the period, either let the T_1 plants self-pollinate to produce homozygous T_2 genome-edited lines, or back-cross the plants to a non-transformed, wild-type plant to produce heterozygous T_2 plants.
5. When necessary, grow heterozygous T_2 plants to produce T_3 lines by selfing. Identify homozygous genome-edited Cas9-negative plants with the abovementioned genotyping method.
6. Observe phenotype of genome-edited lines (*see* **Note 9**).

4 Notes

1. We use BpiI manufactured by Thermo Scientific because we have found that BbsI restriction enzyme from New England Biolabs is unstable at −20 °C.
2. It is advantageous to design primers that are different lengths away from their closest gRNAs; for example, the forward primer may be 75 bp upstream from gRNA 1, and the reverse primer may be 125 bp downstream from gRNA 2. This is useful for the T7E1 assay described below.
3. Use ultracompetent cells for good efficiency.
4. It is possible to have bands of other sizes due to genomic DNA contamination. As long as 3.9 kb and 690 bp fragments are present, proceed to Level 2 cloning.
5. Elution buffers can contain salts that will produce electrical arcs during electroporation of *Agrobacterium*.
6. Since CRISPR edits can result in very small differences in band size, it is advantageous to have clear, unsaturated bands when run on a gel. We recommend modifying amount of template and number of PCR cycles or amount of DNA added to the gel until bands are no longer oversaturated.
7. Sequenced PCR fragments with deletions will produce clear electropherogram peaks until the deletion site, at which point double peaks will be present throughout the remainder of the sequence. Once you have determined that a deletion is present, ligate the PCR product into an entry vector, and sequence the vector to characterize the deletion more clearly.

8. It is not necessary for samples with multiple bands to undergo the T7E1 assay because it is already known that a large deletion is present.
9. Phenotype may be observed in the T_1, T2, and T_3 generations because it is possible that different gene edits and gene dosages can cause a gradient of phenotypic severity across a population.

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Chapter 14

Genome Editing in Potato with CRISPR/Cas9

Satya Swathi Nadakuduti, Colby G. Starker, Daniel F. Voytas, C. Robin Buell, and David S. Douches

Abstract

Cultivated potato, *Solanum tuberosum* Group Tuberosum L. ($2n = 4x = 48$) is a heterozygous tetraploid crop that is clonally propagated, thereby resulting in identical genotypes. Due to the lack of sexual reproduction and its concomitant segregation of alleles, genetic engineering is an efficient way of introducing crop improvement traits in potato. In recent years, genome-editing via the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 system for targeted genome modifications has emerged as the most powerful method due to the ease in designing and construction of gene-specific single guide RNA (sgRNA) vectors. These sgRNA vectors are easily reprogrammable to direct *Streptococcus pyogenes* Cas9 (*Sp*Cas9) to generate double stranded breaks (DSBs) in the target genomes that are then repaired by the cell via the error-prone non-homologous end-joining (NHEJ) pathway or by precise homologous recombination (HR) pathway. CRISPR/Cas9 technology has been successfully implemented in potato for targeted mutagenesis to generate knockout mutations (by means of NHEJ) as well as gene targeting to edit an endogenous gene (by HR). In this chapter, we describe procedures for designing sgRNAs, protocols to clone sgRNAs for CRISPR/Cas9 constructs to generate knockouts, design of donor repair templates and use geminivirus replicons (GVRs) to facilitate gene-editing by HR in potato. We also describe tissue culture procedures in potato for *Agrobacterium*-mediated transformation to generate gene-edited events along with their molecular characterization.

Key words Potato, CRISPR/Cas9, Plant genome-editing, Targeted mutagenesis, Single guide RNA, *Agrobacterium*-mediated transformation, Tissue culture

1 Introduction

Cultivated potato, *Solanum tuberosum* Group Tuberosum L. ($2n = 4x = 48$) is an autotetraploid with a highly heterozygous genome [1, 2]. Due to its high allelic diversity, complex segregation, and reduced sexual fertility, potato is vegetatively propagated, and as a consequence genetic engineering is an efficient way to introduce crop improvement traits. In recent years, genome-editing by CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated systems9) technology derived from bacterial type-II CRISPR/Cas immune system has

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emerged as the most powerful technology for targeted DNA manipulations [3]. This technology can be used for precise modification of gene targets including knocking out or editing a gene. By utilizing a single chimeric guide RNA (sgRNA), which contains a 20-nt spacer sequence for DNA recognition, the Cas9 endonuclease is directed to make double stranded breaks (DSB) in DNA at the target site adjacent to a protospacer adjacent motif (PAM). PAM requirement for the most commonly used *Streptococcus pyogenes* Cas9 is 5′-NGG-3′ adjacent to selected protospacer sequence. DSB repair in the cell is mediated by the cell's native non-homologous end-joining (NHEJ) pathway which can lead to gene knockouts or by precise homologous recombination (HR) to edit genes. CRISPR/Cas9 technology has been successfully implemented in potato for targeted mutagenesis (knockout gene function) as well as gene targeting (edit an endogenous gene) [4–6]. In this chapter, we describe a detailed procedure for designing sgRNAs to target specific genes of interest. In addition, protocols to clone sgRNAs into suitable vectors to make plant expression CRISPR/Cas9 constructs to generate knockout mutations in potato plants are described, along with methods to design donor repair templates and use geminivirus replicons (GVRs) to facilitate gene-editing by HR in potato. The use of GVRs has been shown to increase gene-targeting frequencies (where a donor template is supplied) by improving the frequency of HR [7]. GVRs result in an increase in the number of donor templates many-fold within the cell due to replication of GVR to high copy numbers and also cause the cell to transition into S-phase. Both circumstances increase gene-targeting. We also describe tissue culture procedures in potato for *Agrobacterium*-mediated transformation to generate gene-edited events along with molecular screening of these events.

2 Materials

All media, solutions, containers, and equipment must be sterilized by standard autoclaving methods. All plant growth regulators and antibiotics must be filter sterilized and stocks are made so that the final concentration can be achieved by adding 1 mL of reagent to 1 L of the media prepared.

1. General propagation medium: Dissolve 4.43 g of Murashige and Skoog (MS) basal medium with vitamins and 30 g of sucrose in 800 mL of ddH_2O. Once dissolved, bring the volume up to 1 L and adjust the pH to 5.8. Then add 7 g of phytoagar and microwave to melt the phytoagar. Once homogeneous, dispense 50 mL into a Magenta box making 20 boxes per liter or 10 mL into a tube to make 100 tubes. All boxes/tubes must be capped and autoclaved for 30 min at 121 °C temperature under 15 lb $in.^{-2}$ (~103.5 kPa) pressure.

2. Liquid MS medium: Dissolve 4.43 g MS basal medium with vitamins and 30 g of sucrose in 800 mL of ddH_2O. Once dissolved bring the volume up to 1 L, adjust the pH to 5.8. All liquid media is autoclaved using the same conditions listed above. pH of all the reagents/media are adjusted to 5.8 using 1 N NaOH or HCl.
3. Thyamine HCl (1 mg/mL): Dissolve 0.05 g Thyamine hydrochloride in 50 mL ddH_2O. Filter sterilize, dispense into 1 mL aliquots, and store at −20 °C. Filter sterilization is done using 0.2 μm Whatman sterile filters.
4. Zeatin riboside (ZR, 0.8 mg/mL): Dissolve 0.04 g ZR in several drops of 1 N NaOH. Bring the volume up to 50 mL with ddH_2O. Filter sterilize, dispense into 1 mL aliquots, and store at −20 °C.
5. Gibberellic acid A3 (GA3, 2 mg/mL): Dissolve 0.05 g GA3 in 1 mL 95% EtOH. Bring the volume up to 25 mL with ddH_2O. Filter sterilize, dispense into 1 mL aliquots, and store at −20 °C.
6. 2,4-Dichlorophenoxyacetic acid (2,4-D, 2 mg/mL): Dissolve 0.05 g 2,4-D in several drops of 1 N NaOH and bring the volume up to 25 mL with ddH_2O. Filter sterilize, dispense into 1 mL aliquots, and store at −20 °C.
7. Cefotaxime (Cef, 250 mg/mL): Dissolve 2.5 g of cefotaxime sodium salt in 5 mL ddH_2O and bring the volume up to 10 mL with ddH_2O. Filter sterilize, dispense into 1 mL aliquots, and store at −20 °C.
8. Timentin (Tim, 150 mg/mL): Dissolve 1.5 g of timentin in 5 mL ddH_2O and bring the volume up to 10 mL with ddH_2O. Filter sterilize, dispense into 1 mL aliquots, and store at −20 °C.
9. Kanamycin (Km, 50 mg/mL): Dissolve 0.5 g of kanamycin monosulfate in 5 mL ddH_2O and then bring the volume up to 10 mL with ddH_2O. Filter sterilize, dispense into 1 mL aliquots, and store at −20 °C.
10. Ampicillin (Amp, 50 mg/mL): Dissolve 0.5 g of ampicillin sodium salt in 5 mL ddH_2O and then bring the volume up to 10 mL with ddH_2O. Filter sterilize, dispense into 1 mL aliquots, and store at −20 °C.
11. Gentamicin (Gm, 50 mg/mL): Dissolve 0.5 g of gentamicin sulfate salt in 5 mL ddH_2O and then bring the volume up to 10 mL with ddH_2O. Filter sterilize, dispense into 1 mL aliquots, and store at −20 °C.
12. Streptomycin (Sm, 50 mg/mL): Dissolve 0.5 g of streptomycin sulfate salt in 5 mL ddH_2O and then bring the volume up to 10 mL with ddH_2O. Filter sterilize, dispense into 1 mL aliquots, and store at −20 °C.

13. X-Gal (32 mg/mL): Dissolve 0.16 g of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) in 5 mL of dimethylformamide. The stock solution is stored at −20 °C in a glass container protected from light by wrapping in aluminum foil for 6–12 months. If the solution turns pink it must be discarded. Sterilization is not required.
14. STEP I and STEP II media plates: Dissolve 4.43 g of MS basal medium with vitamins and 30 g of sucrose in 800 mL of ddH_2O in a flask. Once dissolved, bring the volume up to 1 L and adjust the pH to 5.8. Then add 7 g of phytoagar, cover with aluminum foil, and autoclave. Cool the media to 55 °C and then add:
    (a) STEP I media/Callus induction medium (CIM): 0.9 mg/L thiamine–HCl, 0.8 mg/L ZR, and 2 mg/L 2,4-D.
    (b) STEP II media: 0.9 mg/L thiamine–HCl, 0.8 mg/L ZR, 2 mg/L GA3, 150 mg/L Tim, 250 mg/L Cef, and 50 mg/L Km.
15. Shoot induction medium#1 (SIM#1): 0.9 mg/L thiamine–HCl, 0.8 mg/L ZR, 4.0 mg/L of GA3.
16. Shoot induction medium#2 (SIM#2): 0.9 mg/L thiamine–HCl, 0.8 mg/L ZR, 2 mg/L GA3. A liter of media can be used to pour 30 presterilized disposable petri plates. The plant growth regulators must be added after autoclaving and cooling the medium to 55 °C.
17. Root induction medium (RIM): Dissolve 4.43 g of MS basal medium with vitamins and 30 g of sucrose in 800 mL of ddH_2O. Once dissolved, bring the volume up to 1 L, adjust the pH to 5.8, add 7 g of phytoagar or 2 g of phytogel/gelzan and sterilize in an autoclave. After cooling the medium to 55 °C, add appropriate antibiotic (50 mg/L Km) for selection along with 150 mg/L Tim and 250 mg/L Cef. In a set of presterilized tubes, aseptically pour 10–12 mL into each tube.
18. LB medium: Dissolve 10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl in 800 mL of ddH_20 in a glass bottle. Mix well, bring the volume up to 1 L and adjust the pH to 7.0 and sterilize in an autoclave.

    For solid LB plates, before autoclaving add 12 g of agar. Cool the media to 55 °C and then add appropriate antibiotics to the medium (50 mg/L Km or 50 mg/L Amp) and pour into presterilized disposable petri plates. For LB plates that include X-Gal, 40 μL of X-Gal stock solution (at room temperature) along with 40 μL of 100 mM IPTG solution is spread on the premade LB plates with a sterile spatula. Allow the plates dry in a 37 °C incubator before use. To prepare a batch of LB plates, 1 mL of X-Gal stock solution along with 1 mL of 100 mM

IPTG can be added to 1 L liquid LB medium along with other antibiotics.

19. Acetosyringone solution (74 mM): Dissolve 145 mg acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone) in few drops of dimethyl sulfoxide (DMSO) and bring the volume up to 10 mL with ddH_2O and filter sterilize. Use freshly prepared solution.
20. Vectors pDIRECT_22A (Addgene #91133; ABRC #CD3-2667), pMOD_B2515 (Addgene #91072; ABRC #CD3-2613), Vector pMOD_C2515 (Addgene #91083; ABRC #CD3-2622), pMOD_A0101 (Addgene #90998; ABRC #CD3-2547), pTRANS_220d (Addgene #91114; ABRC # CD3-2651), pMOD_C0000 (Addgene #91081; ABRC # CD3-2620).
21. Restriction enzymes: *Esp*3I, *Aar*I, *Bae*I.
22. T4 Polynucleotide Kinase (NEB, M0201S), T4 DNA ligase (NEB, M0202S).
23. Wizard® Plus SV Minipreps DNA Purification Systems (Promega, A1330).
24. Gibson Assembly Master Mix (NEB).
25. Chemically competent DH5α *E. coli* cells.
26. S.O.C. liquid medium (2% tryptone, 0.5% yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulfate, 20 mM glucose).
27. Phusion High-Fidelity DNA Polymerase (ThermoFisher).
28. Wizard® SV Gel and PCR Clean-Up System (Promega).
29. DNeasy Plant Mini Kit (Qiagen).
30. Zero Blunt TOPO PCR cloning kit (Invitrogen).
31. Other lab equipment: A thermocycler, 37 °C shaking and static incubator, 42 °C water bath, a NanoDrop spectrophotometer for DNA quantification, 1.5 mL centrifuge tubes, 0.2 mL PCR tubes, agarose gel equipment, and DNA ladders.

3 Methods

3.1 Construction of the CRISPR/Cas9 Expression Vector

In this section, we describe the detailed procedure for designing sgRNAs for target genes and constructing the CRISPR/Cas9 expression vectors for *Agrobacterium*-mediated plant transformation. Here, we describe procedures for knockout of target genes using a sgRNA cloned into a pDIRECT vector, two sgRNAs cloned using modular assembly of vectors, and gene-targeting to edit an endogenous gene by supplying a donor DNA template using GVR.

3.1.1 Selection of 20-bp Target (Spacer) sgRNA Sequence

5′-$N_{(20)}$-NGG-3′ (targeting template strand) or for 5′-CCN-$N_{(20)}$-3′ (targeting non-template strand) can be selected using web-based tools such as CRISPR RGEN tools (http://www.rgenome.net/) or CRISPR-P 2.0 (http://crispr.hzau.edu.cn/CRISPR2/), which have the *Solanum tuberosum* Group Phureja (PGSC v4.03) potato genome sequence. For manual selection, the sgRNA target site (*see* **Notes 1** and **2**):

(a) Must be specific/unique to the gene of interest.

(b) Must immediately precede the 5′-NGG PAM (*see* **Note 3**).

(c) Must be in 5′ exonic regions encoding a functional domain so as to disrupt gene function (*see* **Note 4**).

(d) Must take off-targets into account and be minimized, which can be done by BLAST search and using Cas-off-finder (http://www.rgenome.net/cas-offinder/).

(e) For tetraploid potato, allele information for the target gene must be considered. For this purpose, the gene must be cloned and sequenced to determine the allelic composition. To knockout the gene, sgRNA from a conserved region targeting all alleles must be selected (*see* **Note 5**).

3.1.2 Synthesis and Annealing of Oligonucleotides

1. Design and synthesize forward and reverse oligonucleotides. These oligonucleotides contain the 20-bp spacer sequence without the –NGG PAM sequence and must include overhangs specific for an RNA Pol III promoter. For sgRNA vectors containing the Arabidopsis U6 promoter (AtU6), oligonucleotides are designed as diagramed below, where Ns adjacent to 5′–GATT constitute the spacer sequence that is in the same strand as the PAM and other strand is complementary strand.

```
5′-GATTGNNNNNNNNNNNNNNNNNN
          | | | | | | | | | | | | | | | | | | ||
          NNNNNNNNNNNNNNNNNNNNCAAA-5′
```

2. Phosphorylate the forward and the reverse oligonucleotides using the following reaction:

   (a) 3 μL of 100 μM sense gRNA oligonucleotide (forward oligonucleotide).

   (b) 3 μL of 100 μM antisense gRNA oligonucleotide (reverse oligonucleotide).

   (c) 3 μL T4 DNA ligase buffer (contains ATP).

   (d) 2 μL T4 polynucleotide kinase.

   (e) 19 μL water.

   Incubate the reaction for 1 h at 37 °C and then add 4 μL of 0.5 M NaCl. Alternatively, phosphorylated oligonucleotides may be purchased.

3. Anneal the oligonucleotides, which can be done in a thermocycler with the following cycle: 95 °C/5 min + ramping down to 85 °C at −2 °C/s + ramping down to 25 °C at −0.1 °C/s +4 °C hold OR by boiling the reaction in a water bath for 3 min and letting it cool down gradually.
4. Dilute the reaction 25 times (1 μL oligo mixture + 24 μL water).

3.1.3 Cloning of sgRNAs into sgRNA Expression Vectors

Cloning a sgRNA into Plant Expression pDIRECT T-DNA Vector

For rapid construction of CRISPR/Cas9 reagents for targeted gene knockouts, the pDIRECT vector such as pDIRECT_22A which has a T-DNA vector backbone, a kanamycin (Km) selectable marker, guide RNA expression cassette, and Cas9 nuclease (Addgene #91133; ABRC #CD3-2667) can be used (*see* **Note 6**). This plasmid is designed for *Agrobacterium*-mediated transformation.

1. Directly clone the phosphorylated and annealed sgRNA oligonucleotides into pDIRECT_22A vector via the following Golden Gate reaction:
   (a) 50 ng of pDIRECT_22A.
   (b) 1 μL 25× diluted phosphorylated and annealed oligonucleotides.
   (c) 0.4 μL *Aar*I oligonucleotide (comes with the *Aar*I enzyme).
   (d) 0.5 μL *Aar*I restriction enzyme.
   (e) 2 μL 10× T4 DNA ligase buffer.
   (f) 1 μL T4 DNA ligase.
   (g) Water up to 20 μL.
2. Place the Golden Gate reaction into a thermocycler with the following cycle: 37 °C for 5 min, 16 °C for 10 min, 37 °C for 15 min, 80 °C for 5 min.
3. Transform competent *E. coli* competent cells (such as DH5α) and plate on LB plates containing 50 mg/L Km and 32 mg/L X-gal.
4. PCR screen white colonies using sense sgRNA oligonucleotide as forward primer and 5′-CGAACGGATAAACCTTTTCACG-3′ binding to vector backbone as reverse primer.
5. Confirm correct clones by Sanger sequencing using AtU6_F primer 5′-AGATAATCTTCAAAAGGCCCCTGG-3′.

Cloning of Dual sgRNAs into a T-DNA Vector for Multiplexed Genome Editing

The approach of using two sgRNAs from the same target gene to delete a larger portion of a gene might be desirable as it simplifies screening for edited plants. Therefore, an alternative method is described here for cloning two sgRNA spacer sequences using

modular vector assembly and then subsequently creating a Cas9-containing T-DNA vector. A modular vector set for diverse range of genome-editing applications and online resources to aid in vector selection and construct design are available (http://cfans-pmorrell.oit.umn.edu/CRISPR_Multiplex/) [8]. Briefly, Module A vectors may be used for Cas9 expression cassettes, Module B vectors may be used to clone either single or multiple sgRNAs, and Module C vectors may be used to add additional sgRNA cassettes or donor templates for gene targeting. Finally, a T-DNA transformation backbone can be selected to assemble Module A, B, and C plasmids into this final plant transformation vector. One of each module type (A, B, and C) must be used for assembly into the final T-DNA vector.

1. Set up the following Golden Gate reaction:
   (a) 50 ng of pMOD_B2515 (module B plasmid).
   (b) 1 μL 25× diluted phosphorylated and annealed oligonucleotides.
   (c) 0.5 μL *Esp*3I restriction enzyme.
   (d) 2 μL 10× T4 DNA ligase buffer.
   (e) 1 μL T4 DNA ligase.
   (f) Water up to 20 μL.
2. Place the Golden Gate reaction into a thermocycler with the following cycle: 37 °C for 5 min, 16 °C for 10 min, 37 °C for 15 min, 80 °C for 5 min.
3. Transform competent *E. coli* cells (such as DH5α) and plate on LB plates containing 50 mg/L Amp.
4. Pick two clones for miniprep, followed by sequencing confirmation with AtU6_F primer 5-′-AGATAATCTTCAAAAGGCCCCTGG-3′.
5. Repeat **steps 1–4** to clone the second sgRNA spacer into pMOD_C2515 (Addgene #91083; ABRC #CD3-2622) simply by substituting pMOD_C2515 for pMOD_B2515 in steps mentioned above.
6. Assemble the T-DNA vector by setting up the following reaction:
   (a) 75 ng pTRANS_220d (transformation backbone).
   (b) 150 ng module A plasmid (pMOD_A0101).
   (c) 150 ng module B plasmid (pMOD_B2515).
   (d) 150 ng module C plasmid (pMOD_C2515).
   (e) 0.4 μL *Aar*I oligonucleotide (comes with the *Aar*I enzyme).
   (f) 0.5 μL *Aar*I restriction enzyme.

(g) 1 μL T4 DNA ligase.

(h) 2 μL 10× T4 DNA ligase buffer.

(i) Water up to 20 μL

7. Place the reactions in a thermocycler with the following cycle: 10× (37 °C for 5 min then 16 °C for 10 min), 37 °C for 15 min, 80 °C for 5 min and 4 °C hold.
8. Transform CcdB-sensitive E. coli (such as DH5α) and plate on LB containing Km 50 mg/L.
9. Screen colonies by PCR use a forward primer 5′-GTTGGATCTCTTCTGCAGCA-3′ (HSP terminator) and a reverse primer 5′-GTAAAACGACGGCCAGT-3′ (M13).
10. Miniprep and confirm constructs by sequencing. The Golden Gate junctions spanning module B to C and module C to T-DNA can be sequenced using AtU6_F primer 5′- AGATAATCTTCAAAAGGCCCCTGG-3′, junctions spanning T-DNA to module A using primer 5′-CTTTACACTTTATGCTTCCGGCTC-3′ and module A to B using primer 5′-CATCCATCTCTTCACCCTTACCAAC-3′ (*see* **Note 7**).

Cloning of DNA Donor into GVR Viral Vector for Gene Targeting

Gene-targeting to modify or edit endogenous genomic regions by supplying an external donor template is described here. Donor templates are designed to create specific changes in the genome of a target organism. These changes can range from a single nucleotide change to targeted insertion of several kilobases of DNA. Donor templates typically have ~1000 bp to 500 bp of sequence each of left homology arm and right homology arm (together called donor arms) identical to the genome targeted for editing/modification. The sequence in between the donor arms contains the desired modifications that we aim to make in the genome. A critical design feature that should be considered is to incorporate changes in the donor template sequence such that the sgRNAs used to cut the genome will not anneal to the donor template.

There are a variety of ways to construct a donor template. They range from amplifying the genomic fragment from species that already contains our desired modifications, or by incorporating the modifications into the oligonucleotide primers used to amplify the donor arms. Alternatively, the donor template sequence with intended edits can be synthesized by a number of companies such as Integrated DNA Technologies (https://www.idt.com/) or GenScript (https://www.genscript.com/). These donor arms are assembled with each other via Gibson assembly.

1. PCR amplify different parts of the donor template using overlapping primers for Gibson assembly with *Bae*I overhangs (Fig. 1a). To design primers:

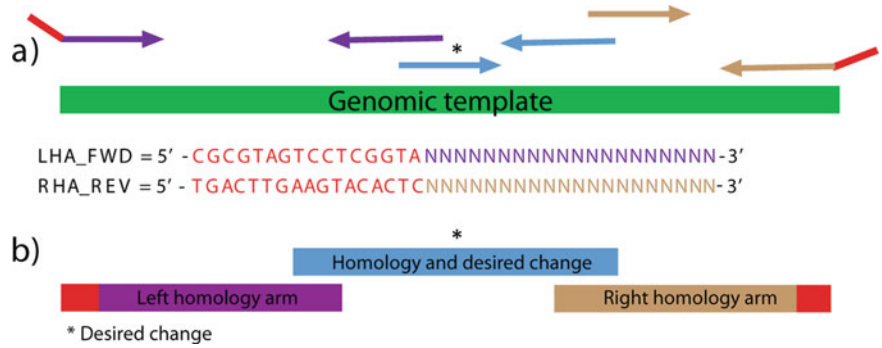

Fig. 1 Schematic of Gibson assembly to create gene targeting donor templates. (**a**) Arrows indicate primers to amplify the fragments to be assembled by Gibson Assembly. Identical colors indicate primer pairs to be used together. Red "tails" of most distal primers are identical to the *Bae*I digested pMOD_C0000 vector, the details of overhang sequences are given. LHA_FWD is a forward primer sequence that overlaps with *Bae*I digested module C vector. Replace N's with a primer binding to the 5′ end of the left homology arm. RHA_REV is a reverse primer, replace N's with sequence complementary to 3′ end of right homology arm. (**b**) PCR products from reactions using the primers shown in (**a**). Overlap (identity) between oligos and resulting PCR products are at least 20 bp long. *LHA* left homology arm and *RHA* right homology arm

2. Gel-purify the fragments of correct size and measure the DNA concentrations. These donor template fragments (Fig. 1b) will be assembled into the *Bae*I site of the pMOD_C0000 (Addgene #91081; ABRC # CD3-2620), an empty backbone vector by Gibson assembly using a commercially available Gibson assembly master mix (NEB), according to the manufacturer's instructions (*see* **Note 8**).
3. To prepare pMOD_C0000 for Gibson assembly set up the following digestion reaction:
   (a) 2 μg of pMOD_C0000 (empty backbone vector).
   (b) 1× restriction buffer.
   (c) 1 μL *Bae*I restriction enzyme.
   (d) Water up to 20 μL.
4. Incubate for 1 hr. at 25 °C.
5. After complete digestion, gel-purify the linearized backbone with the Wizard® SV Gel and PCR Clean-Up System and measure the DNA concentration.
6. Set up the following Gibson assembly reaction to assemble donor template fragments into pMOD_C0000:
   (a) 50 ng of the *Bae*I digested pMOD_C0000.
   (b) Equimolar amounts of each donor fragment, each three times higher molar concentration than the module C backbone (*see* **Note 9**).
   (c) 10 μL of 2× Gibson assembly master mix (NEB).
   (d) Water up to 20 μL.

7. Incubate for 1 h at 50 °C.
8. Transform 5 μL of the reaction into *E. coli* (DH5α or similar) and plate on LB with 50 mg/L of Amp.
9. Correct clones can be screened by colony PCR using 5′ GGAA-TAAGGGCGACACGGAAATG-3′ (AmpR) and a donor template specific primer.
10. The correct clones must be confirmed by sanger sequencing using the AmpR primer and/or primers in the donor template.
11. Assemble this module C plasmid containing the donor template along with module A vector pMOD_A0101 (Cas9-expression) and module B vector pMOD_B2515 with the sgRNA cloned (*see* **Note 10**) and selected transformation backbone pTRANS_221 (Addgene #91115; ABRC #CD3–2652) to create and assemble GVR using the steps described in section **step 6** of cloning of dual sgRNAs into a T-DNA vector above (replace pTRANS_220d with pTRANS_221 which has the bean yellow dwarf virus (BeYDV) replication feature within the T-DNA) (*see* **Note 11**).
12. For colony PCR screening, use forward primer (HSP terminator)

    5′-GTTGGATCTCTTCTGCAGCA-3′ and a reverse primer (BeYDV)

    5′- GCAATCCTGACGAAGACTGGATGT-3′
13. Golden Gate junctions can be sanger sequenced using the same primers used for assembling pTRANS_220d (*see* **Note 12**).

3.2 Introducing the Cloning Plasmids into Agrobacterium

Electroporation using Biorad-micropulser may be used to introduce the cloning plasmids into *A. tumefaciens* strains LB4404 or GV3101(pMP90) (Fig. 2a), which is performed as follows.

1. *Agrobacterium*-electro-competent cells are thawed on ice.
2. For each DNA sample to be electroporated, 40 μL of cells is mixed with 0.5–1 μg of plasmid in a tube, mixed by gentle tapping.
3. This mixture is transferred to a chilled electroporation cuvette and incubated on ice for 5 min.
4. The cuvette is pulsed in an electroporator for 5 ms at 2.5 kV/0.2 cm cuvette gap and 400 Ω resistance.
5. Immediately after the pulse, 800 μL of S.O.C media is added, and the cells are incubated in a shaker at 30 °C and 200 rpm for 2 h.
6. Aliquots of electroporated cells are plated on LB plates containing appropriate selective media (50 mg/l Km) and incubated for 48 h at 30 °C.

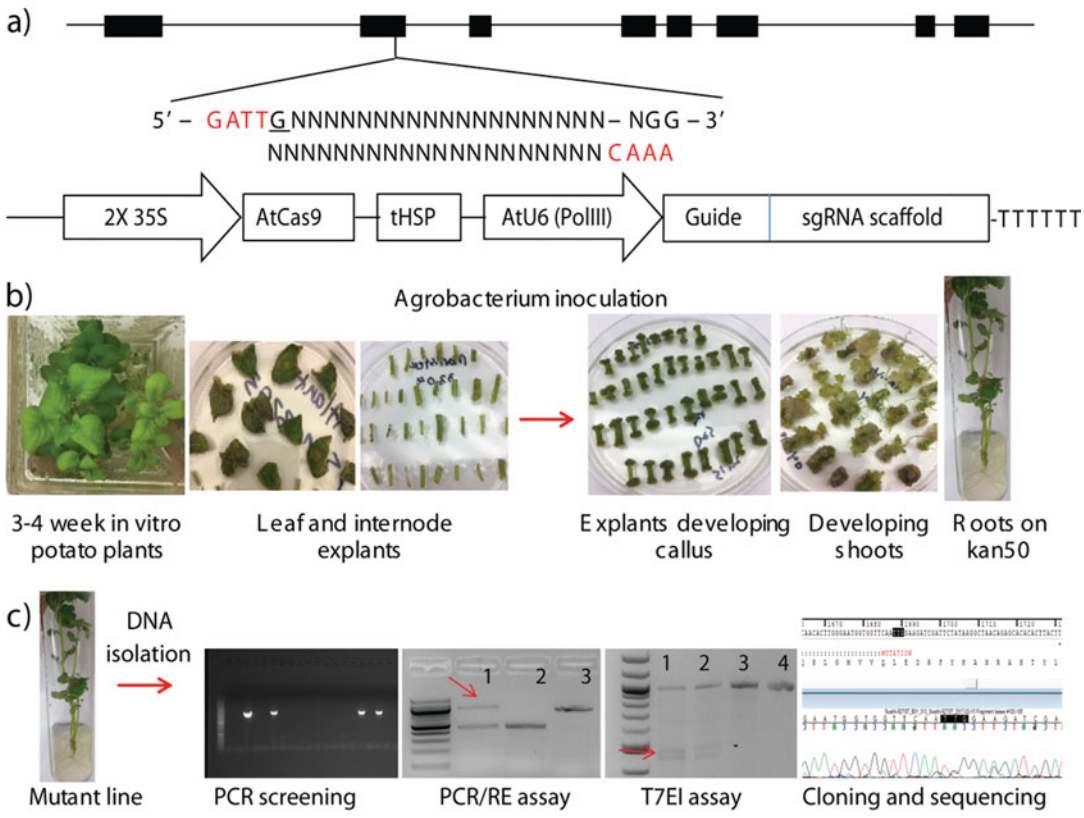

Fig. 2 Illustration showing the key procedures to generate and evaluate CRISPR/Cas9 mediated genome-editing events in potato**. (a**) Construction of the CRISPR/Cas9 expression vector. The structure of target gene is shown and sgRNA is selected from the second exon. "N" represents the 20 bp spacer sequence. The overhangs in red are specific for AtU6 PolII promoter. The first nucleotide must be G for the sequence transcribed from the U6 promoter. –NGG in the 3′ end is the protospacer adjacent motif (PAM) sequence required for *SpCas9.* Cloning of sgRNAs into CRISPR/Cas9 expression vector for plant transformation. (**b**) *Agrobacterium*-mediated plant transformation and regeneration in potato. 3–4-week-old in vitro propagated potato plants in a Magenta box are shown. Explants are prepared from leaf and stem internodes and placed on callus induction media. Callus growth observed from the explants. After 6–8 weeks, depending on the genotype shoots emerge and grow on shoot induction media. 1–2 cm shoots are excised and transferred to root induction media. The lines that develop rooting and have growth on antibiotic containing selection media are selected as candidates for molecular screening. (**c**) Molecular characterization of the genome-edited events. Genomic DNA is isolated from the candidate-mutagenized lines. PCR screening is carried out to confirm T-DNA insertion or gene targeting. Loss of restriction site assay is carried out on the PCR product amplified from the target region. Arrow indicates the resistant band in mutant line representing targeted mutagenesis. T7 endonuclease I assay performed on the same PCR product shows cleaved bands at the heteroduplex regions. Arrow represents the expected cleavage products in mutant lines. Cloning of the PCR product and sequencing results give us details of the insertions/deletions in the mutagenized lines

3.3 Agrobacterium-Mediated Plant Transformation and Regeneration in Potato

Agrobacterium-mediated transformation is the most common and routine method to transform potato plants. Here, we describe the protocols routinely used and that have been employed for a wide range of tetraploid and diploid potato genotypes in our laboratory including Atlantic, Desirée, MSUE149-5Y, Snowden, and Yukon Gold. Transformation protocols may vary depending on the genotype (*see* **Note 13**) [9, 10].

3.3.1 In Vitro Propagation and Regeneration

1. In vitro grown plant material is used for transformation experiments to maintain sterile conditions (*see* **Note 14**). In vitro propagation is carried out by subculturing the nodes containing axillary buds, five nodes widely spaced in each Magenta box grown at 22 °C under 16/8 h light/dark photoperiod and average light intensity of 300 μmol m^{-2} s^{-1}.
2. Four-week-old plants must be used for the experiments. For every new genotype used for a transformation experiment, it is a prerequisite to establish an efficient regeneration system for that genotype from leaf and stem explants prior to attempting transformation.
3. Regeneration from explants is not only genotype specific but also depends on the various combinations of plant growth regulators in the culture medium. To establish regeneration protocol for a new species, stem and leaf explants can be placed on CIM plates for a week and transferred to SIM#1 media plates for 2 weeks. The explants must then be transferred to SIM#2 media plates once in every 2 weeks until shoots appear.
4. Rates of regeneration can be determined by the number of regenerated shoots out of the total number of explants in each plate. Phytohormone concentrations must be adjusted if necessary depending on the genotype.

3.3.2 Explant Preparation and Pre-culture

1. Excise leaves and stems from 3 to 4-week-old healthy in vitro plants.
2. Thick stem internodal segments devoid of axillary buds must be cut (*see* **Note 15**) and placed horizontally on STEP I media for stem explants.
3. For leaf explants, top immature/young leaves must be selected (*see* **Note 16**). The base (petiole end) and tip of the leaf explant must be discarded. The remainder of the leaf should be immediately transferred to STEP I media plates with adaxial surface touching the medium. A bigger leaf can be cut into 2–4 pieces (Fig. 2b).
4. STEP I plates containing explants must be wrapped using micropore tape and placed under lights in regular growth conditions at 22 °C under 16/8-h light/dark photoperiod for 2 days.

3.3.3 Agrobacterium Inoculation and Cocultivation

1. A few days before each transformation experiment (*see* **Note 17**), streak a fresh plate of the desired *Agrobacterium* culture (s) from −80 °C glycerol stock onto LB agar plates containing appropriate antibiotics (Km 50 mg/L for the plasmids outlined here). Incubate the plate at 30 °C for 2 days.
2. Inoculate 3 mL of LB media with specific antibiotics (50 mg/L Km and 50 mg/L Gm for GV3101 strain or 50 mg/L Sm for LBA4404) using a single colony from the freshly streaked plate. Shake the cultures at 250 rpm in a 30 °C shaker for 2 days.
3. Add 1 mL of the liquid *Agrobacterium* culture into a sterile flask with 50 mL of LB with selective antibiotics and shake at 250 rpm in a 30 °C shaker for 4–6 h or overnight. This step is to ensure the bacterial cultures are in the active log phase of growth; grow at least four flasks of 50 mL culture each.
4. Spin down the cultures at 5000 rpm (5152 × *g*) for 10 min and resuspend the pellet in 25 mL of liquid MS medium. Determine the $OD_{\lambda 600}$ and adjust it to 0.6 using the media. Add 20 μL of freshly prepared acetosyringone stock to 40 mL of the diluted *Agrobacterium* culture.
5. Dispense 25 mL of the culture into several empty sterile petri dishes. Gently transfer the explants into the petri dishes making sure that both sides of the leaves are exposed to the bacterial solution. Incubate for 20 min at room temperature with occasional swirling.
6. Shake off excess liquid and blot-dry the explants in between sterile filter paper. This is a very critical step to avoid overgrowth of *Agrobacterium*.
7. Transfer the explants to fresh STEP I media plates (using the forceps we can disrupt the agar to gently place the tissue securely on the media). Seal the plates using micropore tape and incubate the plates for 2 days at 22 °C in the dark.
8. After 2 days of cocultivation, collect all the explants into 50 mL falcon tubes and rinse with 40 mL of sterile ddH_2O containing 30 μL stocks for each of Tim and Cef. Wash the explants until no turbidity is observed in the water (*see* **Note 18**).
9. Blot-dry the explants after rinsing on a sterile filter paper and place them on STEP II media plates containing antibiotic selection. Seal the plates with micropore tape and place in the growth incubator under 16/8-h light/dark photoperiod and average light intensity of 300 μmol m^{-2} s^{-1}. Initially cover the plates with 2–3 layers of cheese cloth until 4 days and then uncover them to full light. Transfer the explants to fresh STEP II media plates every 7–10 days.

10. Shoot primordia start to emerge after ~4 weeks. When the shoots are at least 1–2 cm, gently cut them slightly above the base of the callus making sure no callus tissue is cut and transfer them to RIM tubes with selection by inserting the cut ends of the shoots into the medium.
11. Shoots that produce well-formed deep roots in the medium are the first indication of a positive transformation event. Shoots that fail to form substantial root systems on the selective medium are very often escapes (*see* **Note 19**).

3.4 Molecular Characterization of the Genome-Edited Events

Positive transformation events must be sorted out first based on the root development and general plant growth in selection media (*see* **Note 20**). All the plants with root growth on 50 mg/L Km containing RIM must be selected for molecular characterization. DNA extraction from the leaves of the selected mutant lines may be performed using DNeasy Plant Mini Kit (Qiagen).

3.4.1 PCR Screening

1. 10 ng of genomic DNA may be used in a PCR reaction to amplify regions within the T-DNA using primers specific to Cas9 or kanamycin to screen for the presence or integration of T-DNA region in the mutant lines (Fig. 2c). Primers that recognize all the alleles must be used for screening.
2. PCR amplicons obtained from such primers can be used for high throughput sequencing.
3. To further characterize the mutations induced, PCR amplicons from the candidate events can be cloned and at least 20 clones must be sanger sequenced to detect mutations in one or more of the alleles.
4. For gene-targeted lines, the DNA samples must be genotyped for the presence of right and left recombination junctions. For this purpose, a forward primer outside of the donor template and a reverse primer within and unique to the donor template region must be selected.
5. All PCR products are resolved on an agarose gel. Selected PCR products are excised, purified and cloned into a cloning vector such as pCR4 cells from the Zero Blunt TOPO PCR cloning kit (Invitrogen) and transformed into *E. coli*. Plasmids are isolated, and Sanger sequenced.

3.4.2 Targeted Mutagenesis Detection Assays

10 ng of genomic DNA from mutant lines along with the wild type must be used in a PCR reaction to amplify 500–800 bp of the target region that harbors mutations using Phusion High-Fidelity DNA Polymerase (NEB). The PCR products are purified using Promega

Wizard SV gel and PCR clean-up system and 1 μg of the product can be used for various assays below:

PCR Loss of Restriction Enzyme Site (PCR/RE Assay)

A restriction site in the target locus coincident with the nuclease cleavage site that may be disrupted by a nuclease-induced mutation is a candidate for the PCR/RE assay. Targeted mutagenesis by CRISPR/Cas9 disrupts the site thereby making the mutant amplicons resistant to enzyme digestion. Include wild-type samples as negative controls in the digestion reaction set up as follows:

(a) 1 μg of the purified PCR product.

(b) 5 μL of 10× enzyme buffer.

(c) 2 μL of restriction enzyme.

(d) Water up to 50 μL.

The digested products are run on a 2% agarose gel. Presence of uncleaved resistant band in the CRISPR/Cas9 treated samples after restriction enzyme digestion and not in the negative control indicates targeted mutagenesis (Fig. 2c). The resistant band can be purified and cloned for sequencing to confirm the presence of mutations and determine the sequence of the mutagenized region in different alleles.

T7 Endonuclease I Assay (T7EI Assay)

This assay relies on the cleavage of heteroduplex DNA by T7 endonuclease I (*T7E*I). The PCR products containing wild-type and mutant alleles are denatured and renatured to form heteroduplexes. *T7E*I recognizes and cleaves non-perfectly base-paired DNA. The assay is performed as per the manufacturer's instructions (NEB).

1. PCR products are denatured at 95 °C.
2. Cool down the PCR products slowly to reanneal using a ramp PCR from 95 to 85 °C at −2 °C/s and 85 to 25 °C at −0.1 °C/s.
3. Annealed PCR products must be incubated with *T7E*I (NEB) at 37 °C for 1 h.
4. Analyze the products via 2% (w/v) agarose gel electrophoresis (Fig. 2c).
5. PCR bands may be quantified using ImageJ software (https://imagej.nih.gov/ij/) and the mutagenesis frequencies for genome-editing nucleases can be estimated using % gene modification = $100 \times (1 - (1\text{-fraction cleaved})^{1/2})$ [11].
6. Cloning and sequencing the region of mutagenesis gives details of insertion/deletions (*see* **Note 21**).

4 Notes

1. The Arabidopsis U6 RNA polymerase III promoter used to express the sgRNA prefers a guanine (G) nucleotide as the first base of its transcript. Therefore, an extra G must be appended to the 5′ of the sgRNA 20-nt spacer sequence, if it does not begin with G. G is not included in the vector backbone and should therefore be included in the oligonucleotides.
2. Must not have an *Esp3*I or an *Aar*I restriction site in the sequence (presence of these restriction sites complicates downstream cloning).
3. Avoid stretch of T bases in the sgRNA spacer sequence (e.g., TTTT is a Pol III terminator).
4. Avoid 3′ end of coding sequence or introns, which may have less effect on gene function.
5. Having restriction enzyme sites within the target sequence at the Cas9 endonuclease-cutting site will facilitate detection using a PCR/RE assay. If not, *T7E*I assay can be used as well.
6. This method sacrifices some flexibility if changes are to be made to the T-DNA vector design later. Using pDIRECT_22A, adding a second sgRNA or a donor template is not possible.
7. It is usually sufficient to confirm via sequencing only the junctions between each of the Module plasmids and the junction between Module A and the transformation backbone and the junction between Module C and the transformation backbone. Diagnostic restriction enzyme digests may also be used to confirm correct clones.
8. While designing primers for Gibson assembly, Tm of the overlap should be at least 48 °C. If Tm is below 48 °C, the overlap can be extended until the Tm is at least 48 °C.
9. Optimized cloning efficiency is 50–100 ng of vectors with two- to threefold of excess inserts. Use five times more of inserts if size is less than 200 bp. Total volume of unpurified PCR fragments in Gibson Assembly reaction should not exceed 20%. If greater numbers of fragments are assembled, additional Gibson Assembly Master Mix may be required.
10. Donor templates can also be assembled into pMOD_C2515 if the use of two sgRNAs is desired. If pMOD_C2515 is used, the oligonucleotides cloned into the vector cannot have a *Bae*I restriction enzyme site in them. It is highly recommended that if pMOD_C2515 is used, cloning the oligonucleotides precede the cloning of the donor template. In this case, all reactions are performed as described above.

11. When GT donor templates cloned into pMOD_C0000 contain *Aar*I sites, skip the last digestion step at 37 °C for 15 min to prevent digestion of the final product at the *Aar*I site in the donor sequence which is not removed in the assembly process unlike the *Aar*I sites that flank the A, B, and C cassettes.
12. If pMOD_B2515 and pMOD_C2515 derivatives are used in construction of a T-DNA vector, use primers from spacer sequence in pMOD_B2515 and pMOD_C2515-derived plasmids for sequencing instead of AtU6_F as that primer will anneal to AtU6 promoter in both plasmids and give mixed sequencing results.
13. Potato transformation is genotype-dependent, which sometimes limits the adaptability of a single protocol across different genotypes [9, 10].
14. Healthy and vigorous plants are critical for successful transformation experiments.
15. While using stem explants, utmost care must be taken to avoid axillary buds as they can give rise to early non-transgenic shoots.
16. Leaves that are mature from the bottom of the plant must be avoided for transformation. Sharp scalpel must be used to prepare explants to make a clean cut so that ends of the explants are not crushed.
17. The first step in transformation experiment is to have plant material ready and before they reach 4 weeks, streak fresh *Agrobacterium* plate 4 days prior. Start the cultures the same day as we prepare explants for pre-culture.
18. If overgrowth of *Agrobacterium* is observed, the explants should be rinsed again as before and blotted dry.
19. It is always safe to have positive and negative controls included in each step to ensure the selection is applied. For example, a wild-type explant that has not been treated with plasmid-containing *Agrobacterium* should not produce rooting in Km containing selection media.
20. It is important to make sure that the potential transformants have roots that are going into the medium, not just on the surface of the medium or growing away from the medium.
21. One limitation of *T7E*I assay is that mutants containing identical alleles of all mutagenized loci (mutagenized homoduplexes) will appear to be wild type.

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Chapter 15

Visual Assay for Gene Editing Using a CRISPR/Cas9 System in Carrot Cells

Magdalena Klimek-Chodacka, Tomasz Oleszkiewicz, and Rafal Baranski

Abstract

The development of the Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas9) system has advanced genome editing and has become widely adopted for this purpose in many species. Its efficient use requires the method adjustment and optimization. Here, we show the use of a model carrot callus system for demonstrating gene editing via CRISPR/Cas9 targeted mutagenesis. The system relies on the utilization of carrot tissue accumulating anthocyanin pigments responsible for a deep purple cell color and generation of knockout mutations in the flavanone-3-hydroxylase (*F3H*) gene in the anthocyanin biosynthesis pathway. *F3H* mutant cells targeted by Cas9/gRNA complexes are not able to synthesize anthocyanins and remain white, easily visually distinguished from purple wild-type cells. Mutations are either small indels or larger chromosomal deletions that can be identified by restriction fragment analysis and sequencing. This feasible system can also be applied for validating efficiency of CRISPR/Cas9 vectors.

Key words *Agrobacterium*-mediated transformation, *Daucus carota*, Callus, Anthocyanins, Flavanone-3-hydroxylase, F3H

1 Introduction

Carrot is a model species used in plant cell and tissue culture in vitro, and is amenable to genetic transformation [1]. Recent publication of the carrot genome [2] has opened new prospects for genetic research in this species and development of new genotypes with altered metabolism. Therefore, we have utilized clustered regularly interspersed short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system to demonstrate its feasibility for editing genes in the carrot genome [3].

Genome editing with the CRISPR/Cas9 has become a routine technique applied in animal and plant research [4, 5]. This system was developed based on the native immune system found in bacteria and archaea. The system consists of two components, Cas9 and sgRNA. The Cas9 enzyme possesses two nuclease domains, RuvC

Yiping Qi (ed.), *Plant Genome Editing with CRISPR Systems: Methods and Protocols*, Methods in Molecular Biology, vol. 1917, https://doi.org/10.1007/978-1-4939-8991-1_15,

and HNH, which are responsible for cleavage of both DNA strands. The second component of CRISPR system, sgRNA, guides the enzyme to a specific locus through sequence complementary. Currently used sgRNAs are hybrid oligonucleotides, which are created by joining two sequences into a single molecule, i.e., crRNA recognizing target DNA and tracrRNA activating the CRISPR complex [6]. A range of Cas9 protein variants are now available for genome editing. A selection of the Cas9 enzyme determines the target site sequence that must be proximal to the protospacer adjacent motif (PAM), a short, usually 3–5 nt conserved sequence located at the 3′ end of the target site [7]. Genome editing results from the repairing of double-stranded breaks in the target locus by a non-homologous end-joining (NHEJ) or homology-directed repair (HDR) pathways. NHEJ is the most common pathway often leading to imperfect repair, thus generating small indel mutations. The alternative system, HDR, requires delivery of the template sequence that allows for precise fragment insertion or host sequence correction. The efficiency of HDR is very low [8]. The first reports demonstrating successful edition of human genome using the CRISPR/Cas9 system were published in 2013. Now this system is extensively and effectively employed for genome edition in human cells, mice, and other species, including crop plants like maize, rice, and other cereals. The research mainly focused on induction of small indel mutations for gene knockout, but also on deleting long chromosome fragments or inserting new coding sequences. Such genetic changes may result in new phenotypes [9].

We choose the carrot flavanone-3-hydroxylase (*F3H*) gene to generate mutations that abolish anthocyanin biosynthesis. The occurrence of anthocyanin pigments in plant tissue is the result of a multistep flavonoid biosynthesis pathway. At its early step, naringenin chalcone is converted to naringenin, which is then hydroxylated to dihydrokaempferol, eriodictyol, or dihydrotricetin. The later three compounds are precursors of anthocyanidins such as peonidin, petunidin, pelargonidin, malvidin, cyanidin, and delphinidin. Anthocyanins are derived in a series of glycosylation and methylation reactions [10]. The *F3H* gene catalyzes naringenin, eriodictyol, and dihydrotricetin hydroxylation and thus is critical for metabolite flux in the pathway [11]. Mutant cells with knockout *F3H* gene are not capable of anthocyanin biosynthesis and do not accumulate these pigments [3, 12]. In consequence, mutant cells are easily visually distinguished from wild-type purple cells. Therefore, cell culture characterized by purple color due to anthocyanins accumulation can be considered as a model system for demonstrating CRISPR/Cas9 editing approach and for validating different CRISPR systems. Two components are required to demonstrate the protocol here, a CRISPR/Cas9 vector with gRNAs targeting the carrot *F3H* gene and carrot callus accumulating anthocyanins cultured in vitro. The advantage of this protocol is its simplicity

since mutant phenotype is revealed based on color change of carrot cells. The presented protocol of using *Agrobacterium tumefaciens*-mediated CRISPR/Cas9 system can also be used to target other carrot genes. However, mutant selection in those cases may have to rely on molecular detection rather than color change in carrot cells.

2 Materials

2.1 Callus Transformation

1. *Agrobacterium tumefaciens* LBA4404 possessing a binary plasmid pMDC32-AteCas9-DcF3H-gR3, gR4 (Fig. 1, *see* **Note 1**).
2. Stable carrot callus culture (*see* **Note 2**).
3. Lysogeny broth (LB) (*see* **Note 3**).
4. Liquid carrot callus growth medium (BI): Gamborg B5 macro- and microelements with vitamins [13], 1 mg/L 2,4-D, 0.0215 mg/L kinetin, 30 g/L sucrose, pH 5.8, sterilized by autoclaving (121 °C, 1 atm for 25 min).
5. Solid BI medium: BI medium solidified with 2.7 g/L phytagel sterilized by autoclaving (121 °C, 1 atm for 25 min) (*see* **Note 4**).
6. Antibiotic stock solutions: dissolve antibiotic in distilled water, filter (0.22 μm) sterilize. Final concentrations in the stock solution:
   (a) 200 mg/mL cefotaxime, store at −20 °C.
   (b) 100 mg/mL timentin, store at −20 °C.
   (c) 50 mg/mL kanamycin, store at −20 °C.
   (d) 100 mg/mL hygromycin B, store at 4 °C.
7. 100 mM acetosyringone stock solution: dissolve 19.62 mg in 1 mL 96% ethanol and store at −20 °C.
8. Disposable 1 cm spectrophotometer cuvettes.
9. 2 mL PCR tubes with a round-shaped bottom.
10. 100 mL Erlenmeyer flasks.
11. Sterile Petri dishes Ø 90 mm.
12. Sterile Petri dishes Ø 60 mm.
13. Sterile filter paper discs Ø <60 mm.
14. Sterile tweezers.
15. Centrifuge with a rotor capable to spin 2 mL PCR tubes.
16. Spectrophotometer capable of measuring at 600 nm wavelength using cuvettes, e.g., Nanodrop 2000c (Thermo Fisher Scientific) spectrophotometer.
17. Flow hood, either with a horizontal or vertical flow, for sterile work.
18. 26 °C shaking and static incubators.

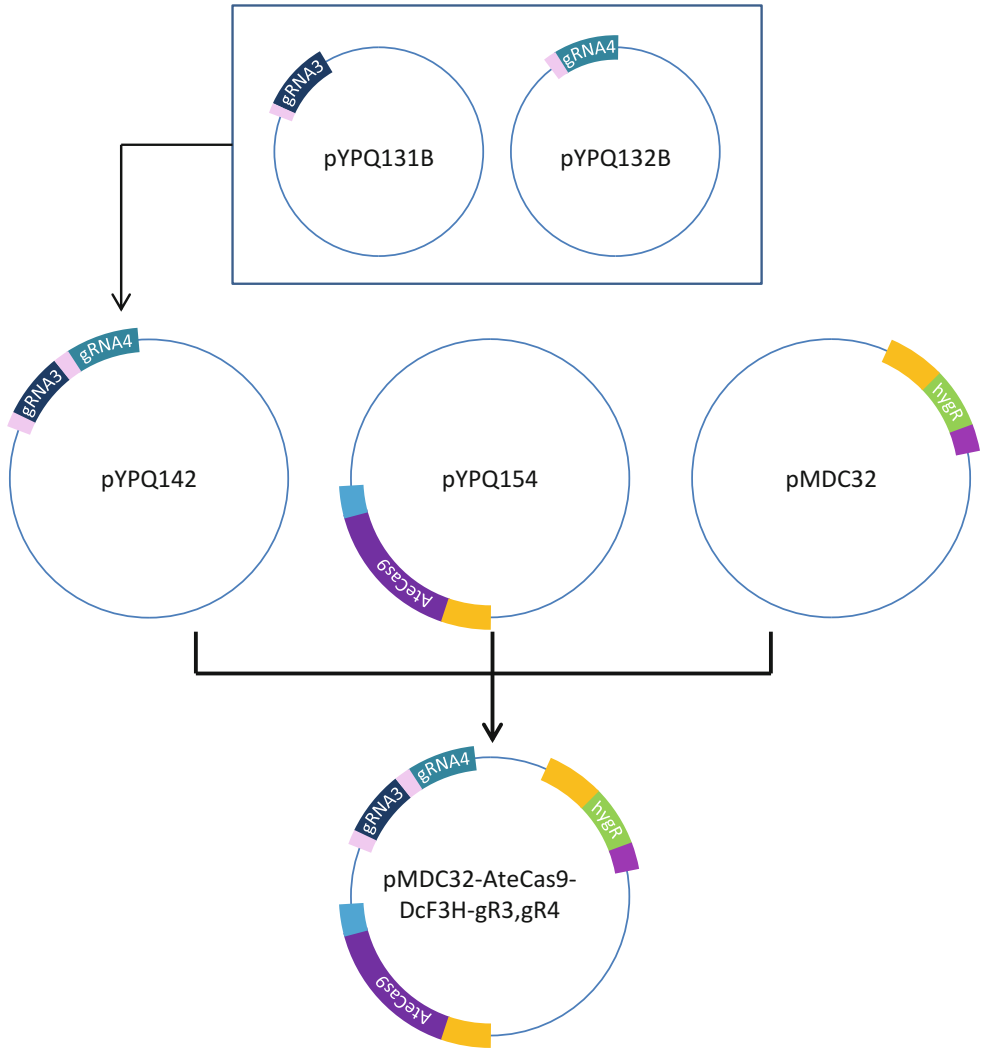

Fig. 1 Construction of the pMDC32-AteCas9-DcF3H-gR3,gR4 T-DNA transformation vector used in this protocol [14]. Both forward and reverse synthetic oligos for gRNA3 and gRNA4 are annealed and ligated into entry vectors (pYPQ131B and pYPQ132B, respectively) at the *Bsm*BI site downstream the AtU3 promoter. Then, both gRNA cassettes are assembled in a Golden Gate reaction to the recipient pYPQ142 vector. In the third reaction, pYPQ142-gRNA3&gRNA4, pYPQ154, and the destination vector pMDC32 [16] are assembled using the Gateway Multisite LR recombination to create the final vector, in which the AteCas9 gene is under CaMV 35S promoter. All pYPQ CRISPR/Cas9 toolbox vectors [14] are available from Addgene (https:/www.addgene.org/browse/article/15693)

2.2 Molecular Analyses

1. Oligonucleotides for PCR reaction and sequencing (Table. 1 and Fig. 2).
2. PCR reagents: *Taq* polymerase with a buffer, dNTPs.
3. *Nco*I enzyme (HF, NEB) and CutSmart buffer.
4. Standard electrophoresis reagents (agarose, TBE buffer, DNA m.w. marker, SYBRGreen).
5. Silica microcentrifuge columns for DNA purification from agarose gel (e.g., Wizard® SV Gel, Promega).
6. Plasmid minipreparation kit (e.g., PCR Clean-Up system, Promega).
7. Plasmid with 3′-T overhangs, ligase, and buffer (e.g., pGEM-T kit, Promega).
8. Lysogeny broth (LB) liquid and solid (1% agar).
9. SOC liquid medium: 2% tryptone, 0.5% yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulfate, 20 mM glucose.
10. *E. coli* DH5α chemically competent cells (*see* **Note 5**).
11. 50 mg/mL ampicillin stock solution, 0.22 μm filter sterilized, store at −20 °C.
12. 50 mg/mL X-gal stock solution, dissolved in DMF, filter (0.22 μm) sterilized, store at −20 °C.
13. 23.83 mg/mL IPTG solution, dissolved in sterile water, filter (0.22 μm) sterilized, store at −20 °C.
14. PCR tubes for 0.2 mL, 1.5 mL, and 2 mL.
15. Petri dishes Ø 90 mm.
16. Retsch Mixer Mill MM400 (Retsch GmbH, Haan, Germany) equipped with adaptors for 2 mL tubes and 3 mm beads, or a handheld homogenizer.
17. Spectrophotometer for measuring DNA concentration.
18. Thermocycler.
19. Electrophoresis unit and power supply.
20. UV transilluminator.
21. 37 °C shaking and static incubators.
22. Heat block for PCR tubes.
23. Software for sequence analysis.

3 Methods

3.1 Preparation of Agrobacterium Inoculum

1. Prepare *A. tumefaciens* strain LBA4404 transformed with the pMDC32-AteCas9-DcF3H-gR3,gR4 binary vector (Fig. 1, *see* **Note 1**).

Table 1
List of primers

Purpose	Primer name	Oligonucleotide sequence 5′–3′	Annealing temp. (°C)	Expected product length (bp)
Verification of transgenic events	35S-Cf3	CCACGTC TTCAAAGCAAGTGG	60	576, 903
	Cas9-R	TTGGGTGTCTCTCG TGCTTC		
	Aph-F	AAGGAATCGGTCAA TACACTACATGG	60	398
	Aph-R	AAGACCAA TGCGGAGCATA TACG		
Nested PCR of the carrot F3H gene fragment	F3H_FO	GAGAAACTCCGG TTCGATATG	56	709
	F3H_RO	CTGAACAGTGA TCCAGGTTT		
	F3H_FM	CGTGTTATCG TTGGGATCGG	56	538
	F3H_RM	AGCAAGAGCGTAA TTGTGCC		
Sequencing	F3H_FI	ATCACTTTAAAAAGG TTATCAGGG	56	–

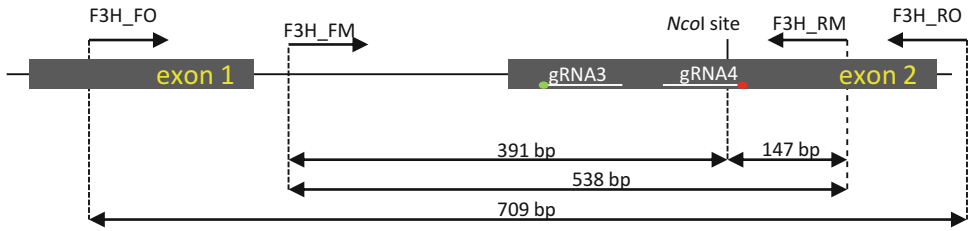

Fig. 2 Localization of the two target sites at the carrot *F3H* gene and primers. Both gRNAs target the exon 2 of the *F3H* gene with 120 bp distance between the two PAM sequences. *Nco*I restriction site is localized within the gRNA4 target sequence proximal to PAM. PAM sequences are marked by green and red dots for gRNA3 and gRNA4, respectively. The first round of a nested PCR with F3H_FO and F3H_RO primers results in a 709 bp product. Based on this fragment as template, the second round of PCR with F3H_FM and F3H_RM primers results in a 538 bp fragment. Digestion of the final PCR product with the *Nco*I nuclease results in two restriction fragments, 391 bp and 147 bp, if no mutation is generated at the *Nco*I restriction site, or a 538 bp product if the *Nco*I restriction site is abolished by the induced mutations

Steps 2–5 are done 1 day before transformation; the remaining steps should be done just before transformation.

2. Pour 20 mL of LB to a sterile 100 mL Erlenmeyer flask.
3. Add 20 μL kanamycin stock solution.
4. Inoculate *A. tumefaciens* LBA4404 cells carrying pMDC32-AteCas9-DcF3H-gR3,gR4 from a fresh culture on LB plate (*see* **Note 6**).
5. Incubate the flask with vigorous shaking at 26 °C in the dark for up to 24 h.
6. Centrifuge overnight *A. tumefaciens* cell culture for 5–10 min at 5000 × *g* at RT.
7. Remove supernatant by decanting (*see* **Note 7**).
8. Resuspend the bacteria pellet in 1 ml of liquid BI medium.
9. Take 100 μL of bacteria suspension and mix with 900 μL BI medium to get 10× dilution in a spectrophotometer cuvette.
10. Measure optical density at 600 nm (OD_{600}) of the prepared 10× dilution using a spectrophotometer.
11. Make the inoculum in a fresh tube by diluting bacteria with BI medium to acquire the final $OD_{600} = 0.5$.
12. Add acetosyringone stock solution to acquire 100 μM concentration in the inoculum.

3.2 Callus Transformation

1. Prepare Ø 6 cm Petri dishes with solid BI medium and place one sterile filter paper disc on the surface of the solidified medium (*see* **Note 8**).
2. Pick small callus clumps using tweezers and place them gently on the filter paper in prepared Petri dishes.
3. Gently apply freshly prepared *A. tumefaciens* inoculum over callus using a pipette. Callus should be completely immersed in the inoculum.
4. Incubate callus in the inoculum for 20 min at RT.
5. Gently rotate plate every few minutes to mix the inoculum without disturbing callus clumps.
6. Remove the inoculum from the Petri dish by pipetting it out while not disturbing callus.
7. Pick up callus clumps with tweezers and touch it to a piece of dry sterile filter paper for a few seconds to remove excess inoculum (*see* **Note 9**).
8. Gently move callus clumps to Petri dishes with solid BI medium and place them on the filter paper on top of the medium surface.
9. Incubate plates at 26 °C in the dark for 3 days to allow callus coculture with *Agrobacterium*.

3.3 Agrobacterium Elimination

1. Prepare a fresh antibiotic solution with final concentrations: 800 mg/l cefotaxime and 400 mg/l timentin.
2. Pipette the prepared antibiotic solution over callus to cover it completely (*see* **Note 10**).
3. Keep callus under the antibiotic solution for 20 min.
4. Gently remove the antibiotic solution by pipetting it out. While pipetting, touch a filter paper by the pipette tip near callus to ensure complete removal of the solution.
5. Transfer the whole filter paper with attached callus into a fresh 6 cm Petri dish with solid BI medium containing 400 mg/L cefotaxime and 200 mg/L timentin.
6. Incubate the Petri dishes with calli at 26 °C in the dark for 3 weeks.

3.4 Selection of Transformants

1. Prepare Ø 90 mm Petri dishes with solid BI medium containing 400 mg/L cefotaxime, 200 mg/L timentin, and 25 mg/L hygromycin (*see* **Note 11**).
2. Transfer the whole callus clumps using tweezers and gently spread them on the surface of BI medium.
3. Incubate plates at 26 °C in the dark for 3 weeks.
4. Prepare Ø 90 mm Petri dishes with solid BI medium supplemented with 25 mg/L hygromycin, 200 mg/l cefotaxime, and 100 mg/L timentin.
5. Pick white or pink calli (putative mutants) and place them onto fresh BI medium with 25 mg/L hygromycin, 200 mg/L cefotaxime, and 100 mg/L timentin (*see* **Note 12**).
6. Also transfer the remaining purple colored calli to fresh BI medium with 25 mg/L hygromycin, 200 mg/L cefotaxime, and 100 mg/L timentin. This allows recovering more transgenic calli, which may require longer time to visually identify mutants.
7. Incubate plates at 26 °C in the dark. Transfer the growing calli to fresh BI medium with 25 mg/L hygromycin every 3 weeks.
8. Grow calli on such selection medium for at least 2 months to select transformed calli.

3.5 Validation of Transgenic Events

1. Isolate genomic DNA from small amount of calli (i.e., 10 mg) which are white or pink in color. Dilute genomic DNA to 5 ng/μL (*see* **Note 13**).
2. Confirm the presence of T-DNA by PCR with primer pairs: (1) 35S-Cf3 and Cas9-R (for amplifying the fragment of 35S promoter and Cas9 gene, respectively), (2) aph-F and aph-R (for amplifying hygromycin resistance gene) (Table 1, *see* **Note 14**).

3. Run 1% agarose gel electrophoresis with SYBRGreen for 2 h. Visualize on a UV transilluminator.

3.6 PCR and Restriction Fragment Length Polymorphism (RFLP) Analysis of F3H Mutants

1. Set up the first round of a nested PCR with F3H_FO and F3H_RO primers (Fig. 2).
2. Dilute amplified products ×100 and use the solution as the DNA template for the second round of nested PCR with primers F3H_FM and F3H_RM. Set the final reaction volume to at least 25 μL (*see* **Note 15**).
3. Perform a fast agarose gel electrophoresis with 5 μL of the PCR reaction solution to check the presence of PCR products.
4. Digest 10 μL of the PCR products using 0.25 μL *Nco*I enzyme, 2 μL of CutSmart buffer in 20 μL final volume. Incubate at 37 °C for 3 h. Deactivate the enzyme at 80 °C for 20 min.
5. Run 1% agarose gel electrophoresis with SYBRGreen with or loading 5 μL of the PCR amplified products from the second round of PCR (undigested sample) and 20 μL of the digested sample in separate lanes. Visualize the results on a UV transilluminator (Fig. 3, *see* **Note 16**).

3.7 Identifying Mutations Induced by Cas9 at F3H

1. Aided with a UV transilluminator, isolate the gel containing the two uncut bands from each sample respectively (the longer one of ~538 bp carries putative mutations at gRNA4 and the slightly shorter one carries putative deletions induced by gRNA3 and gRNA4; *see* Fig. 2). Purify DNA from the gel fragments using a gel purification kit.
2. Ligate gel-purified products into a TA cloning plasmid harboring 3′-T overhangs at the cloning site (e.g., pGem®-T, Promega).
3. Transform competent *E. coli* cells with the recombinant plasmid (*see* **Note 17**).
4. Pick three white colonies with pipette tips and set up the colony PCR with T7 and Sp6 primers.
5. Run PCR products by gel electrophoresis to confirm the successful cloning.
6. Inoculate two confirmed *E. coli* colonies for each construct into liquid LB medium containing 50 mg/L ampicillin. Incubate with vigorous shaking at 37 °C overnight and then purify plasmid DNA with a miniprep kit.
7. Sequence plasmids with the F3H_FI primer (Table 1) and analyze results by sequence alignment with reference wild-type *F3H* sequence (Acc. No. XM_017385173 at NCBI) using an available software (e.g., ApE, BioEdit) to detect mutations at gRNA3 and gRNA4 target sites.

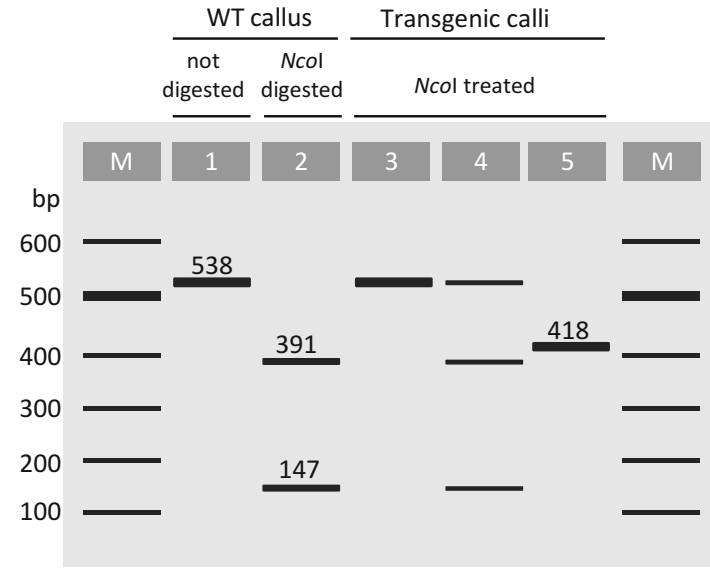

Fig. 3 A schematic diagram illustrating the analysis of targeted mutations by PCR and restriction fragment length polymorphism (RFLP) assays. Wild-type, non--transgenic control (lanes 1–2) and transgenic calli (lanes 3–5). Lanes: 1—undigested PCR product; 2—completely digested product; 3—undigested product due to the loss of *Nco*I restriction site; 4—partial digestion due to a monoallelic mutation or mosaic mutations; 5—shorter PCR product (e.g., 418 bp) due to chromosomal deletions by gRNA3 and gRNA4

4 Notes

1. The assembled T-DNA binary vector based on the pMDC32 destination vector (Fig. 1) [14] contains two gRNAs (gRNA3—ATTAGAGCCCGGGACTACT, gRNA4—AAGTTTTGTCAGAGGCCAT), Arabidopsis codon-optimized Cas9 gene (AteCas9 from pYPQ154) and hygromycin resistance gene. Two gRNAs were designed to target the second exon of the carrot *F3H* gene (NCBI Acc. No. XM_017385173, Fig. 2).
2. To visualize the effect of the *F3H* gene editing, a purple carrot cell line that accumulates high levels of anthocyanin is used. Such a cell line can be induced from commercial 'Anthonina,' 'deep purple,' or other carrot varieties developing purple storage roots. Callus is induced by incubating root discs on BI medium (*see* Subheading 2.1, **step 5**) for 2–3 months. During this time period, transfer root discs to a fresh medium after the first month and then transfer developing callus to a fresh medium for subculturing every 3 weeks. Callus culture should be well established, homogeneously displaying purple color and well growing. Callus growing 1–2 weeks on a fresh medium is recommended for transformation.

3. A richer medium is recommended for other *Agrobacterium* strains that have a slower growth rate (e.g., YEP: 10 g/L bacto-peptone, 10 g/L yeast extract, 5 g/L NaCl with 2 mL/L 1 M $MgSO_4$ added after autoclaving).
4. Callus growth media can be solidified with 3% commercial agar (cell and tissue culture grade). However, the use of phytagel instead of agar results in better carrot callus growth.
5. Other *E. coli* strains commonly used for DNA cloning can be used.
6. *A. tumefaciens* culture in liquid LB can be stored at 4 °C and used for culture later. For this purpose, put the flask with bacteria culture at a shaker (vigorous shaking at 26 °C in dark) for 1 h and then collect 100 μL of culture to inoculate 20 mL fresh liquid LB in a new Erlenmeyer flask.
7. While centrifuging bacterial culture, use tubes with a round-shaped bottom. Decanting supernatant from round bottom tubes is easier than from cone-shaped tubes. Centrifuged bacteria create a dense pellet at the tube bottom and there should be no loss of pelleted bacteria when decanting supernatant.
8. The choice of the Petri dish size is based on the amount of calli to be transformed. Callus clumps used for transformation should be around 0.5 cm in diameter. About five such callus clumps can be accommodated in a Ø 60 mm Petri dish.
9. Callus clumps may have loose structure and break in the inoculum into smaller pieces. In this case, use tweezers to gather small calli together to form a bigger clump.
10. *Agrobacterium* colonies growing around calli are usually slightly visible after 3 days of coculture. However, sometimes *A. tumefaciens* grows very fast and creates thick biofilm difficult to eliminate using antibiotics. Calli overgrown by *A. tumefaciens* will most likely die. Overgrowth of bacteria may be due to long coculture time or insufficient removal of the inoculum during the transformation procedure. Although it is sometimes possible to remove the biofilm with a pipette tip, but it is recommended to repeat transformation by reducing cocultivation time to 2 days.
11. For most carrot callus lines, 25 mg/L hygromycin concentration should be sufficient. However, depending on carrot variety, callus structure, and growth rate, a higher concentration may be required.
12. It is essential to pick single growing callus without touching others.
13. Independent callus clumps should be used for genomic DNA isolation. We use Rogers and Bendich CTAB method [15], which allows for high quality DNA isolation from a small amount of plant material. A modification to the original protocol involves the use of the Retsch Mixer Mill MM400 (Retsch

GmbH, Haan, Germany) for tissue homogenization. Fresh callus sample is each placed in a 2 mL eppendorf tube with two beads (Ø 3 mm) and 100 μL CTAB buffer, and is then ground with 25 beats/s at RT for 3 min. Then additional 600 μL CTAB buffer is added and further steps are done according to the original protocol. Other homogenizing methods, e.g., using a handheld homogenizer, may also be used.

14. Set up the PCR reaction with standard reagents (e.g., 10 μL final reaction volume with 1 μL DNA, 0.5 μL 10 μM primers, 5 μL PCR Mix, containing dNTPs and Taq polymerase) and with thermal conditions as follows: initial denaturation at 95 °C for 4 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C or 60 °C (depending on the primer sequences, Table 1) for 30 s, elongation at 72 °C for 1 min and the final extension step at 72 °C for 5 min.
15. A nested PCR is recommended to avoid off-target amplification due to similarity of the *F3H* gene sequence to other regions in the carrot genome.
16. Digestion of wild-type *F3H* sequence with *Nco*I yields 147 bp and 391 bp long fragments (Fig. 3). As a consequence of the CRISPR/Cas9 modification of DNA at the gRNA4 target site, the *Nco*I restriction site is lost and an undigested 538 bp product should be seen. However, small Indels or large deletions are often generated. To detect products of different lengths, we usually apply long 4–6 h electrophoresis at a low voltage. A long resolving time allows for better separation of the DNA products differing by several nucleotides.
17. For *E. coli* transformation, we use standard protocol with heat shock at 42 °C. In brief, remove competent cells stored in 100 μL aliquots at −80 °C and add the entire volume of a ligation mixture (usually 20 μL), and incubate on ice for 30 min with delicate mixing every 5 min. At the same time prepare 1.5 mL tubes with 1 mL of SOC medium (without antibiotics) and a heat block set to 42 °C. Place tubes with bacteria in the heat block for 40 s, transfer on ice, and then pipet the whole volume into the previously prepared tubes with SOC medium. Incubate at 37 °C for 1 h with vigorous shaking. Spread 100 μL of transformed bacteria onto Petri dishes with a solid LB containing 50 mg/L ampicillin (20 μL stock solution per 20 mL LB), 119 mg/L IPTG (100 μL stock solution per 20 mL LB), and 80 μg/mL X-Gal (32 μL stock solution per 20 mL LB). Centrifuge the remaining volume of transformed bacteria at 5000 × *g* for 5 min, discard 920 μL of the supernatant, resuspend the pellet in the remaining volume, and spread all onto another LB plate with the antibiotics for incubation at 37 °C overnight.

Acknowledgments

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Chapter 16

Genome Editing in Soybean with CRISPR/Cas9

Junqi Liu, Samatha Gunapati, Nicole T. Mihelich, Adrian O. Stec, Jean-Michel Michno, and Robert M. Stupar

Abstract

CRISPR/Cas9 mediated genome editing technology has experienced rapid advances in recent years and has been applied to a wide variety of plant species, including soybean. Several platforms have been developed for designing and cloning of single CRISPR targets or multiple targets in a single destination vector. This chapter provides an updated working protocol for applying CRISPR/Cas9 technology to target a single gene or multiple genes simultaneously in soybean. We describe two platforms for cloning single CRISPR targets and multiplexing targets, respectively, and reagent delivery methodologies. The protocols address crucial limiting steps that can limit CRISPR editing in soybean hairy roots, composite plants, and tissue culture-based regenerated whole plants. To date, transgenic soybean plants with mutagenesis in up to three target genes have been obtained with this procedure.

Key words CRISPR/Cas9, Hairy root transformation, Composite plant, *Agrobacterium*-mediated whole plant transformation, Gradient polyacrylamide gel electrophoresis (PAGE)

1 Introduction

Genome editing with the CRISPR/Cas9 system has become a routine technology for precision genomics. There are a wide variety of platforms available to facilitate target design and cloning. In soybean (*Glycine max* (L.) Merr.), high-throughput hairy root transformation [1] is valuable for rapidly assessing mutagenesis efficiency of various CRISPR/Cas9 platforms. Furthermore, transformation of composite plants (i.e., plants with a wild-type shoot and transgenic roots) have proved to be efficient for studying certain root phenotypes (e.g., nodulation or nutrient uptake) without encountering the long period of tissue culture-based plant regeneration process [2]. However, genome engineering in whole soybean plants still requires reagent delivery through genetic transformation. While DNA-free and transformation-free genome editing have been demonstrated in some plant species [3–6], the whole-plant genome editing studies published to date in soybean

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have all delivered reagents through stable T-DNA transgenesis [7–12].

In this chapter, we describe platforms for cloning a single CRISPR target and multiple targets in one binary T-DNA vector [13, 14] (*see* **Note 1**). Furthermore, we report simplified procedures for high efficiency transformation of the CRISPR transgenes to different soybean cell types, including hairy roots, composite plants, and whole plants. We also describe protocols for detecting new mutations in these cells. For low abundance mutations in somatic tissues, detection can be accomplished using enrichment CAPS-PCR (cleaved amplified polymorphic sequences-PCR). For multiple mutations in individual transgenic plants, detection can be accomplished by resolving heteroduplex DNA using gradient polyacrylamide gel electrophoresis (PAGE) analysis of PCR amplified target regions [15]. This method accelerates the initial screening of a large number of regenerated plants. Finally, with the combination of an improved whole plant transformation pipeline and a Csy4/Cas9 vector system for multiplexing CRISPR targets, targeted mutagenesis of up to three genes (or more) on different chromosomes can be achieved in a single transgenic plant.

Taken together, this chapter is an updated working protocol for applying CRISPR/Cas9 technology to target a single gene or multiple genes simultaneously in soybean. It is worth noting that there are several alternative tools and protocol variants that may be effective at achieving similar outcomes. Furthermore, at the time of this writing, there is no reliable system for regenerating soybean plants from protoplast and there have been no published accounts of ribonucleoprotein (RNP) delivery resulting in a modified soybean whole-plant. Given the pace in which new discoveries are made in the field of genome engineering, it would not be surprising to see new construct development and soybean reagent delivery methods (such as RNP) in the near future that render the methods outlined in this chapter obsolete. Thus, this chapter merely provides details for the methods that have worked in our lab to date. But, to borrow (and revise) an old phrase, there is more than one way to edit a soybean gene.

2 Materials

2.1 Cloning CRISPR Targets

1. Bacterial strains: DH5α competent cells, TOP10 One-Shot competent cells.
2. Media: LB liquid broth and agar plates (+ampicillin, 100 μg/mL). YEP liquid and agar plates (+kanamycin, 50 μg/mL).
3. Plasmid vectors: pBS_AtU6:gRNA, pMDC32/d35S/gmco-Cas9, pTRANS230d, pMOD_A0521, pMOD_2103, pMOD_C2906 (*see* **Note 1**).

4. CRISPR target specific oligos were designed based on CRISPR targets (*see* **Note 1**).
5. Enzymes and PCR Kits: Hind III, Pst I, Qiagen Hot Start PCR Master Mix, KOD high-fidelity PCR Kit, Aar I, Esp 3I, Sap I, T4 DNA ligase, T7 DNA ligase, 2× Rapid T4 DNA ligation buffer, 10× T4 DNA ligation buffer, 2× T7 ligation buffer.

2.2 Hairy Root Transformation

1. *Agrobacterium rhizogenes* NCPPB2659 (also known as strain K599) freeze-thaw competent cells (*see* **Note 2**).
2. ½ Murashige & Skoog (MS) liquid medium (pH 5.7) [16]: Add 2.15 g MS basic salts to 1 L of ddH_2O to dissolve and adjust pH value to pH 5.7 with 1 M KOH. Autoclave and store at room temperature (RT).
3. Whatman filter paper and Envision brown single fold paper towels: Wrap Whatman filter papers and paper towels with aluminum foil. Autoclave in liquid cycle and store at RT.
4. Commercial bleach (with 5.25% sodium hypochlorite).
5. Corning bioassay dishes (245 cm × 245 cm).

2.3 Agrobacterium-Mediated Whole Plant Transformation

1. YEP Broth: Add about 400 mL deionized (DI) water to a beaker. Add 5 g Bacto Peptone, 2.5 g yeast extract, and 2.5 g NaCl. Adjust pH to 7.0 and bring volume to 500 mL with DI water. Autoclave and cool to RT.
2. Liquid cocultivation media (LCCM) (1 L): Add 800 mL DI water to a beaker. Add 0.31 g Gamborg's B-5 Basal Salt Mixture, 30 g sucrose, and 3.9 g MES hydrate. Bring pH to 5.4 with 1 M KOH. Bring volume to 996 mL with DI water. Aliquot 249 mL into four 500 mL bottles. Autoclave and cool to RT. In flow hood, to each 249 mL, add 250 μL Gamborg's B5 vitamins (1000× stock), 18 μL of 5 mg/mL 6-BAP, 9.6 μL of 1 mg/mL Gibberellic Acid, and 250 μL of 200 mM Acetosyringone in DMSO.
3. Cocultivation media (CCM) (500 mL): Add about 400 mL DI water to a beaker. Add 0.155 g Gamborg's B-5 Basal Salt Mixture, 15 g sucrose, and 1.95 g MES hydrate. Bring pH to 5.4 with 1 M KOH. Bring volume to 496 mL with DI water. Transfer to 1 L autoclavable bottle and add 3 g of Difco noble agar or phytagel. Autoclave and cool to 50 °C. In flow hood, add 500 μL Gamborg's B5 vitamins, 835 μL of 5 mg/mL 6-BAP, 19.2 μL of 1 mg/mL Gibberellic Acid, 250 μL of 1 M Sodium Thiosulfate, 500 μL of 500 mM Dithiothreitol, 500 μL of 200 mM Acetosyringone in DMSO, and 500 μL of freshly prepared 50 mg/mL L-Cysteine. Pour into 15 × 100 mm petri plates in flow hood and let cool. Place a sterile Whatman filter paper on each plate after solidified.

4. Liquid shoot initiation media (LSIM) (500 mL): Add about 400 mL DI water to a beaker. Add 1.6 g Gamborg's B-5 basic salts, 15 g sucrose, and 0.293 g MES hydrate. Bring pH to 5.6 with 1 M KOH. Bring volume to 498 mL with DI water. Autoclave and cool to RT. In flow hood, add 200 μL of 200 mg/mL Ticarcillin, 500 μL of 200 mg/mL Cefotaxime, and 500 μL of 100 mg/mL Vancomycin.
5. Shoot initiation media I (SIM I) (500 mL): Add about 400 mL DI water to a beaker. Add 1.6 g Gamborg's B-5 Basal Medium with Minimal Organics, 15 g sucrose, and 0.293 g MES hydrate. Bring pH to 5.6 with 1 M KOH. Bring volume to 496 mL with DI water. Transfer to 1 L autoclavable bottle and add 4 g of Difco noble agar or phytagel. Autoclave and cool to 50 °C. In flow hood, add 200 μL of 200 mg/mL Ticarcillin, 500 μL of 200 mg/mL Cefotaxime, 500 μL of 100 mg/mL Vancomycin, 835 μL of 5 mg/mL 6-BAP, and 269 μL of 2 mg/mL Kinetin. Pour into 25 × 100 mm petri plates half full in flow hood and let cool.
6. Shoot initiation media II (SIM II) (500 mL): Same recipe as SIM I but after autoclaving and cooling to 50 °C, also add the appropriate selection agent (such as 500 μL of 5 mg/mL Glufosinate).
7. Shoot elongation media (SEM) (500 mL): Add about 400 mL DI water to a beaker. Add 2.2 g MS salts, iron stock, B5 vitamins, 15 g sucrose, and 0.293 g MES hydrate. Bring pH to 5.6 with 1 M KOH. Bring volume to 495 mL with DI water. Transfer to 1 L autoclavable bottle and add 4 g of Difco noble agar or phytagel. Autoclave and cool to 50 °C. In flow hood, add 200 μL of 200 mg/mL Ticarcillin, 500 μL of 200 mg/mL Cefotaxime, 500 μL of 100 mg/mL Vancomycin, 19.2 μL of 1 mg/mL Gibberellic Acid, 500 μL of 50 mg/mL Asparagine, 500 μL of 100 mg/mL L-Pyroglutamic Acid, 50 μL of 1 mg/mL Indole-3-acetic Acid (IAA), 500 μL of 1 mg/mL Zeatin Acid, and selection agent (such as 300 μL of 5 mg/mL Glufosinate). Pour into 25 × 100 mm petri plates half full in flow hood and let cool.
8. Rooting Media (RM) (500 mL): Add about 400 mL DI water to a beaker. Add 1.55 g Gamborg's B-5 Basal Salt Mixture, 10 g sucrose, and 0.293 g MES hydrate. Bring pH to 5.6 with 1 M KOH and bring volume to 497 mL with DI water. Transfer to 1 L autoclavable bottle and add 4 g of Difco noble agar or phytagel. Autoclave and cool to 50 °C. In flow hood, add 200 μL of 200 mg/mL Ticarcillin, 500 μL of 200 mg/mL Cefotaxime, 500 μL of 100 mg/mL Vancomycin, and 58 μL of 1 mg/mL Indole-3-butyric Acid (IBA). Pour into Combiness filter boxes about 2 cm thick in flow hood and let cool with lid slightly open. Close lids and store and store at RT.

2.4 Detection of Mutations

1. Enrichment CAPS-PCR: 2× PCR Hot Start Master Mix (i.e., all components for PCR except primers and template DNA), Restriction enzymes specific to each CRISPR target.
2. Gradient PAGE analysis of heteroduplex DNA: Premade 4–20% polyacrylamide gel, 1× TBE buffer, 5× Novex TBE loading buffer, Apex Safe DNA gel stain.

3 Methods

3.1 Cloning of a Single CRISPR Target

The structure of this CRISPR/Cas9 construct in binary T-DNA vector is depicted in Fig. 1a [*see* ref. 13].

1. Cloning of a CRISPR target into pBS_AtU6:gRNA expression vector: Mix 5 μL forward primer (100 μM), 5 μL reverse primer (100 μM), and 2.2 μL 5× KOD buffer. Incubate at 50 °C for 2 h. Dilute the annealed ds-oligo 100-fold and ligate with Bbs I-digested pBS_gRNA by mixing 1 μL pBS_gRNA vector (50 ng/μL), 1 μL ds oligo (1.0 μM), 2.5 μL 2× rapid ligation buffer, and 0.5 μL T4 DNA ligase. Incubate at RT for at least 1 h. Thaw a tube of 50 μL DH5α competent cells on ice. Add the full 5 μL of the ligation reaction to the competent cells and mix gently by pipetting up and down several times. Incubate on ice for 30 min. Heat shock for 1 min at 42 °C and immediately put on ice for 2 min. Add 500 μL LB liquid medium and incubate at 37 °C for 1 h. Plate out 200 μL on LB+ agar plates containing 100 μg/mL ampicillin. Incubate the plates overnight at 37 °C. Pick four single colonies to inoculate 10 mL LB + Ampicillin (100 μg/mL) liquid medium and grow the culture in a shaking incubator overnight at 37 °C. Perform a plasmid DNA prep for each culture according to the QIAGEN Plasmid Mini KIT instructions. Sequence plasmid DNA with the forward primer (5′-TCGTTGAACAACGGAAACTCGAC-3′) from the AtU6 promoter.
2. Sub-cloning of gRNA cassette into a binary T-DNA vector pMDC32/GUS/Cas9: Digest 2 μg pBS_gRNA plasmid with Hind III and Pst I to release the gRNA expression cassette by mixing 2 μg plasmid DNA, 2 μL CutSmart buffer, 1 μL HindIII, 1 μL Pst I, and sterile water in a 20 μL volume. Incubate for 2 h at 37 °C. Heat-inactivate the enzymes by incubating for 20 min at 80 °C. Move 1.0 μL directly from the heat-inactivated digestion mix to a new tube. Add 1.0 μL HindIII/Pst I-digested pMDC32/GUS/Cas9 vector (50 ng/μL), 2.5 μL 2× rapid ligation buffer, and 0.5 μL T4 DNA ligase. Incubate at RT for at least 1 h. Transform TOP10 one-shot chemical competent cells as described above for heat shock transformation (*see* Subheading 3.1,

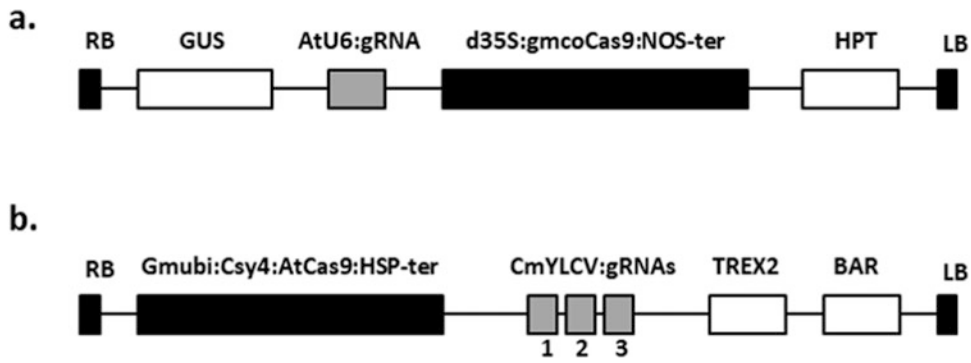

Fig. 1 Representative structures of CRISPR/Cas9 components in T-DNA binary vectors. (**a**) Construct with a single CRISPR target in an AtU6/gRNA cassette and *Glycine max* codon-optimized Cas9 nuclease (gmcoCas9) driven by the d35S CaMV promoter in the pMDC32 binary T-DNA vector; GUS: constitutively expressed GUS reporter gene; HPT: hygromycin phosphotransferase gene as selective marker. T-DNA borders: left border (LB) and right border (RB). (**b**) Construct with triplex gRNAs interspaced by Csy4 nuclease cleavage sites. The Csy4/Cas9 fusion is driven by the CmYLCV promoter. TREX2: constitutive expression cassette of an exonuclease gene; BAR: BASTA resistance gene. T-DNA borders: left border (LB) and right border (RB)

step 1). Plate out 100 μL on LB + kanamycin (50 μg/mL) agar plate and incubate overnight at 37 °C. Pick four single colonies for plasmid DNA prep as described above. Once the plasmids are prepped, digest 1 μg plasmid DNA with Hind III/Pst I and run on an agarose gel to verify that gRNA cassette is cloned.

3. *Agrobacterium* transformation by "freeze-thaw" procedure: Thaw a 100 μL batch of *Agrobacterium rhizogenes* (NCPPB2659) competent cells on ice. Add 1 μg of pMDC32/GUS/Cas9/gRNA plasmid DNA and mix gently by pipetting up and down several times. Freeze in liquid nitrogen for exactly 1 min and transfer to 37 °C for 5 min or until the cells are completely thawed. Immediately add 500 μL LB liquid medium and mix by inverting the tube several times. Incubate at 28 °C for 1–3 h. Plate out 100 μL on LB agar plates (+kanamycin 50 μg/mL). Incubate the plates at 28 °C for 3 days. Pick single colonies to inoculate 10 mL of LB + kanamycin (50 μg/mL) to prepare a glycerol stock of transformed *Agrobacterium* for long-term storage.

3.2 Cloning of Multiple CRISPR Targets

A three-step procedure is carried out in this example for cloning three CRISPR targets in a single vector (below referred to as a "triplex") (Fig. 1b) [*see* ref. 14].

1. Amplification of gRNA spacers: Prepare a master mix for four PCR reactions with 4 μL of pMOD_B2103 plasmid DNA (1 ng/μL), 20 μL 10× KOD buffer, 20 μL 10 mM dNTP, 20 μL 5 mM $MgSO_4$, 4 μL KOD Polymerase, and 120 μL sterile ddH_2O. Aliquot 40 μL to four tubes with the following

primer combinations, respectively: Reaction #1 (CmYLCV +Csy_gRNA1); Reaction #2 (rep_gRNA1 + Csy-gRNA2); Reaction #3 (rep-gRNA2+Csy-gRNA3); Reaction #4 (rep-gRNA3 + Csy-E). Set up thermocycler with the following conditions: 96 °C for 3 min, 30× (96 °C for 10 s, 60 °C for 15 s, 70 °C for 10 s), 70 °C for 10 min. All primers were diluted to 100 μM and 5 μL primer was added in a 50 μL reaction. The pMOD_B2103 plasmid DNA is used as template to amplify all gRNA spacers.

2. Assembly of triplex targets in pMOD_B2103. Prepare a 20 μL reaction with: 3.0 μL pMOD_B2103 plasmid DNA (50 ng/μL); 1.0 μL 10× diluted gRNA spacers (from each of four reactions); 10 μL 2× T7 ligase buffer; 1.0 μL Sap I; 1.0 μL Esp 3I; 1.0 μL T7 DNA ligase. Spin down briefly and mix thoroughly by pipetting up and down. Set up a thermocycler for the following conditions: 20× (37 °C for 5 min; 25 °C for 10 min). Use 5 μL to transform TOP10 One-Shot chemical competent cells. Grow LB + ampicillin (100 μg/mL) culture from colonies for plasmid DNA with the QIAGEN plasmid mini kit. Verify positive clones by sequencing plasmid DNA with the forward primer (5′-CTAGAAGTAGTCAAGGCGGC-3′).

3. Cloning of triplex CRISPR targets into a binary DNA vector. Cloning into destination vector pTRANS_230d: 1 μL (50 ng/μL) pTRANS_230d, 1 μL (150 ng/μL) pMOD_A0521, 1 μL (150 ng/μL) pMOD_B2103_triplex, 1 μL (150 ng/μL) pMOD_C2906, 0.4 μL Aar I oligo, 0.5 μL Aar I, 1.0 μL T4 DNA ligase, 2.0 μL 10× T4 DNA ligase buffer, and 12 μL sterile ddH_2O. Set the thermocycler for the following conditions: 10× (37 °C for 5 min then 16 °C for 10 min), 37 °C for 15 min. Use 5.0 μL of this reaction to transform TOP10 One-Shot competent cells as described for heat shock transformation (*see* Subheading 3.1, **step 1**). Plate out on LB + kanamycin (50 μg/mL) agar plates. Incubate overnight at 37 °C. Screen for positive clones by colony PCR with forward primer (5′-GTTGGATCTCTTCTGCAGC-3′) and reverse primer (5′-GTAAAACGACGGCCAGT-3′). Inoculate LB + kanamycin (50 μg/mL) liquid medium with positive colonies for a plasmid DNA prep. Use plasmid DNA to transform *Agrobacterium* competent cells as described in the "freeze-thaw" transformation procedure (*see* Subheading 3.1, **step 3**).

3.3 Hairy Root Transformation

1. Seed sterilization: Place soybean seeds in a 250 mL flask. Add 150 mL 20% (v/v) commercial bleach. Incubate for 8 min at RT on a shaking platform (at 80 rpm). Extensively rinse six times (6 × 10 min) with sterile water.

2. Imbibition/germination: Place sterilized seeds on two layers of filter paper presoaked with ½ × MS liquid medium (pH 5.7) [16] in Petri dishes in a culture room under 18:6 (light:dark) photoperiod at 25 °C. Typically, five- to seven-day-old seedlings are used for preparing explants to induce hairy roots.
3. *Agrobacterium rhizogenes* culture: Inoculate 50 mL LB liquid medium (+kanamycin 50 μg/mL) with a single colony. Incubate with rigorous shaking at 28 °C until OD600 nm reached 0.5–1.0. Centrifuge at 4000 rpm for 20 min at RT. The bacteria will form a pellet. Pour off the remaining liquid (treat this liquid with bleach solution to kill any remaining bacteria). Resuspend the pellet with ½ × MS medium (pH 5.7). Adjust the volume to reach an OD600 nm of 0.2–0.8 for optimal hairy root induction.
4. Explant preparation and infection: Excise cotyledons with a scalpel at approximately 3 mm above the cotyledonary node. Then cut the adaxial side (i.e., the flat side) multiple times at 1–3 mm depth to introduce multiple wounds (*see* Fig. 2b). Place the wounded cotyledonary explants in Petri plates. Add *Agrobacterium rhizogenes* suspension (OD 600 nm, 0.2~0.8) to the plates and make sure all explants are submerged. Incubate at RT for 20 min with gentle shaking at 80 rpm. Transfer inoculated cotyledons (adaxial side up) on a single layer of filter paper presoaked with ½ × MS liquid medium in Petri plates. Incubate in the dark at 25 °C for 3 days.
5. Hairy root induction and elongation: Subsequent incubation should be 24 °C under an 18:6 (light:dark) photoperiod. Add ½ MS liquid (pH 5.7) to the Petri dishes regularly to keep the underlying filter paper moist.
6. Assessment of hairy root transformation using vital GUS staining of hairy roots: Remove hairy roots and the attached cotyledonary explants carefully from Petri dishes. Rinse with sterile water. Submerge into vital GUS staining solution (X-Gluc, 1 mg/mL in ½ × MS liquid medium, pH 5.7). Incubate at RT until blue color developed in hairy roots.

3.4 Regeneration of Composite Plants

1. "Double inoculation" of hypocotyl: Seed treatment and germination procedure is the same as described above (in Subheading 3.3, **steps 1** and **2**). Cut off the root/hypocotyl approximately 0.5–1.0 cm below the cotyledonary node from the germinated seedlings with a scalpel dipped in an *Agrobacterium* suspension (cutting at a diagonal angle of 45°, leaving both cotyledons intact). Make a single puncture approximately 2 mm beneath the cotyledonary node with the same scalpel re-dipped with *Agrobacterium* suspension for a second inoculation site (*see* Fig. 2f; **Note 3**). Subsequent incubation

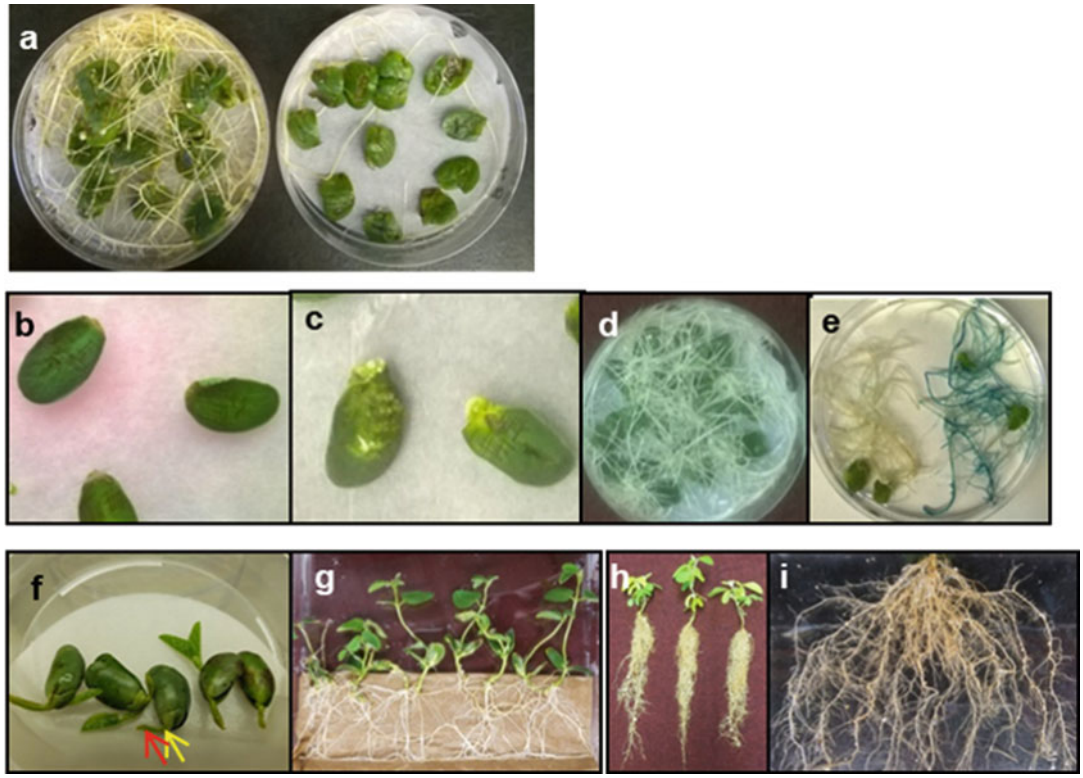

Fig. 2 Hairy root transformation and regeneration of composite plants. (**a**) Hairy roots induced on cotyledons by *A. rhizogenes* (NCPBB2659) clones with high virulence (left) contrasted with low or no virulence (right). (**b**) Cotyledons with multiple wounds. (**c**) Emerging hairy roots 7–10 days after inoculation. (**d**) Massive hairy root formation after incubation for 3 weeks. (**e**) Hairy roots attached to cotyledonary explants after GUS staining. GUS-positive roots were considered as transgenic (with CRISPR/Cas9 constructs) and were subject to mutation screening analysis; GUS-negative hairy roots were either non-transgenic or transgenic, but without transgene expression. (**f**) Double inoculation at the hypocotyl to generate composite plants. Arrows indicate wounding sites at the cotyledonary node (yellow) and diagonal cut at the hypocotyl (red). (**g**) Composite plants in large square plates with ½ × MS medium-soaked paper towels. (**h**) Composite plants after transplanting to vermiculite/perlite for 3 weeks. (**i**) Root architecture of a composite plant inoculated with *Bradyrhizobium japonicum* (USDA 110)

procedures are the same as described for hairy root induction (*see* Subheading 3.3) on cotyledons.

2. Maintenance of composite plants: After incubation for 1–2 weeks, transfer composite plants with emerging hairy roots to larger square plates (245 cm × 245 cm) for further root elongation (*see* Fig. 2g).
3. Transplanting of composite plants: Transplant large composite plants (with 5–10 cm hairy roots in length) to vermiculite/perlite (50/50) mix to produce a larger root mass. These large composite plants can be inoculated with the proper

Bradyrhizobium japonicum strain (e.g., USDA 110) for studying nodulation or other root phenotypes (*see* Fig. 2h–i, **Note 4**).

3.5 Whole Plant Transformation

This whole plant transformation procedure is modified from previously published protocols [17, 18].

1. **Seed sterilization**: Fill Petri dishes (half-full) with soybean seeds and place the Petri dishes (with lid open) in a glass desiccator in a fume food (*see* **Note 5**). Add 150 mL of commercial bleach to a glass beaker in the desiccator. Slowly add 3.5 mL of 12 M HCl to the bleach. Cover the desiccator immediately and incubate overnight (up to 24 h, *see* **Note 6**). Open the desiccator and cover the lids of Petri dishes before transferring to a Laminar flow hood. Open the lids of the Petri dishes so that the trapped residual chlorine gas in the soybean seeds will evaporate; keep the lids open for 30 min. Wrap the Petri dishes with Micropore surgical tape and store at RT.
2. **Preparation of *Agrobacterium* inoculum**: A 150 μL glycerol stock of *Agrobacterium rhizogenes* (18r12) harboring the construct of interest is used to directly inoculate 150 mL of YEP (+kanamycin 100 μg/mL). Incubate with rigorous shaking at 250 rpm for 2 days or until an OD600 nm of approximately 1.0 is reached. Precipitate the *Agrobacterium* culture by centrifugation at 4000 rpm for 20 min. Resuspend pellet in 30 mL LCCM medium and adjust the volume (to OD600 of approximately 1.5).
3. **Preparation of explants and infection**: In the Laminar flow hood, add sterile water to cover sterilized seeds in a Petri dish. Incubate at RT for 20 h in the dark. Transfer imbibed seeds to sterile Petri dishes. Make a longitudinal cut along the hilum to separate the cotyledons. Remove the seed coat and excise to the embryonic axis, but do not remove completely (*see* Fig. 3a). Transfer explants to Petri dishes and add *Agrobacterium* inoculum to cover all explants. Cover the lids and incubate at RT for 30 min on a shaking platform (with gentle shaking at approximately 80 rpm).
4. **Cocultivation**: In a Laminar flow food, place the infected explants on CCM medium overlaid with a layer of filter paper with the flat adaxial side facing down. Wrap the Petri dishes with parafilm and incubate at 24 °C under 18:6 (light:dark) photoperiod (with light intensity at 150 μmol s^{-1} m^{-2}) for 5 days (*see* Fig. 3a).
5. **Shoot induction and selection**: After 5 days of cocultivation, transfer the explants to Petri dishes and add LSIM medium to cover the explants. Add agent to terminate residual agrobacterium (e.g., 500 μL of 500 mg/mL carbenicillin). Incubate for

Fig. 3 *Agrobacterium*-mediated whole plant transformation. (**a**) Half seed explants. The upper explant has an intact embryonic axis while the lower sample shows partial excision (cutting) of the embryonic axis. (**b**) Explant in cocultivation media to facilitate cell transformation after infection with *Agrobacterium rhizogenes* (strain 18r12) inoculum. (**c**, **d**) Shoot induction stage for differentiation of transformed cell clusters for shoot initiation. (**e**) Shoot elongation. (**f**) Rooting plantlets. (**g–i**) Transplanting and adapting to standard growth conditions in the growth chamber and greenhouse. (**j**) Healthy pods bearing T1 seeds from plants grown in the greenhouse

30 min with shaking (80 rpm) (*see* **Note 7**). Place the explants on shoot induction media (SIM I) agar plates with the cotyledonary nodes imbedded in the agar media and the adaxial side of explants facing up (*see* **Note 8**). Wrap the plates with Micropore surgical tape and incubate at 24 °C under a 18:6 (light:dark) photoperiod for 14 days. Transfer the explants to SIM II media with appropriate selection agent (5 mg/L Glufosinate for constructs with the *BAR* gene). Cut and discard large shoots and make a fresh cut at the base of the shoot pad flush to the medium, and make sure the cut surface is imbedded in the medium with the differentiating region flush to the surface. Incubate for 14 days at 24 °C under a 18:6 (light:dark) photoperiod to select transformed shoots (*see* Fig. 3c, d).

6. **Shoot elongation**: After four total weeks of cultivation on shoot induction medium (SIM), transfer the explants to shoot elongation media (SEM) with appropriate selection agents. Remove the cotyledons from the explants and make a fresh cut at the base of the cell cluster/shoot pad. Transfer the cell cluster/shoot pad to SEM media with the cut side down (*see* **Note 9**). Wrap the plates containing explants with micropore tape and incubated at 24 °C for 2–8 weeks under a 18:6

(light:dark) photoperiod. The newly emerged tissues are transferred to fresh shoot elongation medium every 2 weeks or as needed, until stems have elongated beyond 2.5 cm (*see* Fig. 3e, **Note 10**).

7. **Rooting of transgenic plants**: Excise shoots of at least 2–3 cm in length from the cell cluster/shoot pad and transfer to rooting media (RM) in a Combiness filter box. For optimal root formation, the shoot and cell cluster pad are dipped into 1 mg/mL IBA for 30–60 s prior to culturing on rooting media. Incubate for 1–2 weeks at 24 °C under a 18:6 (light:dark) photoperiod, or until two or more roots have developed (*see* Fig. 3f, **Note 11**).
8. **Plant hardening and screening**: Remove rooted plants from RM after 1–2 weeks (or when healthy growing roots are observed and before plants reach the top of the filter box) and wash off any remaining medium with tap water. Transplant the plants into jiffy pots with moist metro mix potting soil. Pots should be placed in flat tray, covered with a humidome, and grown at 24 °C under a 18:6 (light:dark) photoperiod for at least 1 week. The plantlets should be watered as needed. Gradually open the vent holes in the humidome to allow aeration (*see* **Note 12**). Upon attaining the required growth of the plantlets, completely remove the humidome cover and transfer plantlets with at least two healthy trifoliates, along with peat pot, to an 8-in. pot filled with a 50/50 mix of metro mix and field soil. Allow the plantlets to grow in the growth chamber and greenhouse under standard conditions (*see* Fig. 3g–j).

3.6 Detection of Mutations

1. **Enrichment CAPS-PCR**: Predigestion of genomic DNA: Add 10 μL (20 ng/μL) genomic DNA, 2 μL 10xCutsmart buffer, 1 μL restriction enzyme (specific to the CRISPR target), and 7 μL sterile ddH2O. Incubate for at least 3 h or overnight at 37 °C. Take 1 μL of the digested DNA to set up PCR reactions (*see* **Note 13**): 1 μL predigested genomic DNA, 10 μL 2× Hot-Start Master mix, 2 μL forward primer (100 μM), 2 μL Reverse primer (100 μM), 5 μL sterile ddH_2O. Thermocycler conditions: 96 °C for 5 min, 30× (96 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min), 72 °C for 10 min. Following the reaction, the PCR buffer and fragments can be removed using a Qiagen PCR cleanup kit. The PCR product is then digested again with the same restriction enzyme used in the predigestion step above. This product can be resolved on a 1.5% agarose gel (*see* Fig. 5a). While enrichment CAPS-PCR is effective for detecting low abundance mutations in somatic cells (e.g., hairy roots), standard CAPS-PCR (without enrichment) may be preferred for some whole-plant applications (*see* **Note 14**).

2. **Gradient PAGE analysis of Heteroduplex DNA**: Amplify the target region from genomic DNA using PCR (*see* **Note 15**). Denature 10 μL of the PCR product by incubating at 96 °C for 5 min. Cool down the reaction at RT for 15 min for renaturation; this step allows for the imperfect renaturation of mutated sequences with wild-type sequences (or mutated sequences with other polymorphic mutated sequences in plants carrying biallelic mutations), which produces a novel product on the gel. Add 2 μL of 5× gel loading buffer. Set up PAGE 4–20% gels and fill the chamber with 1× TBE buffer. Remove the comb and white sealant tape carefully from the premade gel. Place the gel into the tray and clip tightly to avoid leaking. Add 200 mL of 1× TBE buffer in the middle chamber. Rinse the wells with 1× TBE gel running buffer before loading. After loading all samples, fill the exterior chamber with 600 mL of 1× TBE buffer. Run the gel at a constant voltage of 200 V for approximately 1.5 h (or until the xylene color of the loading dye reaches the bottom of the gel). Take the gel from the apparatus and place the gel in 100 mL of deionized water with 10 μL of safe DNA stain for 20 min with gentle shaking at 80 rpm. Replace the staining solution with 200 mL deionized water to destain the gel for 20–30 min. The resolved PCR products can be visualized under a UV-transilluminator (*see* Fig. 5b).

4 Notes

1. Designing CRISPR targets is not included in this book chapter. This process is greatly simplified by using web tools. Resources for designing/cloning single CRISPR targets can be found at http://stuparcrispr.cfans.umn.edu/CRISPR; resources for designing/cloning multiple CRISPR targets can be found at http://cfanspmorrell.oit.umn.edu/CRISPR_Multiplex. There are also several other web tools that are publicly available for target design. Vector information is also available at http://www.addgene.org.
2. Maintenance and screening of *Agrobacterium rhizogenes* (NCPPB2659) clones. The *Agrobacterium rhizogenes* strain (NCPPB2659), also commonly known as K599 [19], was imported from the National Center for Plant Pathogenic Bacteria (NCPPB) as vacuum-packaged dry powder. To isolate single clones, the dry powder was suspended in sterile water and streaked on LB agar plates (without antibiotics). After a 3-day incubation at 28 °C, single colonies were picked to inoculate LB liquid medium and grown at 28 °C for 2 days with rigorous shaking. The liquid culture was mixed with 50%

glycerol at a ratio of 2:1 (v/v) for long-term storage of glycerol stocks. All selected single clones were tested for virulence in hairy root induction. It is important to note that improper propagation/storage of *Agrobacterium* stock could lead to acquisition of novel antibiotic resistance or a drastic decrease/loss of hairy root-inducing ability (*see* Fig. 2a). Therefore, multiple batches of the original glycerol stock were prepared and then stored at −80 °C to avoid sequential propagation in the long term. It has been reported that *Agrobacterium rhizogenes* strain (NCPPB2659), when disarmed by homologous recombination, lost the ability to induce hairy roots [19].

3. Double-inoculated explants are kept in Petri plates wrapped with parafilm. High humidity in sealed Petri plates can lead to a higher number of hairy roots emerging from both the diagonally cut cross section and the punctured site at the hypocotyl. On average, a high transformation efficiency (approximately 90%) is routinely obtained (*see* Fig. 2g).
4. Nodulation of composite plants: Inoculated composite plants that are maintained in plates do not exhibit nodule formation in a test of more than 40 inoculated composite plants. In contrast, all composite plants will develop nodules if inoculation occurs after transplanting to vermiculite/perlite (50/50) mix (*see* Fig. 2h–i).
5. The *Agrobacterium*-mediated whole plant transformation protocol in this book chapter is modified from previously reported procedures [17, 18]. Major steps are depicted by representative images (*see* Fig. 3). Seeds collected from plants grown in the greenhouse or growth chamber are preferred for transformation. Seeds from plants in the open field may contain contaminants which are very difficult to remove. To prevent contamination, select clean and well-developed seeds. If you are working with different genotypes, label them properly and cover with transparent tape.
6. Be sure to use appropriate personal protective equipment (especially safety goggles) when working with strong acids and chlorine gas. Extended periods of sterilization (longer than 24 h) with chlorine gas may cause lower germination rates.
7. After cocultivation of explants with *Agrobacterium* inoculum, two washing steps (30 min each) are recommended prior to placing explants on SIM for the onset of shoot induction.
8. Orientation of the seed halves is important. The flat side of the seed halves should face up at a 30–45° angle to make sure that cotyledons are imbedded into the media.
9. Complete removal of dead tissue from the cell clusters at the subculturing stage is crucial for improving transformation efficiency (*see* Fig. 4). In this case, increased shoot production is

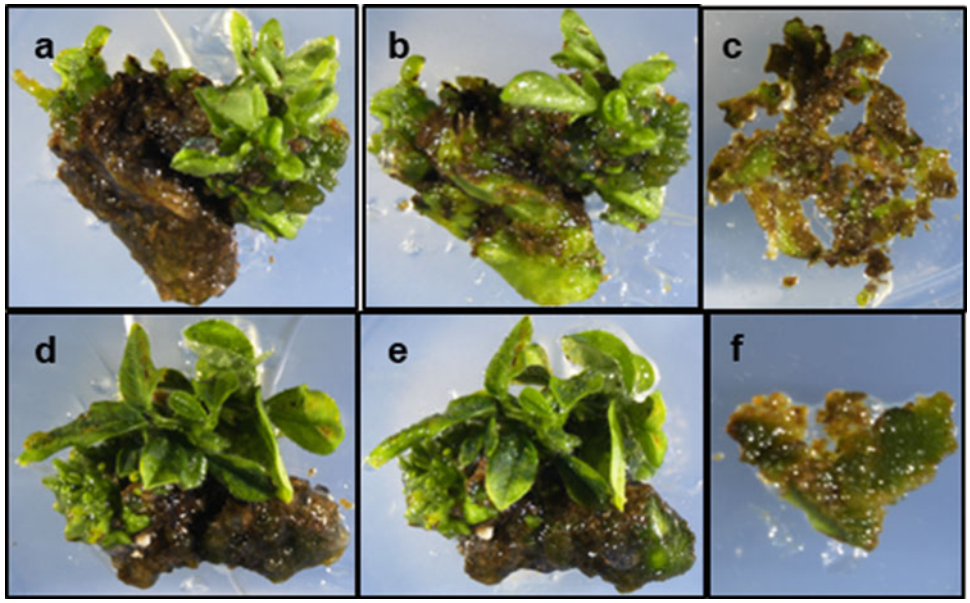

Fig. 4 Trimming of cell clusters and removal of dead tissues during whole-plant transformation. (**a–c**) Complete dead tissue removal. Part (**a**) shows the sample before the removal of dead tissue. Part (**b**) shows the cell clusters after the complete removal of dead tissue. Part (**c**) shows the amount of dead tissue removed. **(d–f)** Partial dead tissue removal. Part (**d**) shows the sample before the removal of dead tissue. Part (**e**) shows the cell clusters after the partial removal of dead tissue. Part (**f**) shows the amount of dead tissue removed

observed following complete removal compared to cell clusters that are transferred without trimming or with only partial removal of dead tissue.

10. Subculturing periodically is also a limiting step. Shoot elongation appears to be more efficient if transferred at 2-week rather than 3-week intervals. Delayed transfer could lead to a drastic reduction in transformation efficiency. Also note that the shoot length of 2.5 cm is measured from the base of the shoot to the apical meristem and does not include any petiole or leaf length.
11. Based on our experience, LED light is not recommended for the rooting process as it appeared to be somewhat detrimental to the survival of rooting plantlets.
12. A humidome is helpful for maintaining high humidity, which is important for the survival of plantlets. However, opening the vents occasionally in the humidome helps keep the plantlets aerated and healthy. It is noteworthy that some groups prefer a longer daylength in the tissue culture and plant hardening stages, to ensure adequate vegetative growth prior to flowering.
13. PCR conditions are optimized according to the desired PCR products prior to CAPS-PCR [20].

14. Enrichment CAPS-PCR is normally performed on somatic tissues (e.g., hairy roots) that are expected to have mostly wild-type alleles; in other words, this protocol "enriches" for mutated sequences by digesting the wild-type alleles with a restriction enzyme prior to PCR. For stable whole-plant events, one may prefer to use standard CAPS-PCR, as mutated alleles may be as abundant (or more abundant) than the wild-type alleles in the template DNA. To do standard CAPS-PCR, simply follow the above protocol, but do not perform the initial restriction enzyme digestion step prior to the PCR.
15. PCR conditions are optimized according to the desired PCR products prior to heteroduplex analysis by gradient PAGE [15]. Lower numbers of PCR cycles (e.g., 30 cycles) are

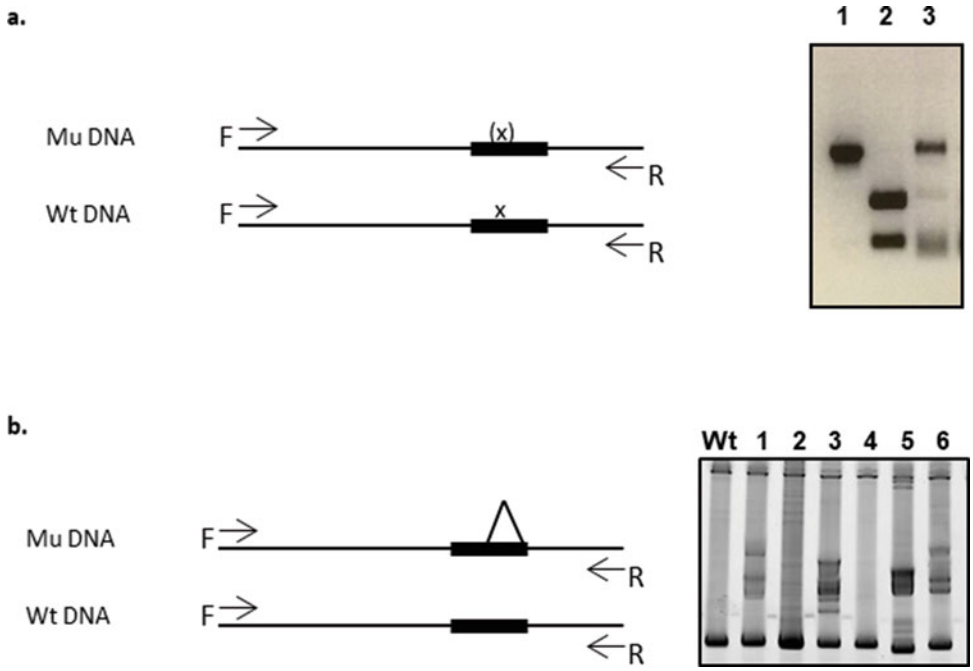

Fig. 5 Detection of mutations. (**a**) Enrichment CAPS PCR to detect mutations of low abundance. On the left: The diagram depicts PCR amplification with primers flanking the target region (F: forward primer and R: reverse primer). The restriction site (x) in the CRISPR target was disrupted in the mutated (Mu) DNA and can no longer be recognized by the restriction enzyme. Meanwhile, the restriction site remains intact for the un-mutated wild-type (Wt) DNA. On the right: The gel image shows the separation of PCR products: (Lane 1) Un-digested PCR products as control. (Lane 2) Wild-type DNA is completely digested. (Lane 3) Mutant DNA is resistant to digestion, resulting in a strong band of similar size compared to the control. (**b**) Gradient PAGE analysis of PCR amplified target regions. On the left: After denaturation and renaturation of PCR products, the mutated (Mu) DNA forms a "bulge" when renatured to the wild-type (Wt) amplicon due to the presence of mutations, typically small deletions. On the right: Major bands at the bottom represent wild-type (perfectly renatured) dsDNA (lanes Wt, 2, and 4). Additional bands with slower mobility indicate heteroduplex dsDNA formed by imperfect renaturation between Mu-Wt amplicons or polymorphic biallelic mutated amplicons (lanes: 1, 3, 5, and 6)

preferred. Purified and salt-free template DNA is also preferred, to obtain clear resolution (*see* Fig. 5). This optimization of PCR conditions minimizes the occurrence of nonspecific PCR products, which could also display altered mobility and lead to false-positive results. It is noteworthy that mutations of very low abundance in somatic tissues may escape detection due to the limited sensitivity of staining/destaining gels. In rare cases, if both alleles for a given target are mutated and harbor the same mutation (e.g., a 10 bp deletion) in a T0 plant, the renaturation of mutant DNA during the heteroduplex analysis will result in the formation of perfect dsDNA, which will display approximately the same mobility as the wild-type dsDNA. Furthermore, the segregation of a mono-allelic or polymorphic biallelic T0 plant will result in progeny that may become homozygous for a mutated allele, and will also result in the formation of perfect dsDNA and display the same mobility as the wild-type dsDNA in the heteroduplex assay. Users should be aware of this possibility when using the heteroduplex approach to identify mutations.

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Chapter 17

Genome Editing in Citrus Tree with CRISPR/Cas9

Hongge Jia, Xiuping Zou, Vladimir Orbovic, and Nian Wang

Abstract

CRISPR/Cas9 has been widely employed to edit genome in most of the organisms, including animal, plant, fungus, and microbe. Here we describe the modification of citrus gene *CsLOB1* in transgenic citrus by Cas9/sgRNA, a two-component system derived from CRISPR-Cas9. Transgenic citrus plants can be created by Agrobacterium-mediated epicotyl transformation.

Key words Cas9/sgRNA, Genome editing, Citrus, Agrobacterium-mediated epicotyl transformation, *CsLOB1*

1 Introduction

Genome editing, which can be achieved by zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas9, is indispensable for basic science research, biotechnology advance, and medicine innovation [1]. To simplify the utilization process, the CRISPR/Cas system has been adjusted from a three-component to a two-component Cas9/single guide RNA (sgRNA) system [2]. Cas9/sgRNA is composed of Cas9 nuclease protein and a synthetic sgRNA containing a fusion of the crRNA and tracrRNA elements. In the case of genome editing, Cas9/sgRNA just requires minor changes in the sgRNA for specific targeting, whereas both ZFNs and TALENs demand elaborate design and assembly of individual DNA-binding proteins for each DNA target. Due to its simplicity and affordability, Cas9/sgRNA is becoming more and more popular. To date, Cas9/sgRNA technology has been successfully used for genome editing in several plant species, including citrus [3–7], Arabidopsis, rice, and so on [8–12]. By taking advantage of Cas9/sgRNA, we successfully edited Duncan grapefruit *CsLOB1* in transgenic citrus, established by Agrobacterium-mediated epicotyl transformation [6].

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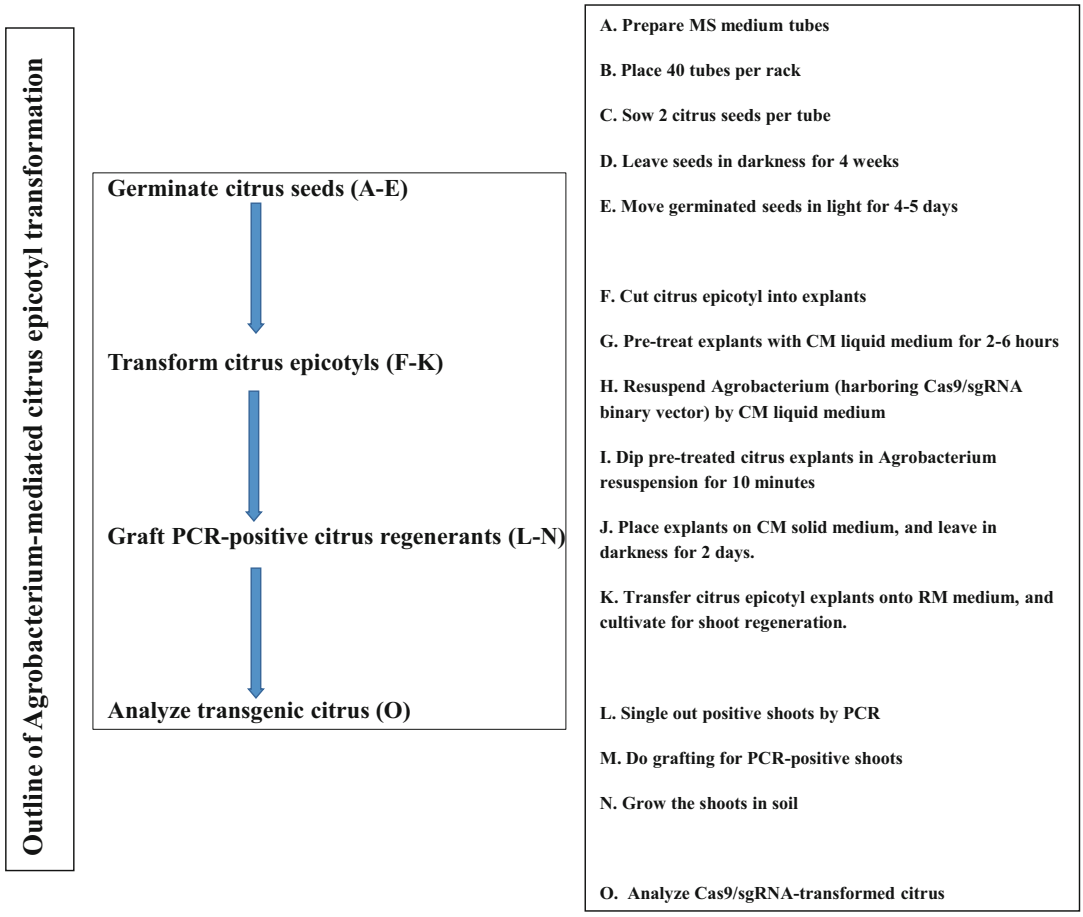

Fig. 1 Outline of Agrobacterium-mediated citrus epicotyl transformation. A quick protocol is outlined to indicate how to produce transgenic citrus from citrus epicotyl by using *Agrobacterium*-mediated method. The detailed protocol is presented in text

Several kinds of citrus tissues, including epicotyl, callus, protoplast, and mature branch, can be used as explants to establish transgenic citrus [13]. Here Duncan grapefruit epicotyls are used as explants, and Agrobacterium-mediated transformation is employed to produce transgenic citrus plants (Fig. 1).

2 Materials

Recombinant *Agrobacterium* EHA105 harboring binary vector, GFP-p1380N-Cas9/sgRNA: cslob1, is used for Agrobacterium-mediated citrus epicotyl transformation (Fig. 1) (*see* **Note 1**). Prepare all media and solutions using distilled-deionized water (DDW). Prepare and store all media and solutions at room temperature. All chemical reagents and bio-hazard materials must be disposed according to disposal regulations.

1. Binary vector GFP-p1380N-Cas9/sgRNA: cslob1: The fragment, RB-CsVMV-GFP-35 T-CaMV 35S-Cas9-NosT-CaMV 35S-sgRNA-NosT-NosP-NptII-35 T-LB, locates between right border and left border of T-DNA (*see* **Note 2**).
2. Murashige and Skoog Basal medium with Vitamins (MS): Macronutrients, micronutrients, vitamins, organics (*see* **Note 3**).
3. Germination MS (GM): MS, 25 g/L sucrose, 3 g/L Gelzan. Before adding Gelzan, adjust pH to 5.8 with 1 M KOH. Autoclave at 121 °C for 25 min.
4. Liquid coculture medium (CM): MS, 2 mg/L 6-BA, 0.5 mg/L IAA, 30 g/L sucrose (*see* **Note 4**). Adjust pH to 5.8 and autoclave at 121 °C for 25 min. Before using, add acetosyringone (AS) to a final concentration of 100 μM.
5. Solid CM: MS, 2 mg/L 6-BA, 0.5 mg/L IAA, 1 mg/L 2,4-D, 30 g/L sucrose, 3 g/L Gelzan (*see* **Note 4**). Before adding Gelzan, adjust pH to 5.8. Autoclave at 121 °C for 25 min. Cool down around 60 °C, and add AS to a final concentration of 100 μM before making CM plates.
6. Regeneration medium (RM): MS, 2 mg/L 6-BA, 0.5 mg/L IAA, 30 g/L sucrose, 3 g/L Gelzan (*see* **Note 4**). Before adding Gelzan, adjust pH to 5.8. Autoclave at 121 °C for 25 min. Cool down to around 60 °C, and respectively add Kanamycin and Cefotaxime to the final concentration of 50 and 250 mg/L before making RM plates (*see* **Note 5**).
7. 20% bleach solution: Mix 200 mL Clorex bleach, which contains about 5.25% NaClO, with 800 mL sterilized DDW.
8. YEP: Dissolve 10 g Peptone, 10 g Yeast extract, and 5 g NaCl in 1 L DDW. Adjust pH to 7 and autoclave at 121 °C for 25 min.

3 Methods

Use 70% ethanol to clean working area and hands. Work in hood and use sterilized tools to prevent citrus tissue culture from contamination.

3.1 Citrus Seed Planting

1. Halve fresh Duncan grapefruit to collect seeds, then peel seed coat (*see* **Note 6**).
2. Place the coat-peeled seeds into a plastic box, add 20% bleach solution. Keep shaking at 180 rpm for 15 min.
3. In the hood, drain out the bleach, and rinse three times with sterilized DDW. Pour seeds into petri dish containing sterilized paper towel. Prepare MS medium tubes (40 tubes/rack), and

use sterilized half-curved forceps to plant two seeds per tubes. In the hood, cover the tubes and place back.

4. Wrap the racks with aluminum foil, and leave them in dark room for 4 weeks at room temperature. Uncover the racks and leave the Duncan grapefruit seedlings in the light for 4–5 days at room temperature before carrying out *Agrobacterium*-mediated citrus epicotyl transformation.

3.2 Agrobacterium-Mediated Citrus Epicotyl Transformation

1. first day, recover and grow *Agrobacterium* EHA 105 harboring GFP-p1380N-Cas9/sgRNA: cslob1 overnight at 28 °C in 3 mL of YEP (*see* **Note 7**). Second day, dilute 5 μL of overnight culture in 50 mL of fresh YEP medium containing antibiotics, and grow overnight at 28 °C, with shaking at 200 rpm. Third day, harvest recombinant Agrobacterium cells at 4000 × *g* and resuspend in liquid CM to adjust the final concentration as $OD_{600} = 0.6$ (*see* **Note 8**), which is *Agrobacterium* resuspension used for epicotyl transformation later.
2. In the hood, use half-curved forceps to pick up citrus seedlings from tubes and place on sterilized paper plates. Slantingly cut citrus epicotyls to a length of around 1 cm by using sharp sterile scalpel. Dip the epicotyl explants in liquid CM and shake at 100 rpm for 2–6 h.
3. After pre-culture, use straight forceps to collect explants from CM and put onto paper towel. After drying for 2 min, dip the epicotyl explants to the Agrobacterium resuspension for 10–20 min.
4. After cocultivation, take the explants out and leave them on the sterilized paper towel for 1 min. Place the explants on solid CM plates. Wrap the plates and incubate in darkness for 2 or 3 days at 25 °C.
5. Transfer the epicotyl explants from solid CM to RM plates and culture in growth chamber till citrus shoot regeneration. Change fresh RM for the explants every 3 weeks.
6. Collect regenerated Duncan grapefruit shoots and grow on GM containing antibiotics (50 mg/L Kanamycin and 250 mg/L Cefotaxime) (*see* **Note 9**).

3.3 PCR Analysis of Regenerated Citrus Shoots for Grafting

1. Use 99% ethanol to clean EMS-Core (ID 0.50 mm, OD 0.80 mm), leave it to dry in hood.
2. Mark the number on PCR tubes to correspond to regenerated citrus shoots and include two tubes for "positive" and "negative."
3. Prepare PCR solution. Use 25 μL solution each sample per PCR tube (*see* **Note 10**). Place the tubes into ice.

4. Place the regenerated shoots on a plate and punch leaves by using EMS-Core (*see* **Note 11**). Eject the leaf tissues into corresponding PCR tubes.
5. Program the PCR thermal Cycler based on primers and carry out the PCR program.
6. Prepare agarose gel, and use it to check PCR products (*see* **Note 12**). Single out the PCR-positive regenerated citrus shoots.
7. Do grafting for the PCR-positive shoots. When the shoots grow big enough, move them to soil after labeling.
8. Leave them in a tray covered by a plastic cap. Grow under the light at room temperature.

3.4 Analysis of Cas9/sgRNA-Transformed Citrus Plants

1. Extract transgenic Duncan grapefruit genomic DNA and do PCR to amplify the edited sequence. Conduct ligation and *E. coli* transformation. Pick up colonies for sequencing to confirm targeted modification (*see* **Note 13**).
2. If there is expected mutation in PCR products after colony sequencing, send the samples for targeted next-generation sequencing (NGS). Based on the targeted NGS results, GFP-p1380N-Cas9/sgRNA: cslob1-mediated mutation rate is calculated, and indel genotypes are analyzed.
3. If necessary, analyze the off-targets of Cas9/sgRNA-directed editing by whole genome sequencing, given that the citrus cultivar whole genome is available.
4. Analyze the expected phenotypes of gene-edited Duncan grapefruit when they grow big enough.

4 Notes

1. Since it usually takes several months to produce transgenic citrus by *Agrobacterium*-mediated epicotyl transformation, Xcc-facilitated agroinfiltration can be initially employed to test the binary vector function [4, 6]. By using Xcc-facilitated agroinfiltration, data can be obtained in 2 weeks.
2. The binary vector is described before [6]. GFP is used as a visual reporter to facilitate Cas9/sgRNA-transformed citrus selection. Make sure that the targeted sequence of GFP-p1380N-Cas9/sgRNA: cslob1 is conserved on both alleles of *CsLOB1* when sgRNA is designed.
3. Murashige and Skoog Basal medium with vitamins can be purchased from some companies. As an example, PhytoTechnology Laboratories provides it with the catalog number M519.

4. It is possible to use different medium recipe to create transgenic citrus. In our hand, another recipe also works well. Liquid CM: MS, 3 mg/L 6-BA, 0.1 mg/L NAA, 0.5 mg/L 2,4-D, 30 g/L sucrose. Solid CM: MS, 3 mg/L 6-BA, 0.1 mg/L NAA, 0.5 mg/L 2,4-D, 30 g/L sucrose, 3 g/L Gelzan. RM: MS, 3 mg/L 6-BA, 0.5 mg/L NAA, 30 g/L sucrose, 3 g/L Gelzan.
5. Based on your own binary vector, appropriate antibiotic is chosen to select the transformed plants. As an example, Kanamycin with a final concentration 50 mg/L is employed to select GFP-p1380N-Cas9/sgRNA: cslob1-transformed Duncan grapefruit. Cefotaxime (250 mg/L) is used to inhibit *Agrobacterium* overgrowth.
6. If seeds are freshly prepared from citrus fruits, treat seeds with 1 M NaOH for 30 min and shake every 5 min. Dry the seeds with paper towel. After NaOH treatment, it is easier to peel the seed coat.
7. The recombinant *Agrobacterium* is usually stored at −80 °C for future use. If there is no such stock, transform competent *Agrobacterium* cells with Cas9/sgRNA binary vector and grow PCR-positive colony for transformation.
8. Do not collect *Agrobacterium* cells and make resuspension too early. You can start to do it at the end of next step, in which the cut citrus epicotyls need to be cocultured in liquid CM for 2–6 h.
9. Since there is GFP fluorescence as for GFP-p1380N-Cas9/sgRNA: cslob1-transformed Duncan grapefruit, it is easy to single out the positive shoots and do grafting without PCR verification by using EMS-Core. If there is no GFP reporter in your binary vector, PCR must be carried out to single out the PCR-positive regenerants before grafting.
10. A PCR master mix is recommended. After adding all of PCR reaction reagents into one tube, mix well by gentle pipetting. Aliquot to the PCR tubes. We use Thermo Scientific Phire Plant Direct PCR Kit to verify transgenic citrus plants, since this kit is designed to amplify DNA directly from plant tissue without requiring DNA purification.
11. Try to pick up citrus leaf samples by using 200 μL tips, if EMS-Core is not available in lab.
12. Ethidium Bromide (EtBr) can be added into agarose gel or running buffer. Handle EtBr very carefully, since it can cause cancer. Never spread EtBr around the lab, and never contaminate other lab stuffs with EtBr either.
13. Suggest to carry out the PCR product direct sequencing for transgenic citrus plants as a critical experiment [5, 14]. After

sequencing, each transgenic citrus line is supposed to have one of the following chromatograms: (a) A single peak is observed, the edited citrus is homologous. (b) A clean double peak is observed, this is supposed to be indicative of monoallelic variation for Cas9/sgRNA-directed modification in citrus. (c) If multiple peaks are observed at the cut-site, this indicates that Cas9/sgRNA-mediated modifications occur in somatic cells, and the transgenic citrus is genetically chimeric at the target locus.

Acknowledgement

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Part VI

CRISPR-Cas12a Editing Systems

Chapter 18

Plant Gene Knockout and Knockdown by CRISPR-Cpf1 (Cas12a) Systems

Yingxiao Zhang, Yong Zhang, and Yiping Qi

Abstract

CRISPR-Cpf1 (Cas12a) is a class II type V endonuclease, which has been used as a genome editing tool in different biological systems. Here we describe a fast, efficient, and user-friendly system for CRISPR-Cpf1 expression vector assembly. In this system, the Pol II promoter is used to drive the expression of both Cpf1 and its crRNA, with the crRNA flanked by hammerhead (HH) and hepatitis delta virus (HDV) ribozyme RNAs for precise crRNA processing. All the components of this system can be modified depending on plant species and experimental goals. Using this system, nearly 100% editing efficiency and 90% gene expression decrease were achieved in rice and *Arabidopsis*, respectively.

Key words CRISPR-Cpf1 (Cas12a), Plant gene knockout, Plant gene knockdown, Gateway cloning

1 Introduction

Clustered Regularly Interspaced Short Palindromic Repeats from *Prevotella* and *Francisella 1* (CRISPR-Cpf1, also known as Cas12a) is a class II type V endonuclease [1], which has been used as a genome editing tool in different biological systems. Genome editing using Cpf1 requires two components, a Cpf1 endonuclease which can achieve pre-crRNA processing, crRNA binding, DNA recognition, and double strand breaks (DSBs) generation, and a crRNA with a 19–20 nt direct repeat sequence and a 23 nt targeting sequence. Characterization of Cpf1 reveals several unique features between Cpf1 and the preexisting CRISPR system using SpCas9 (*Streptococcus pyogenes* Cas9), which could potentially offer several advantages for genome editing [1]. This information is summarized in Table 1. The first identified Cpf1 is from *Francisella novicida* (FnCpf1). However, due to the high genome editing efficiency in mammalian cells, the most commonly used Cpf1s are LbCpf1 (*Lachnospiraceae bacterium* ND2006 Cpf1) and AsCpf1 (*Acidaminococcus sp.* BV3L6 Cpf1). In plants, all three Cpf1s have been used

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Table 1
Comparison between SpCas9 and Cpf1 (Cas12a)

SpCas9	Cpf1	Potential advantages of Cpf1
Recognize G-rich protospacer adjacent motif (PAM) 5′-NGG-3′	Recognize T-rich PAM 5′-TTTN-3′ (5′-TTTV-3′ is preferred in some cases; V = A, C, or G)	Target AT rich region
Cut 3–4 nt upstream of PAM, generate blunt ends	Cut 18–23 bases downstream of PAM, generate cohesive ends with 5 nt 5′ overhang	May facilitate homology-directed repair (HDR) and non-homologous end joining (NHEJ)-mediated gene insertion or replacement
Require both tracrRNA and crRNA, or a single guide RNA (sgRNA) usually about 100 nt	Only require crRNA, usually about 43 nt	Easy for synthesis and multiplex
Require RNase III for RNA processing in bacteria	Cpf1 can process pre-crRNA	Can use CRISPR array for multiplexing
Higher off-target effects, but can use Cas9 nickase to minimize off-target. High fidelity version, such as eSpCas9 and SpCas9-HF1, are available	Lower off-target effects, similar to high fidelity version of Cas9, cannot generate Cpf1 nickase	Minimize undesired off-target mutagenesis

for genome editing, with LbCpf1 showing better overall editing efficiency [2–5].

Like SpCas9, mutations can be introduced into Cpf1 to abolish its DNA nuclease activity, resulting in catalytically dead Cpf1 (dCpf1) [6]. This allows Cpf1 to recruit desired proteins to a certain region of the genome, without introducing DSBs. With the dCpf1 targeting the promoter region of genes of interest, gene expression can be repressed or activated. dCpf1 itself can downregulate genes, potentially due to blocking the transcription initiation and elongation [7]. When using dCpf1 fused with a repressor, genes can also be efficiently repressed [3]. On the other hand, one or more activators can be co-localized with dCpf1 with protein fusion or protein-protein interaction (such as the SunTag system) to upregulate gene expression [8, 9].

In plants, Cpf1-mediated genome editing has been achieved in multiple species, including rice (*Oryza sativa*) [2, 3, 10–13], tobacco (*Nicotiana tabacum* and *N. attenuate*) [2, 14], and soybean (*Glycine max*) [14]. Since the 23 nt targeting sequence is located at the 3′ end of the crRNA, 1–7 extra T nucleotides will be added to the end of crRNA when crRNA expression is driven by Pol III promoter. This could potentially cause mismatch and lower the editing efficiency. To resolve this problem, a tRNA sequence was added to facilitate crRNA processing [13]. However, the 1–4 nt

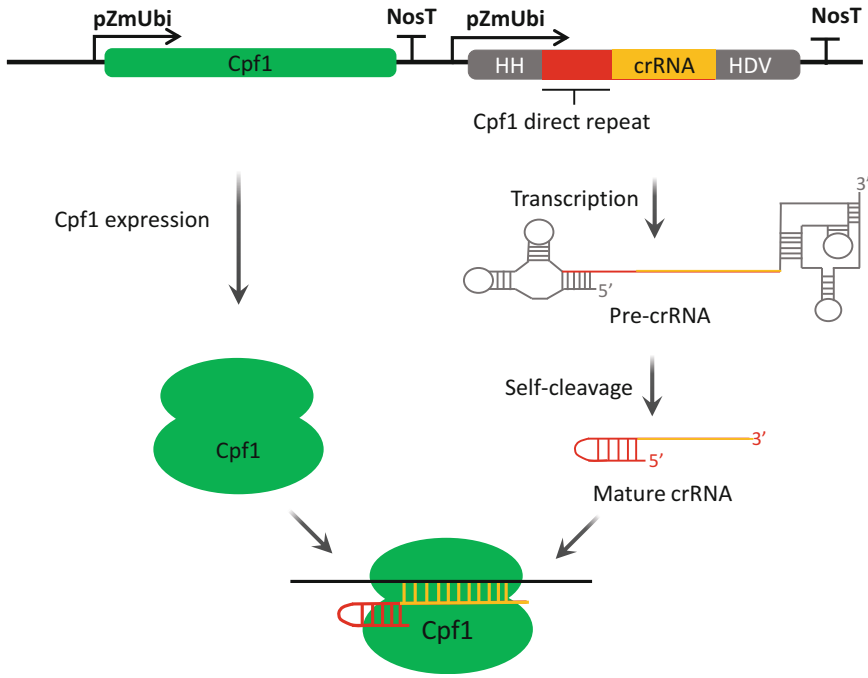

Fig. 1 CRISPR-Cpf1 expression system. Cpf1 and crRNA are under the control of the maize ubiquitin promoter (pZmUbi) and Nos terminator. Pre-crRNA with hammerhead (HH) ribozyme RNA and hepatitis delta virus (HDV) ribozyme RNA is self-cleaved to form mature crRNA, which is then complexed with Cpf1 for genome editing

extra tails are still the limit for efficient genome editing [13]. We used a strong Pol II promoter to express crRNA, which was flanked by hammerhead (HH) ribozyme RNA and hepatitis delta virus (HDV) ribozyme RNA for precise crRNA processing, and achieved nearly 100% editing efficiency in transgenic rice [3] (Fig. 1). This system was also successfully used for gene repression [3].

CRISPR-Cpf1, as an alternative system for genome editing and transcriptional regulation, has expanded the CRISPR toolkit and broadened CRISPR applications. Therefore, a fast, efficient, and user-friendly system for CRISPR-Cpf1 expression vector assembly is needed. In this chapter, a module-based assembly method will be described to express a high efficiency Cpf1 genome editing system in plants [3] (Fig. 2). Relying on the MultiSite Gateway recombination, three modules are required for the final T-DNA vector assembly: (1) a promoterless Cpf1 or Cpf1-repressor entry vector with attL1 and attR5 recombination sites; (2) a crRNA entry vector with attL5 and attL2 sites; and (3) a destination T-DNA vector with a promoter (for Cpf1 expression), a selective marker for transgenic plant selection, as well as attR1 and attR2 sites. Here, we use rice as an example for Cpf1-mediated gene knockout and *Arabidopsis* as an example for gene knockdown. Each module can be modified independently to suit specific experimental purposes in diverse plant species.

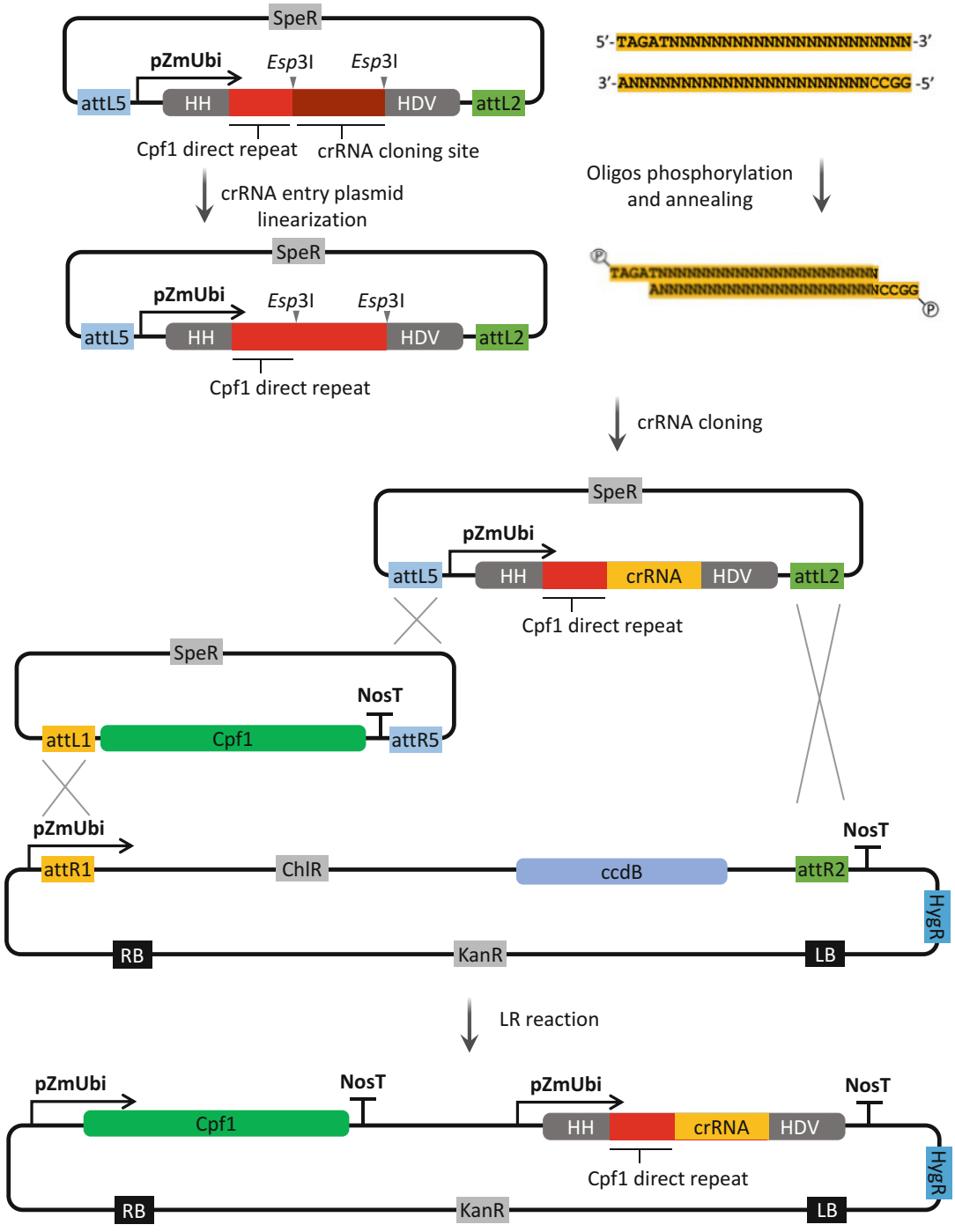

Fig. 2 Overview of the T-DNA vector construction system for CRISPR-Cpf1. crRNA is synthesized as duplexed DNA oligonucleotides, which are phosphorylated, annealed, and cloned into linearized crRNA expression vectors at the *Eps*3I (*Bsm*BI) site. These vectors are assembled with the Cpf1 entry vector and the destination vector to build the final T-DNA vectors by Gateway recombination

2 Materials

1. Annotated genomic sequence of targeted genes. For gene knockout, gene coding sequence is needed. For gene knockdown, usually about 300 bp upstream of transcription start is needed. The genome sequence information for genes used in this study can be found at the National Center for Biotechnology Information (NCBI) (Table 2).
2. Genome editing gRNA/crRNA design software or websites can be used to design crRNA for Cpf1, including CRISPR-P v2.0 [15], CRISPRdirect [16], and Benchling (https://benchling.com). The availability of the whole genome sequence can help minimize the off-target effects.
3. DNA oligonucleotides for crRNA cloning (Fig. 3).
4. Plasmids. All vectors mentioned in this protocol are available from Addgene (https://www.addgene.org): pYPQ141-ZmUbi-RZ-As (no. 86196), pYPQ141-ZmUbi-RZ-Lb (no. 86197), pYPQ202 (no. 86198), pYPQ203 (no. 86207), pYPQ220 (no. 86208), pYPQ223 (no. 86209), pYPQ230 (no. 86210), and pYPQ233 (no. 86211).
5. Molecular grade water.
6. T4 polynucleotide kinase (PNK) and 10× PNK Buffer, 10 mM ATP.
7. Heat block or water bath, magnetic heat plate.
8. 2 mL or 1.7 mL micro centrifuge tubes and 0.2 mL PCR tubes.
9. Restriction enzymes and their reaction buffers: *Esp*3I (*Bsm*BI) and *Eco*RI.
10. Silica column-based gel purification kit, such as the QIAquick Gel Extraction Kit.

Table 2
Information of targeted genes involved in this study

Purpose	Gene	Full name	Gene Locus	NCBI Gene Symbol
Rice gene knockout	*OsPDS*	*Phytoene desaturase*	Os03g0184000	LOC4331854
	OsDEP1	*dense and erect panicle 1*; *Keratin-associated protein 5–5*	Os09g0441900	LOC4347178
	OsROC5	*Homeobox-leucine zipper protein ROC5*	Os02g0674800	LOC4330297
Arabidopsis gene knockdown	*AtMIR159b*	*Micro RNA 159b*	AT1G18075	MIR159b

Target gene	crRNA target sequence with PAM	DNA oligonucleotides
OsPDS	TTTGGAGTGAAATCTCTTGTCTTAAGG	5′-TAGATGAGTGAAATCTCTTGTCTTAAGG-3′ 5′-GGCCCCTTAAGACAAGAGATTTCACTCA-3′
OsDEP1	TTTGCTACTGTTGCAAGTGCTCACCCA	5′-TAGATCTACTGTTGCAAGTGCTCACCCA-3′ 5′-GGCCTGGGTGAGCACTTGCAACAGTAGA-3′
OsROC5	TTTCTGCTTCCTGCAATGCCGGTAGAC	5′-TAGATTGCTTCCTGCAATGCCGGTAGAC-3′ 5′-GGCCGTCTACCGGCATTGCAGGAAGCAA-3′
AtMIR159b	TTTGATTGTATGAATATATGAGTTAGT	5′-TAGATATTGTATGAATATATGAGTTAGT-3′ 5′-GGCCACTAACTCATATATTCATACAATA-3′

Fig. 3 crRNA target sequences and DNA oligonucleotides

11. 37 °C shaker and incubator.
12. Plasmid Miniprep kit, such as the IBI scientific Hi-Speed Mini Plasmid Kit.
13. LB medium: 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride, 1.5% (w/v) agar for making solid LB plates.
14. S.O.C. medium: 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulfate, and 20 mM glucose.
15. Antibiotic stock solutions (1000×): 50 mg/mL spectinomycin and 50 mg/mL kanamycin. Antibiotics are dissolved in water and sterilized using 0.22 μm syringe filter. Stock solutions are aliquoted to 2 mL tubes and stored at −20 °C.
16. DNA quantification equipment, such as the NanoDrop™ One UV-Visible spectrophotometer.
17. Agarose gel electrophoresis equipment and supplies.
18. DNA ladders.
19. Chemically competent cells of *E. coli* strain DH5α and ccdB tolerance *E. coli* strain (such as DB3.1) for destination vector maintenance. Other *E. coli* strains can also be used for cloning if faster growth rate is preferred.
20. Gateway™ LR Clonase™ II Enzyme Mix.

3 Methods

3.1 T-DNA Vector Construction for CRISPR-Cpf1-Mediated Gene Knockout

1. Design three crRNA to target three genes, respectively. The crRNAs used in this study are targeting the first exon of *OsPDS*, the fifth exon of *OsDEP1*, and the fifth exon of *OsROC5* (Fig. 3). A few principles should be taken into consideration when designing crRNAs:
   (a) To knockout a protein-coding gene, a crRNA is optimally located at the beginning of the exon which is predicted to be the functional domain of the protein. In this case, mutations (mostly small deletions) introduced by non-homologous end joining (NHEJ) would be most likely to cause gene knockout.
   (b) Target sequence at the plant genome is usually 23 bp, followed by a LbCpf1 or AsCpf1 PAM sequence 5′-TTTV-3′. The crRNA can target either leading strand or lagging strand.
   (c) If a Restriction Fragment Length Polymorphism (RFLP) method will be used to detect genome editing and measure editing efficiency, a restriction enzyme recognition site should overlap with the Cpf1 cleavage site, which is usually 13–23 bp distal to the PAM site.
   (d) The crRNA editing efficiency can be predicted by crRNA design software or websites. Strong secondary structures and extreme GC content (less than 30% or more than 70%) should be avoided.
2. Synthesize crRNA as two reverse complementary primers or duplexed DNA oligonucleotides. Design the forward primer by adding 5′-TAGAT-3′ at the beginning of the crRNA sequence (5′-TAGATNNNNNNNNNNNNNNNNNNNN-3′).
   Design the reverse primer by adding 5′-GGCC-3′ at the beginning of the reverse complementary sequence of the crRNA and 5′-A-3′ at the end (5′-GGCCNNNNNNNNNNNNNNNNNNNNA-3′).
3. Anneal and phosphorylate DNA oligonucleotides. Dissolve lyophilized DNA oligos into water (*see* **Note 1**) to a final concentration of 100 μM. Phosphorylate DNA oligos using T4 polynucleotide kinase (T4 PNK) (Table 3). Incubate reactions at 37 °C for 30 min (*see* **Note 2**). Anneal phosphorylated oligos by incubating in boiling water. Let water cool down and bring the sample to room temperature. Dilute oligos at a 1:200 ratio for crRNA cloning.
4. Digest crRNA entry plasmids. Use pYPQ141-ZmUbi-RZ-As for AsCpf1 crRNA expression, while use pYPQ141-ZmUbi-RZ-Lb for LbCpf1 crRNA expression. Digest plasmids by

Table 3
Oligonucleotides phosphorylation

Component	Volume
crRNA forward oligo (100 μM)	1 μL
crRNA reverse oligo (100 μM)	1 μL
T4 PNK Reaction Buffer (10×)	1 μL
ATP (10 mM)	1 μL
T4 PNK (10 U/μL)	0.5 μL (5 U)
Water	5.5 μL
Total	10 μL

Table 4
Empty crRNA entry vector digestion

Component	Volume
pYPQ141-ZmUbi-RZ-As or pYPQ141-ZmUbi-RZ-Lb	20 μL (2 μg)
Buffer Tango (10×)	5 μL
DTT (10 mM)	5 μL
Esp3I (BsmBI) (10 U/μL)	2 μL (20 U)
Water	18 μL
Total	50 μL

*Esp*3I (*Bsm*BI) and incubate samples at 37 °C for 1–16 h (Table 4). Clean up digested plasmids using a gel purification kit. Heat inactivation of digestion enzymes is preferred if digested product is not purified immediately after digestion. Running digested plasmids on an agarose gel followed by gel excision and purification is not necessary but can help reduce background (undigested plasmids). Measure DNA concentration of the digestion products using a spectrophotometer.

5. Ligate phosphorylated and annealed DNA oligos into digested crRNA entry plasmids (*see* Table 5 and **Note 3**). Incubate reactions at room temperature for 2 h (*see* **Note 4**). Transform half of the reaction into *E. coli* DH5α competent cells using the heat shock method. Plate cells on LB solid medium supplemented with 50 mg/L spectinomycin and incubate at 37 °C overnight.
6. Pick one or two colonies from each plate and culture in 5 mL LB liquid medium supplemented with 50 mg/L spectinomycin

Table 5
crRNA entry vector ligation

Component	Volume
*Esp*3I (*Bsm*BI) linearized pYPQ141-ZmUbi-RZ-As or pYPQ141 -ZmUbi-RZ-Lb	2 μL (60 ng, 0.02 pmol)
Diluted annealed oligos (1:200 dilution)	2 μL
T4 DNA Ligase Buffer (10×)	2 μL
T4 DNA Ligase (400 U/μL)	1 μL (400 U)
Water	13 μL
Total	20 μL

at 37 °C overnight. Isolate plasmid DNA from each cell culture using a Miniprep kit.

7. Confirm crRNA expression vectors by Sanger sequencing using primers Ubi-intron-F (*see* **Note 5**).
8. Use sequencing confirmed crRNA entry vector, rice codon optimized AsCpf1 (pYPQ220) or LbCpf1 (pYPQ230) entry vector, as well as the destination vector pYPQ203 to set up the three-way Gateway LR reaction (Table 6). Vector pYPQ203 contains the maize ubiquitin promoter (pZmUbi) to drive the expression of Cpf1, as well as a hygromycin resistance gene for transgenic plant selection. Other destination vectors with the desired promoter and selective marker can be also used.
9. Transform all reactions into *E. coli* DH5α competent cells using the heat shock method. Plate cells on LB solid medium supplemented with 50 mg/L kanamycin and incubate at 37 °C overnight (*see* **Note 6**).
10. Pick one or two colonies from each plate and culture in 5 mL LB liquid medium supplemented with 50 mg/L kanamycin at 37 °C overnight. Isolate plasmid DNA from each cell culture. Confirm successful assembly by digestion of the plasmid with *Eco*RI (*see* **Note 7**).
11. Make glycerol stock for digestion-confirmed plasmids by storing cell culture in 25% glycerol at −80 °C. Verified plasmids can be further transformed into Agrobacterium for plant transformation.

3.2 T-DNA Vector Construction for CRISPR-Cpf1-Mediated Gene Knockdown

1. Design one crRNA to target the promoter region of a noncoding RNA, *AtMIR159b*. PAM sequence is 74 bp upstream of the transcription start site (Table 2 and Fig. 3).
2. Design and synthesize crRNA as described in Subheading 3.1, **step 2**. Clone phosphorylated and annealed DNA oligos into

Table 6
Three-way Gateway LR reaction for rice gene knockout

Component	Volume
Cpf1 entry vector (pYPQ220 or pYPQ230)	1.5 μL (150 ng)
crRNA entry vector	1 μL (100 ng)
Destination vector pYPQ203	2 μL (200 ng)
LR Clonase II	1 μL
Total	5.5 μL

pYPQ141-ZmUbi-RZ-As or pYPQ141-ZmUbi-RZ-Lb and confirm the crRNA entry clone as described in Subheading 3.1**, steps 3–7**.

3. Assemble the final T-DNA vectors using the three-way Gateway LR reaction (Table 7). Cpf1 entry vectors for gene knockdown are pYPQ223 (dAsCpf1-SRDX) and pYPQ233 (dLbCpf1-SRDX). Catalytically dead AsCpf1 (dAsCpf1) was generated by introducing a D908A mutation while dLbCpf1 was generated by introducing a D832A mutation. Three copies of the SUPERMAN repressor domain X (SRDX) were fused to the C-terminus of dAsCpf1 and dLbCpf1 through a 5XGS linker. The destination vector used for gene knockdown is pYPQ202, harboring the *Arabidopsis* ubiquitin 10 promoter (pAtUbi10) to drive Cpf1-repressor fusion protein expression, as well as a hygromycin resistance gene for transgenic plants selection.
4. Repeat **steps 9–11** in Subheading 3.1 to generate the final T-DNA vector for plant transformation.

4 Notes

1. All water used in this protocol is sterile molecular grade water.
2. T4 PNK can be heat inactivated at 65 °C for 20 min. If oligos are annealed immediately after phosphorylation, there is no need for heat inactivation of T4 PNK.
3. A control reaction can be added using the same recipe, except without the annealed DNA oligos. The control reaction will indicate the background caused by incomplete digestion of the backbone plasmids and self-ligation.
4. T4 DNA Ligase Buffer need to be thawed completely and resuspended at room temperature. Incubation time can be as short as 10 min at room temperature. Reactions can also be incubated at 16 °C overnight. Ligase can be heat inactivated at

Table 7
Three-way Gateway LR reaction for *Arabidopsis* gene knockdown

Component	Volume
dCpf1 entry vector (pYPQ223 or pYPQ233)	1.5 μL (150 ng)
crRNA entry vector	1 μL (100 ng)
Destination vector pYPQ202	2 μL (200 ng)
LR Clonase II	1 μL
Total	5.5 μL

65 °C for 10 min. If reactions are used for transformation immediately after ligation, heat inactivation is usually not necessary.

5. Primer sequence of Ubi-intron-F is 5′-CCCTGTTGTTTGGTGTTACTTC-3′.
6. If different destination vector is used, use its corresponding antibiotic and the right concentration for selection.
7. Due to the large size of the final T-DNA vectors, confirmation by digestion with *Eco*RI is usually sufficient to verify the plasmids. Since no PCR step is involved, Sanger sequencing-based verification is optional.

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Chapter 19

Editing a Stomatal Developmental Gene in Rice with CRISPR/Cpf1

Xiaojia Yin, Abhishek Anand, Paul Quick, and Anindya Bandyopadhyay

Abstract

CRISPR has arguably been the fastest growing genome editing tool so far. CRISPR/Cas9 (Cas9) has been proved to be efficient and precise in genome editing. However Cas9 has certain limitations. CRISPR/Cpf1 (Cpf1) has been discovered as an alternate approach that can overcome some of those limitations. Cpf1 allows targeting in AT-rich region, creating a staggered cleavage, and cutting at the distal end to the PAM (Protospacer Adjacent Motif) regions. We have successfully tested the efficiency of Cpf1 system in rice using *OsEPFL9* which is a developmental gene known to regulate the stomatal density in leaf. Regulation of stomatal density and patterning is an important factor in regulating plant physiology, especially in improving the plant water use efficiency. We targeted the Exon1 of *OsEPFL9* and the knockout lines were studied for several generations for establishment of stabilized editing, as well as transmission and segregation of edits through generations. The usage of Cpf1 as a genome editing tool to manipulate stomatal patterning may further help us gain more insight of the physiology of rice in stress conditions.

Key words CRISPR/Cpf1, *OsEPFL9*, Genome editing, Rice

1 Introduction

Appearance of stomata was one of the most significant traits in the course of plant evolution that helped plants ascend from aqueous environment to adapt and conquer the land [1]. Stomata are present in almost all land plants [2]. The appearance of stomata dates back to the similar period as the evolution of land plants began. Although their distribution and patterning vary significantly, primarily depending on the habitat of the plants as an adaptive measure, there has been only little change in the stomatal structure since its evolution [2]. However this does not hold true for the developmental stages of the stomata, as the developmental stages have become much more complicated involving several pathways and regulatory factors [3–5].

Xiaojia Yin and Abhishek Anand have contributed equally to this work.

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The secretory peptides encoded by the *EPF/EPFL* (*epidermal patterning factors/EPF-like*) along with their receptor components are among some of the well-characterized factors for stomatal development in Arabidopsis. The positive regulator EPFL9 (also known as STOMAGEN) coordinates with EPF2 and EPF1 which negatively regulate stomatal density and negatively regulate stomatal clustering in Arabidopsis, respectively [6–10]. Overexpression of EPFL9 in Arabidopsis shows increase in stomatal density and the knockdown of EPFL9 shows decrease in stomatal density [7]. Several EPF/EPFL proteins were found to be conserved between dicots and monocots [8, 9]. The distribution and patterning of stomata play a significant role in the physiology of the plants as well as their adaptation to environment such as minimizing water loss. By genetically manipulating the stomatal distribution, we may be able to enhance the adaptability of plants to varied water availability [10, 11]. This can be especially significant for crop plants which have a narrow range of water requirement for optimal growth, such as rice. Rice requires a large amount of water for a certain period for optimal growth. With current changes in environmental conditions, the cultivation of rice frequently suffers from drought, which represents a great risk on food security as a large population depends on rice as a staple diet.

Clustered regularly interspaced short palindromic repeats (CRISPR) and Cas (CRISPR-associated) proteins was discovered as an immune system for bacteria against invading viruses [12]. CRISPR system has become one of the most applied and fastest growing genome editing tool. Till now, an array of CRISPR-associated (Cas) nucleases has been discovered and used for genome editing. There are two classes of CRISPR systems. The first class includes the effector complexes composed of multi protein components and the second class only contains single effector proteins, such as Cas9 [13–15]. Cas9 was the first established CRISPR system and was applied to edit many plant and animal species [16–19]. However, CRISPR/Cas9 still faces certain limitations. Firstly, the trans-activating crRNA (tracrRNA) is required in addition to CRISPR-RNA (cr-RNA) [19, 20]. Secondly, the PAM of Cas9 is NGG that makes it sometimes hard to target an AT-rich genome region. Thirdly, Cas9 cleaves DNA that creates blunt end cleavage which may lead to the more error prone non-homologous end joining (NHEJ) gene repairing processes [18, 21]. The CRISPR/Cpf1 which is also a Class II CRISPR nuclease can overcome some of these limitations. Cpf1 was also discovered as a functional defense molecule in bacteria genomes against foreign DNA molecules [22]. Several characteristics make Cpf1 a complementary nuclease to Cas9, expanding the genome editing toolbox:

- It requires only the crRNA to recognize the target DNA sequence.

- It recognizes the T-rich PAM (TTTN/TTN) and mainly target the AT-rich regions in the genome.
- It cleaves the DNA in a staggered manner leaving a 4–5 nucleotide sticky overhangs.
- It cleaves the DNA at distal end to the PAM which may allow repeated cleavage of mutated target sequence, whereas Cas9 cleaves near to the PAM which does not support repeated cleavage [22].

The first successful use of Cpf1 was editing of *DNMT1* gene in human embryonic kidney cells. It was later used for plant genome editing. Cpf1-mediated mutations were reported at two target genes (*OsPDS* and *OsBEL*) in transgenic rice plants [23]. It was also used as a transcriptional repressor in Arabidopsis. The nuclease domain was deactivated (dCpf1) and three copies of the SRDX transcriptional repressor were fused to the dCpf1 [24]. The FnCpf1 from *Francisella novicida* which uses TTN as PAM was efficiently used to induce targeted mutagenesis in tobacco and rice [25]. A multiplex Cpf1 genome editing approach was reported recently [26]. Four genes from receptor-like kinases gene family (OsRLKs) and four *OsBEL* genes were targeted by FnCpf1 and LbCpf1, respectively [26]. This experiment shows no significant increase in efficiency of cleavage using this multiplex targeting Cpf1 system. However, no off-target effects were reported in this experiment indicating higher fidelity of Cpf1 in multiplex genome editing in plants [26].

We have successfully tested the efficiency of LbCpf1 system in rice using *OsEPFL9* as a marker gene. *OsEPFL9* is a developmental gene known to regulate the stomatal density in leaf. We targeted the Exon1 of *OsEPFL9* and the knockout lines were studied for several generations for studying germline transmission of the targeted mutations and segregation of the Cpf1 transgene. We observed a significant reduction in stomatal counts (more than eightfold) in stable, Cpf1 transgene-free, and homozygous mutants in T2 generation. Here we provide a detailed protocol of using Cpf1 for inducing targeted mutations at *OsEPFL9* in rice [27].

2 Materials

2.1 Generation of the pCambia-LbCpf1 Binary Vector Targeting OsEPFL9

1. *E. coli* strain: DH5α competent cells.
2. Media: Miller's LB Broth and Bacto Agar, plus 50 μg/mL of kanamycin.
3. Plasmid vectors: pCambia-CRISPR_Cas9, pcDNA3-huLbCpf1.
4. Oligos: LbCpf1-gRNA-F and LbCpf1-gRNA-R; Cpf1-F and LbCpf1-R; LbCpf1- NLS-F and Cpf1-NLS-R; LbCpf1-F and

LbCpf1-R; EPFL9-Cpf1-Target-F and EPFL9-Cpf1-Target-R (*see* **Notes 1** and **2**).

5. Enzymes and PCR reagent: AarI, XbaI, BaeI, HindIII, SalI, T4 DNA ligase, Phusion® High-Fidelity DNA Polymerase.
6. GenUP™ Gel Extraction Kit.
7. GenUP™ Plasmid Kit.

2.2 Rice Transformation

1. Immature embryo of *Indica* rice cultivar IR64.
2. *Agrobacterium* strain: LBA4404.
3. Media: YEB Agrobacterium Growth Medium and Bacto Agar.
4. Tools: Scalpel, filter paper, 3 M micropore tape.
5. Infection medium: AA salt Macro 10% v/v, AA MICRO SALTS 0.1% v/v, AA iron 1% v/v, B5 MINOR-1 1% v/v, B5 VITAMIN 0.1% v/v, 100 mM glycine 0.1% v/v, L-glutamine 876 mg/L, aspartic acid 260 mg/L, Arginine 174 mg/L, Casamino acid 500 mg/L, sucrose 20 g/L, and D-glucose 10 g/L, adjust to pH 5.2, add 1 mL of 100 mM acetosyringone in DMSO per liter of medium prior to use.
6. Cocultivation medium: N6 MAJOR-1 2% v/v, N6 MAJOR-2 1% v/v, N6 MAJOR-3 1% v/v, N6 MAJOR-4 1% v/v, B5 MINOR-1 1% v/v, B5 MINOR-2 1% v/v, B5 MINOR-3 1% v/v, B5 MINOR-4 1% v/v, B5 VITAMINS 0.5% v/v, L-glutamine 876 mg/L, aspartic acid 260 mg/L, Arginine 174 mg/L, casamino acid 500 mg/L, L-proline 500 mg/L, sucrose 20 g/L, D-glucose 10 g/L, adjust to pH 5.2, then add 5.5 g/L of type I agarose. Autoclave the medium for 15 min and cool to 50 °C, then add 2 mL of 2,4-D, 1 mL of NAA, 1 mL of BAP, and 1 mL of fresh 100 mM acetosyringone in DMSO to every liter of medium.
7. Resting medium: N6 MAJOR-1 2% v/v, N6 MAJOR-2 1% v/v, N6 MAJOR-3 1% v/v, N6 MAJOR-4 1% v/v, B5 MINOR-1 1% v/v, B5 MINOR-2 1% v/v, B5 MINOR-3 1% v/v, B5 MINOR-4 1% v/v, B5 VITAMINS 0.5% v/v, L-glutamine 300 mg/L, casamino acid 500 mg/L, L-proline 500 mg/L, mannitol 36 g/L, maltose 20 g/L, adjust to pH 5.8, then add 5 g/L of gelrite. After 15 min autoclaving and cooling to 50 °C, add 1 mL of 2,4-D, 1 mL of NAA, 200 μL of BAP per liter of medium.
8. Selection medium: N6 MAJOR-1 2% v/v, N6 MAJOR-2 1% v/v, N6 MAJOR-3 1% v/v, N6 MAJOR-4 1% v/v, B5 MINOR-1 1% v/v, B5 MINOR-2 1% v/v, B5 MINOR-3 1% v/v, B5 MINOR-4 1% v/v, B5 VITAMINS 0.5% v/v, casamino acid 500 mg/L, L-proline 500 mg/L, L-glutamine 300 mg/L, mannitol 36 g/L, maltose 20 g/L, adjust to pH 5.8, then add 5 g/L of gelrite. After 15 min autoclaving and cooling to 50 °C,

add 1 mL of 2,4-D, 1 mL of NAA, 200 μL of BAP, 1 mL of cefotaxime, 1 mL of cabernicillin, and 30 mg of hygromycin per liter of medium.

9. Pre-regeneration medium: B5 MINOR-1 1% v/v, MS-1 2% v/v, MS-2 1% v/v, MS-3 1% v/v, MS-4 1% v/v, MS VITAMINS 0.5% v/v, maltose 30 g/L, sorbitol 20 g/L, adjust to pH 5.8, then add 10 g/L of type I agarose. After 15 min autoclaving and cooling to 50 °C, add 2 mL of kinetin, 500 μL of NAA, 1 mL of cefotaxime, and 50 mg of hygromycin per liter of medium.
10. Regeneration medium: B5 MINOR-1 1% v/v, MS-1 2% v/v, MS-2 1% v/v, MS-3 1% v/v, MS-4 1% v/v, MS VITAMINS 0.5% v/v, sucrose 30 g/L, adjust to pH 5.8 and then add 3 g/L of Gelrite. After 15 min autoclaving and cooling to 50 °C, add 2 mL of kinetin, 1 mL of NAA, 1 mL of cefotaxime, and 50 mg of hygromycin per liter of medium.
11. Yoshida-conventional culture solution (YCS).

2.3 Transgene Screening and Mutation Analysis

1. Taq DNA polymerase PCR reagent.
2. Phusion® High-Fidelity DNA Polymerase.
3. Primers: LbCpf1-F and LbCpf1-R, HptII-F and HptII-R, EPFL9-seq-F and EPFL9-seq-R.
4. Surveyor® Mutation Detection.
5. QIAquick Gel Extraction Kit.
6. For Southern Blot: Restriction enzyme XbaI, agarose, 1× TAE buffer, DIG-labeled molecular weight marker II, Hybond Nylon+ membrane, 20× SSC which has 0.3 M tri-sodium citrate acetate dehydrate in 3 M NaCl with pH 7.0, 2× SSC, PCR DIG Probe Synthesis Kit, Anti-DIG Fab Fragment-AP conjugate, CDP-Star Detection Reagent.

3 Methods

3.1 Generation of the pCambia-LbCpf1 Binary Vector Targeting OsEPFL9

1. Digest 1 μg of the pCambia-CRISPR_Cas9 first with AarI (0.5 μL of AarI, 0.4 μL of 50× oligonucleotide in total of 20 μL). Run the digested product on 0.8% agarose gel and purify the isolated gel with the correct size band. Then digest all purified product with 0.5 μL of XbaI in CutSmart Buffer. Run the digested product on 0.8% agarose gel and purify the isolated gel with the correct size band to remove the Cas9 guide RNA scaffold.
2. Mix 10 μL of 10 μM LbCpf1-gRNA-F and 10 μL of 10 μM LbCpf1-gRNA-R and heat up the mixture to 95 °C on a heat block. Then cool the mixture to room temperature naturally on

bench to form double-stranded oligos carrying compatible sticky end to AarI and XbaI.

3. Ligate AarI-XbaI digested pCambia-CRISPR_Cas9 and 1 μL of the annealed oligo using 0.5 μL of T4 ligase.
4. Transform the ligated product to *E. coli* DH5α using heat shock method. Grow the transformed *E. coli* on LB agar plate containing 50 μg/mL of kanamycin overnight at 37 °C. Transform purified DNA fragment of AarI-XbaI digested pCambia-CRISPR_Cas9 as negative control to see if the digestion is completed. Inoculate and grow single colonies in LB liquid medium containing 50 μg/mL of kanamycin at 37 °C overnight. Isolate the plasmid DNA of this intermediate in the next morning.
5. Digest 500 ng of the intermediate vector with 0.5 μL of HindIII and 0.5 μL of SalI to remove the Cas9 coding sequence and the terminator.
6. PCR amplify the partial coding sequence of the LbCpf1 from cDNA3-huLbCpf1 using the primer Cpf1-F and LbCpf1-R and also PCR amplify the other part of the LbCpf1 coding sequence containing a nuclear localization signal (NLS) and the CaMV terminator from pCambia-CRISPR_Cas9 using primer LbCpf1-NLS-F (carrying the remaining coding sequence of LbCpf1 at 5′) and Cpf1-NLS-R. Perform an overlapping PCR in order to join the two PCR products into one that carries a unique HindIII site upstream of the LbCpf1 cassette and a unique SalI site downstream of the terminator.
7. Ligate the HindIII-SalI digested overlap PCR product with HindIII-SalI digested intermediate vector using 0.5 μL of T4 ligase. Transform the ligated product to *E. coli* DH5α as described above. Screen single colonies using primer LbCpf1-F and LbCpf1-R. Inoculate the PCR positive colony for plasmid DNA isolation.
8. Sequencing verify the plasmid DNA named pCambia-LbCpf1 backbone.
9. Digest 500 ng of pCambia-LbCpf1 backbone vector with 0.5 μL of BaeI and run on 0.8% agarose gel. Purify the isolated gel with the correct size band.
10. Anneal 10 μL of 10 μM EPFL9-Cpf1-Target-F and 10 μL of 10 μM EPFL9-Cpf1-Target-R to form double-stranded oligo carrying compatible end as the unique BaeI site (*see* **Note 3**) in the pCambia-LbCpf1 backbone.
11. Ligate the annealed EPFL9-Cpf1-Target-F and EPFL9-Cpf1-Target-R with the BaeI digested pCambia-LbCpf1 backbone using 0.5 μL of T4 ligase.

12. Transform the ligated product to *E. coli* DH5α and isolate plasmid DNA from a single colony growing in liquid medium. Sequence to verify the plasmid DNA (*see* **Note 4**).

3.2 Rice Transformation

1. Preparation of *Agrobacterium*: Transform the verified pCambia-LbCpf1-EPFL9 to *Agrobacterium* LBA4404 using freeze-thaw method (*see* **Note 5**). Grow the transformed *Agrobacterium* on YEB agar plate containing 50 μg/mL of kanamycin at 28 °C for 2 days. Screen single colonies using LbCpf1-F and LbCpf1-R. Inoculate positive colony into YEB liquid medium containing 50 μg/mL of kanamycin at 28 °C for 18 h. Isolate the plasmid DNA and transform it back to *E. coli* DH5α for sequencing verification. Prepare glycerol stock for transformed *Agrobacterium* and *E. coli*. Streak the *Agrobacterium* glycerol stock on YEB agar containing 50 mg/L of kanamycin 2 days prior to rice transformation. One hour before transformation, take *Agrobacterium* and mix it with 5 mL of infection medium. Adjust the OD_{600} of the *Agrobacterium* suspension to 0.3. Incubate the *Agrobacterium* suspension at 25 °C for 1 h in the dark.
2. Preparation of immature embryos (IEs): We use rice immature embryo system for the transformation [28, 29] (*see* **Note 6**). Collect the immature seeds at milk to soft dough stage (12 days after anthesis). Soak de-hulled immature seeds in 70% ethanol for 1 min and then rinse seeds with sterilized distilled water. Sterilize the immature seeds with 1% sodium hypochlorite solution (containing one drop of Tween 20) in a 50 mL falcon tube for 10 min. Then rinse the seeds with sterilized distilled water until all the sodium hypochlorite is removed. Isolate and place IEs on a cocultivation medium plate (50 IEs per plate). Air-dry the plates for 3 h in laminar flow hood to prevent the overspreading of bacterium.
3. Arrange the IEs on the cocultivation medium plate with the scutellum facing up. Drop 5 μL of *Agrobacterium* suspension on top of each IE. Seal the plates with micropore tape and incubate at 25 °C for 7 days in the dark.
4. Remove the elongated shoots from IEs after the cocultivation using a sterilized scalpel. Gently blot the IEs on sterile filter paper to prevent the overgrowth of bacterium. Transfer blotted IEs to the resting medium with the scutellum side facing up. Incubate the sealed plates at 30 °C for 5 days under continuous illumination.
5. Cut each IE into four pieces and place them on the selection medium containing 30 mg/L of hygromycin with their scutellum side up (10 IEs/plate, i.e., 40 pieces/plate). Group the four pieces from a single IE together on the plate. Incubate the

sealed plates at 30 °C for 10 days under continuous illumination. Then transfer all the cut IEs to fresh selection medium for another 10 days. Then transfer all the IEs again to fresh selection medium for a third time for 10 days.

6. Transfer all resistant calli to pre-regeneration medium and incubate the sealed plates at 30 °C for 10 days under continuous illumination.
7. Transfer the proliferating calli with visible green tissue to the regeneration medium to grow for 10–15 days until roots are about 2 mm long.
8. Transfer the regenerated plantlets on each callus as a bunch to YCS. Separate the plantlets into individual ones when the roots are stably established.

3.3 Transgene Screening and Mutation Analysis

1. Extract the genomic DNA of T0 plants from each plantlet at third leaf stage. Screen the plants with PCR using LbCpf1-F and LbCpf1-R or HptII-F and HptII-R to confirm if the plants are transformed.
2. For T0 plants, subject the transgene PCR-positive plants to indel detection/mutation analysis. PCR amplify the target region with Phusion® High-Fidelity DNA Polymerase using EPFL9-seq-F and EPFL9-seq-R (*see* **NoteS 7** and **8**). Do include one wild-type control in PCR (Fig. 1a). Total volume of each PCR reaction is 15 μL, of which 3.5 μL is to be run on agarose gel to confirm the success of PCR amplification.
3. Mix 6 μL of PCR product from transgenic plants and 6 μL of PCR product from wild-type plants well. Hybridize the mixed PCR product to form DNA heteroduplexes (Fig. 1b) in a PCR machine with hot lid set at 105 °C. Heat the mixture to 95 °C for 10 min and cool the mixture down at 2 °C/s. Hold the temperature for 10 min after every 10 °C decreased until it reaches 25 °C. Digest the hybridized DNA with Surveyor nuclease, following the user's guide. Run the digested product on 2% agarose gel (Fig. 1c). Visible digested bands of expected sizes indicate transgenic plants carrying mutations at the target site (Fig. 1d).
4. Amplify the Surveyor positive samples with Phusion® High-Fidelity DNA Polymerase using EPFL9-seq-F and EPFL9-seq-R. Sequencing verify the PCR products and analyze the sequencing results using TIDE [30] and Poly Peak Parser [31].
5. Analyze the plants identified with targeted mutations with Southern Blot to identify the copy number of the transgene [27]. In order to get transgene-free edited plants, we choose plants with desired edits that have less copies of the transgene to bring to T1 generation.

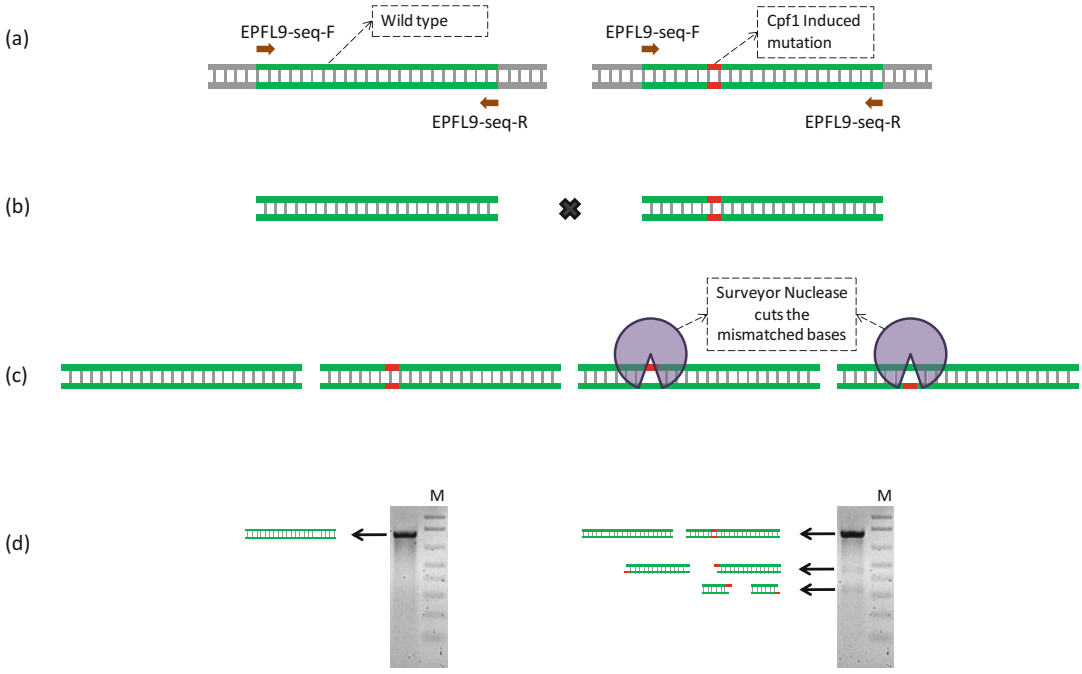

Fig. 1 Indel detection using Surveyor nuclease. (**a**) Target region of wild-type and transgenic plants were both amplified using EPFL9-seq-F and EPFL9-seq-R. (**b**) The mixed PCR product is hybridized to form DNA heteroduplexes. (**c**) The hybridized DNA is digested with Surveyor nuclease. (**d**) Digested product is run on 2% agarose gel. Visible digested bands of expected sizes indicate transgenic plants carrying mutation at the target site

3.4 Selection of Transgene-Free Edited Plants

1. Bring the edited plants to T1 generation. When there are multiple events available, we select three events of relatively less copy number of the transgene (e.g., single copy).
2. PCR screen 30 T1 plants of each event using LbCpf1-F and LbCpf1-R or HptII-F and HptII-R (*see* **Note 9**).
3. Amplify the target region of the transgene PCR-negative plants with Phusion® High-Fidelity DNA Polymerase using EPFL9-seq-F and EPFL9-seq-R. Sequencing verify the high-fidelity PCR products.
4. For the T1 plants carrying the homozygous inherited edits that are transgene PCR-negative, perform Southern Blot to further verify the absence of the transgene.

4 Notes

1. For designing target site, avoid the introns (unless splicing signal can be disturbed and lead to nonsense transcription) and noncoding regions.

2. Target search should be done carefully to eliminate all possibilities for off-target effects. In silico analyses should be done to avoid this. Any off-target editing may generate unpredicted mutations and phenotypes as a consequence. This will not only reduce the accuracy of the method but also complicate further analysis.
3. While cloning the guide RNA and the scaffold to a binary vector, we should be careful about selecting the enzymes. Type IIS restriction enzymes are usually preferred. Firstly, because they have different cutting and recognition sites, the sites will no longer exist in the vector after digestion. Secondly, the ends generated by restriction digestion are not complementary, so the chances of self-annealing of the vectors are eliminated.
4. Sequencing the vector at all stages of cloning is recommended to avoid any error in the final constructed vector.
5. A transient expression of the vector (such as protoplast transient expression) is recommended for testing the editing reagents before stable transformation.
6. All the tissue culture experiments should be performed in aseptic conditions to avoid any contamination.
7. While designing primers for surveyor assay, we should select unique primers flanking the target site. The two primers should have difference in their distance to the target site. About 200–300 bp difference is recommended.
8. All the melting temperatures (Tm) of the primers for surveyor assay should be high to avoid any nonspecific bindings during PCR.
9. If transgene-free homozygous edits were not identified from the 30 T1 plants of some events, more can be grown for further screen.

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Chapter 20

Targeted Mutagenesis Using FnCpf1 in Tobacco

Akira Endo and Seiichi Toki

Abstract

Various CRISPR/Cas9 systems have been extensively applied for targeted mutagenesis to generate mutants that impaired in genes of interest. Clustered regularly interspersed short palindromic repeats (CRISPR) from *Prevotella* and *Francisella* 1 (Cpf1) is new RNA-directed endonuclease possessing some differences as compared to Cas9. Several papers have shown that Cpf1 could be a versatile tool in plant genome engineering. Cfp1 from *Francisella novicida* (FnCpf1) recognizes TTN as its protospacer adjacent motif (PAM). TTN is a shortest PAM among other known Cpf1s such as AsCpf1 or LbCpf1, which use TTTN as PAM. The length of PAM can be the restriction of the number of target sequences. Cpf1 generates cohesive DNA end after the digestion of target sequences. Sticky DNA end is thought to appropriate for in vivo ligation rather than blunt DNA end created by Cas9. Therefore, FnCpf1 is practical for targeted mutagenesis experiments. The application of FnCpf1-mediated targeted mutagenesis to the plant genome engineering could accelerate molecular breeding of crops. Here, we describe procedures for targeted mutagenesis in tobacco using FnCpf1.

Keywords CRISPR/Cpf1, FnCpf1, Targeted mutagenesis, Tobacco

1 Introduction

Sequence-specific nucleases (SSNs) including zinc-finger nucleases (ZFNs), transcription-activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR Associated protein 9 (Cas9) have been developed to engineer genomes of various organisms [1, 2]. Targeted mutagenesis using SSNs are especially favored in non-model plant systems [3, 4]. Polyploidy is often observed in crop genomes such as tobacco, potato, sweet potato, and wheat. In these polyploid genomes, investigation of the function of interested genes may be problematic due to genetic redundancy. To investigate the mutant phenotype of multicopy genes, conventional crossing has been necessary to produce mutants with defect in multiple loci until the emergence of CRISPR/Cas9 or CRISPR/Cpf1 systems. Cas9 and Cpf1 can successfully induce mutations at multiple loci by simple construction of multiplexing vectors [5, 6]. This feature of

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Cas9 and Cpf1 is superior to other SSNs such as ZFNs and TALENs.

Cpf1 is an RNA-directed endonuclease that functions like Cas9 in the bacterial immune system. In recent years, Cpf1 has been applied for animal and plant genome engineering. Cpf1 has three significant differences as compared to Cas9. First, Cpf1 prefers thymine-rich protospacer adjacent motif (PAM) such as TTN or TTTN, whereas Cas9 recognizes Guanidine-rich PAM like NGG or NGRRT. Second, Cpf1 utilizes only CRISPR RNA (crRNA), while Cas9 requires two RNA molecules, crRNA and trans crRNA (tracrRNA), for function. Third, Cpf1 generates cohesive DNA ends after cleavage of target sequences while blunt DNA ends are generated by Cas9.

Three orthologs of Cpf1 including *Acidaminococcus* sp. BV3L6 Cpf1 (AsCpf1), *Lachnospiraceae bacterium* ND2006 Cpf1 (LbCpf1), and *Francisella novicida* Cpf1 (FnCpf1) have been successfully applied to genome engineering of various organisms [7–14]. Although these Cpf1 proteins could similarly induce mutations, FnCpf1 recognized shorter PAM, TTN as compared to the other two Cpf1s, which utilize TTTN as PAM [7]. Since the length of PAM restricts the number of possible target sequences in the genome, Cpf1 recognizing shorter PAM are preferred for targeted mutagenesis. Therefore, we chose FnCpf1 for application to plant genome engineering [10]. Our paper firstly demonstrated that FnCpf1 could successfully induce mutations in both tobacco and rice genomes [10].

In this protocol, we describe the detailed procedures for crRNA design, construction of binary vectors harboring crRNA and FnCpf1, the Agrobacterium-mediated transformation of tobacco, and the detection of targeted mutations.

2 Materials

2.1 Vector Construction

1. pUC19-AtU6::crRNA-*ccd*B is used for construction of the crRNA expression cassette (Fig. 1a). AtU6-26 promoter controls the expression of crRNA in tobacco. The FnCpf1 is codon-optimized for expression in *Arabidopsis thaliana* (At. Opt). Binary vector harboring codon-optimized FnCpf1, pRI-FnCpf1(At. Opt) is applied for targeted mutagenesis of tobacco (Fig. 1b).
2. Restriction enzyme: *Bbs*I, *Asc*I, and *Pac*I (New England Biolabs).
3. Gel Extraction kit (QIAGEN).
4. PCR purification kit (QIAGEN).
5. T4 ligase and ligation buffer (Promega).

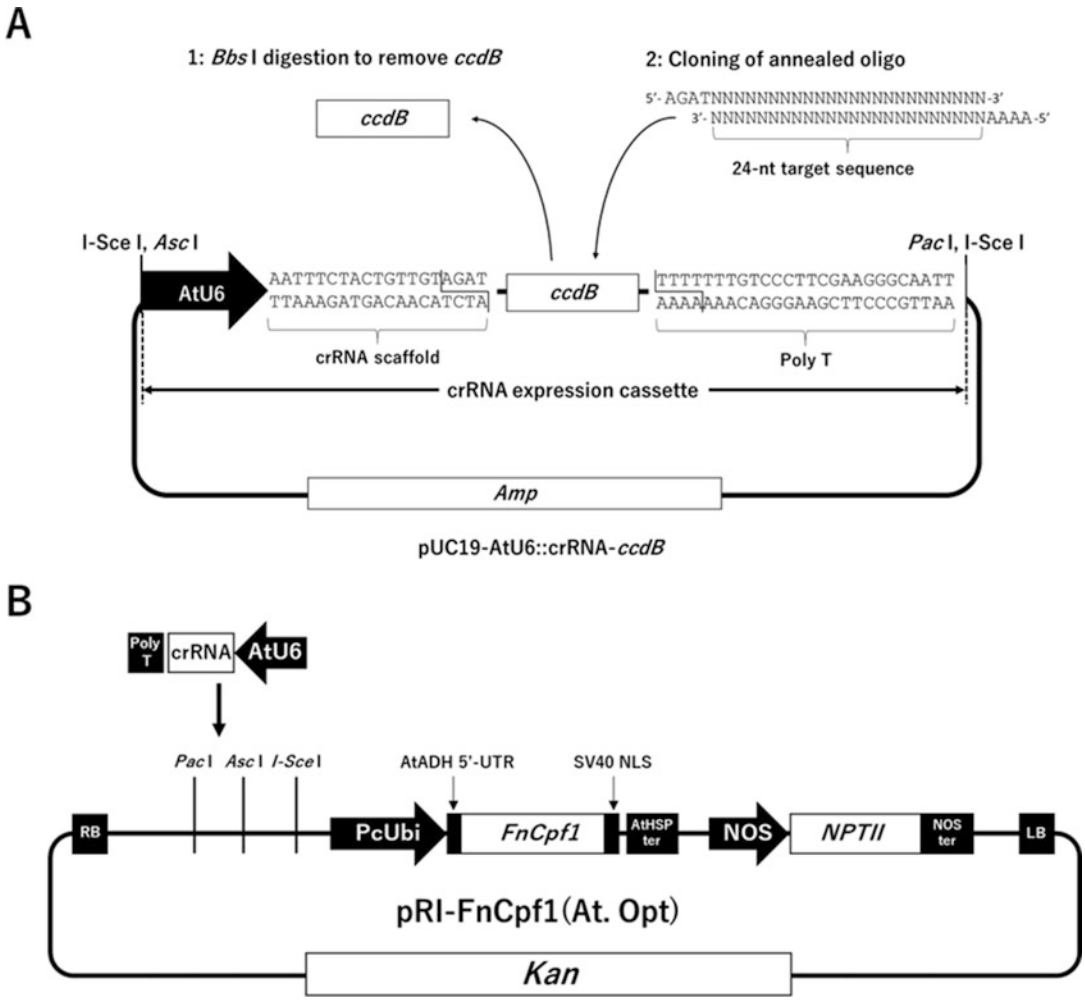

Fig. 1 Vectors used for targeted mutagenesis of tobacco by FnCpf1. (**a**) Cloning of a target sequence into the crRNA expression cassette. First, the restriction enzyme, *Bbs* I, is used for removing the *ccd*B gene from pUC19-AtU6::crRNA-*ccd*B. Second, the annealed oligo is ligated with *Bbs* I-digested vector. Directional cloning of the target sequence is completed by the 5′-overhang of the annealed oligo. *Amp* indicates the ampicillin-resistant gene in *E. coli*. (**b**) The binary vector for targeted mutagenesis in tobacco. Targeted mutagenesis of tobacco is performed by introducing pRI-FnCpf1 (At. Opt) into tobacco. *Kan* and *Spec* represent kanamycin- and spectinomycin-resistant genes, respectively, in bacteria. NPTII: Neomycin phosphotransferase II is kanamycin-resistant gene in plants. The crRNA expression cassette is introduced into the I-*sce*I site or the double-digested sites with *Asc* I and *Pac* I in the vector. PcUbi is ubiquitin promoter from *Petroselinum crispum*. SV40 NLS: SV40 large T-antigen nuclear localization signal. AtADH 5′ -UTR: 5′ untranslated region of *Arabidopsis thaliana ALCOHOL DEHYDROGENASE* gene. AtHSP ter: the terminator of *Arabidopsis thaliana HEAT SHOCK PROTEIN 18.2* gene

6. *E. coli* DH5α competent cells.
7. *E. coli ccd*B survival competent cells (Thermo Fisher Scientific).

2.2 Agrobacterium-Mediated Transformation

2.2.1 Plant and Agrobacterium Strain

1. Tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR-1).
2. *Agrobacterium tumefaciens* LBA4404 (for tobacco transformation).

2.2.2 Stock Solutions for Tobacco Medium

1. Vitamin stock (100×): 10 g/L *myo*-inositol, 50 mg/L nicotinic acid, 50 mg/L pyridoxine HCl, and 1 g/L thiamine HCl. Dissolve the powder in water and sterilized by filtration using 0.22 mm filter. Store at −20 °C.
2. 2 mg/mL NAA: Dissolve in a small amount of 1 N KOH and make up volume with water. Store at 4 °C.
3. 2 mg/mL Benzylaminopurine (BAP): Dissolve in a small amount of 1 N KOH and make up volume with water. Store at 4 °C.
4. 0.5 M 2-morpholinoethanesulfonic acid (MES)-KOH (pH 5.8).

2.2.3 Media for Agrobacterium-Mediated Transformation of Tobacco

1. Coculture medium (liquid 1 L): Add 50 g/L sucrose, 0.1 mL of NAA stock, 1 mL of BAP stock, and 10 mL of 0.5 M MES-KOH (pH 5.8). Bring the medium up to 1 L. Sterilize by autoclaving.
2. MS medium (1 L): Add 30 g/L sucrose, 4.6 g/L MS salt, 10 mL of vitamin stock, 10 mL of 0.5 M MES-KOH (pH 5.8), and 8 g of bacto-agar into water for a final volume of 1 L. Sterilize by autoclaving. When using this medium for rooting, 1 mL of 25 mg/mL meropenem and 1 mL of 50 mg/mL kanamycin should be added when the autoclaved medium has cooled down to around 55 °C. The medium is solidified in sterile plastic Petri dishes.
3. Selection medium (1 L): Add 4.6 g/L MS salt, 30 g/L sucrose, 10 mL of vitamin stock, 0.1 mL of NAA stock, 1 mL of BAP stock, 10 mL of 0.5 M MES-KOH (pH 5.8), and 8 g of bacto-agar into water for a final volume of 1 L. Sterilize by autoclaving. 1 mL of 25 mg/mL meropenem and 1 mL of 50 mg/mL kanamycin are added when the temperature of the medium is down to around 55 °C. The medium is solidified in sterile plastic Petri dishes.

2.3 Detection of Mutations in Transgenic Tobacco

1. DNA extraction kit (e.g., Agencourt Chloropure, BECKMAN COULTER).
2. Thermoresistant DNA polymerase (e.g., KOD FX Neo, TOYOBO).
3. Restriction enzymes depending on the target sequences (New England Biolabs.).

4. Microchip electrophoresis system (e.g., MultiNA, SHIMADZU).

3 Methods

3.1 Selection of Target Sequence for FnCpf1

FnCpf1 utilizes TTN as PAM, placed just upstream of the target sequence. Therefore, a 24-nt sequence just next to TTN can be chosen as the target sequence (*see* **Note 1**). FnCpf1 generates staggered DNA ends and DNA cleavage by FnCpf1 occurs at the 18th base from PAM on the non-targeted strand, and at the 23rd base from PAM on the targeted strand within the 24-nt target sequence. When recognition sites of restriction enzymes are found at the cleavage site of Cpf1, cleaved amplified polymorphic sequence (CAPS) analysis is performed for detecting mutations that were induced by Cfp1. When recognition sites of restriction enzymes were not overlapped with the cleavage site of Cpf1, heteroduplex mobility assay (HMA) is performed to detect mutations. Microchip electrophoresis system is used for resolving PCR product to perform HMA in a high throughput manner.

3.2 Vector Construction

For constructing the crRNA expression cassette, the *ccd*B gene is firstly removed from pUC19-AtU6::crRNA-*ccd*B by the digestion of *Bbs* I (Fig. 1a). Two *Bbs* I sites are located at both the sides of the *ccd*B gene, which is toxic to *E. coli* strains other than the *ccd*B resistance strain. *Bbs* I digestion leaves overhangs complementary to the overhang of the annealed oligos (Fig. 1a). Once a target sequence is selected, oligo annealing is performed with forward and reverse oligos consisting of the 24-nt target site with 4-nt 5′ overhangs fitting the *Bbs* I digestion site. Annealed oligos are cloned into the *Bbs* I-digested vector. crRNA expression cassette on pUC19-AtU6::crRNA is subcloned into the double-digested sites of *Pac* I and *Asc* I located on the pRI-FnCpf1 (At. Opt) (Fig. 1b). The detailed procedure is described as follows.

1. Mix 1 μL of each oligo DNA (100 μM) with 48 μL of H_2O. The mixture is subjected to treatments at 95 °C for 5 min and left at room temperature for 20 min to anneal the oligos.
2. Digest pUC19-AtU6::crRNA-*ccd*B as follows: combine 2 μg of the plasmid DNA, 5 μL of 10× NEB cut smart buffer, 1 μL of *Bbs* I (5 unit / μL), and H_2O up to 50 μL. Incubate at 37 °C for 2–16 h. The digested products are resolved on 1% (w/v) agarose gel in TAE buffer. The linearized vector was purified using a gel extraction kit.
3. Ligate the annealed oligo and the *Bbs* I-digested vector as follows: mix up 2 μL of digested vector (10 ng/μL), 2 μL of annealed oligo, 5 μL of the 2× T4 DNA ligation buffer, and

0.5 μL of T4 DNA ligase. Incubate at room temperature for 30 min. Transform the ligation product with *E. coli* DH5α competent cells according to the manufacturer's instruction.

4. Miniprep plasmid DNA and sequence with an M13 reverse primer to confirm whether the target sequence is correctly introduced in the plasmid for expressing the crRNA.
5. Digest pRI-FnCpf1 (At. Opt) with *Asc*I and *PacI* for cloning the crRNA expression cassette. Purify the linearized vector after electrophoresis with a Gel Extraction kit. Simultaneously, digest the crRNA expression cassette vector (from **step 4**) with *Asc*I and *Pac*I. Gel purify the 668 bp of crRNA expression cassette after agarose gel electrophoresis.
6. Ligate the double-digested binary vector with crRNA expression cassette, and transform the ligation reaction with *E. coli* DH5α competent cells. Miniprep the plasmids and confirm the binary vectors by restriction digestion.
7. Transform resultant binary vector into *A. tumefaciens* LBA4404 by electroporation. Spread transformed cells on LB plate containing 50 mg/L of kanamycin and 25 mg/L of rifampicin. Incubate for 2~3 days at 28 °C.

3.3 Agrobacterium-Mediated Transformation Using Tobacco Leaf Disks

3.3.1 Preparation of Agrobacterium Culture

1. Start a preculture of *A. tumefaciens* LBA4404 harboring FnCpf1 binary vector in 2 mL LB medium supplemented with 50 mg/L of kanamycin. Incubate the culture at 28 °C with constant shaking overnight.
2. Transfer 2 mL preculture to 30 mL LB medium supplemented with 50 mg/L kanamycin. Incubate the culture at 28 °C with constant shaking overnight.
3. Harvest Agrobacterium cells by centrifugation at 3000–4000 *g* for 10 min.
4. Dissolve the bacterial pellet to a final OD600 of 0.5–1.0 in 30 mL coculture medium.

3.3.2 Preparation of Tobacco Leaf Disks

1. Detach fully expanded leaves (10–15 cm width) from 1- to 2-month-old tobacco plants growing in the greenhouse (*see* **Note 2**).
2. Wash the leaf surface with tap water.
3. Submerge two to three leaves in 1 L of 5% commercial bleach for 30 min. Rinse leaves three times in 1 L of sterilized water to wash out bleach (*see* **Note 3**).
4. Transfer surface-sterilized leaves to sterilized filter paper. Prepare leaf disks using a sterile hole punch (8 mm diameter) on the filter paper (Fig. 2a). Avoid leaf margin and mid-rib. Soak leaf disks in coculture medium to avoid drying up before inoculation.

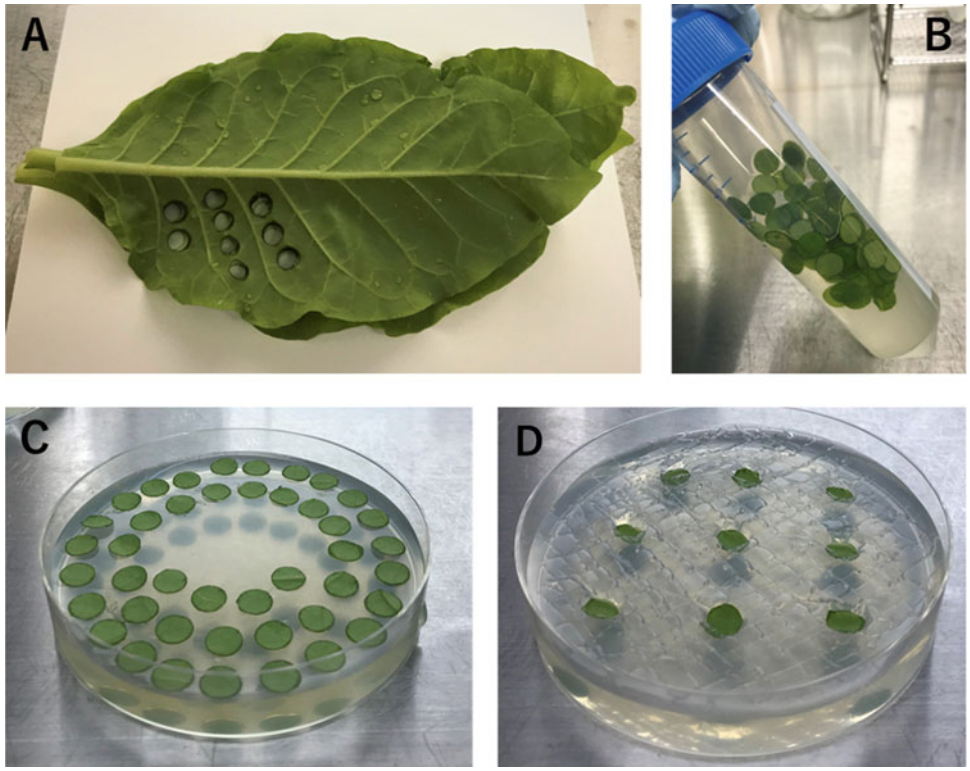

Fig. 2 Transformation of tobacco leaf disks. (**a**) Preparation of tobacco leaf disks using a hole punch. (**b**) Inoculation of leaf disks in 50 mL tube. (**c**) Leaf disks on cocultivation medium after the inoculation step. (**d**) Embedded leaf disks on selection medium

3.3.3 Transformation of Tobacco Leaf Disks

1. Mix approximately 100 leaf disks in 30 mL *Agrobacterium* coculture medium suspension in a 50 mL plastic tube. Incubate at room temperature for 30 min (Fig. 2b) (*see* **Note 4**).
2. Discard resuspended *Agrobacterium* as much as possible and then briefly blot explants on sterile filter paper. Place explants adaxial side up onto MS medium (approximately 40 explants per plate) (Fig. 2c). Seal the plates with parafilm to keep moist. Cocultivate the disks at 24 °C in the dark for 3 days.
3. After 3 days of cocultivation, transfer the disks, adaxial side up, to selection medium containing 50 mg/L kanamycin and 25 mg/L meropenem. Place no more than nine disks per plate to culture. The disks should be completely embedded in the selection medium; the rim of each disk should contact the selection medium to avoid escaper (Fig. 2d) (*see* **Note 5**).
4. Incubate the plates at 28 °C under constant illumination for 24 h. Transfer the explants to fresh selection medium every 2 weeks. Kanamycin-resistant calli will emerge along the rim of the disks after approximately 2 weeks of culture on the selection medium.

5. Separate each callus cluster using a razor blade and transfer callus clusters to fresh selection medium. Multiple shoots arising from a callus cluster are mostly clones; therefore, track shoots arising from the same cluster.
6. Separate vigorously growing shoots by cutting the stem of the shoots using a razor blade. Embed the stem of the shoot in MS medium supplemented with 50 mg/L kanamycin and 25 mg/L meropenem. The shoot is cultured until roots emerge from the cut edge of the shoots under growth conditions identical to **step 4** (*see* **Note 6**).

3.3.4 Plant Care and Seed Harvest

1. Carefully pick plantlets with well-established root systems from MS medium. Wash off any excess agar with tap water and transfer the plantlets to soil. Cover the plantlets briefly with plastic wrap until plantlets are acclimated to the conditions of the plant growth chamber.
2. Grow the plantlets in the plant growth chamber at 22 °C under constant illumination for 24 h.
3. Grow the plants under the normal growth condition to maturity. Collect T_1 seeds from fully mature pods in 1.5 mL plastic tubes. Store the seeds in a desiccator at room temperature.

3.4 Detection of Mutation in Regenerated Tobacco Leaves

DNA samples are isolated from regenerated plants, and patterns of mutation are determined. T_0 plants should be kept growing to collect seeds (T_1 generation) that possess the mutation at the targeted locus to follow-up genetical transmission of FnCpf1-induce mutations to the next generation (*see* **Note 7**).

3.4.1 DNA Isolation

Extract DNA from hygromycin-resistant leaves of kanamycin-resistant regenerated tobacco shoots using a DNA extraction kit (e.g., from Agencourt Chloropure) according to the manufacturer's instruction.

3.4.2 Amplification of the Targeted Sequence

To detect mutation by CAPS or HMA assays, PCR primers are designed to amplify products in the range of 200–300 bp including the targeted sequence in the middle of the product. A typical PCR mixture and PCR program are described as follows:

PCR mixture	
Genomic DNA	1 μL
2× PCR reaction buffer	7.5 μL
2 mM dNTPs	3 μL
5 μM forward primer	1 μL
5 μM reverse primer	1 μL
KOD FX Neo (DNA polymerase)	0.2 μL

(continued)

PCR mixture	
Total	15 μL

PCR program	
1: 95 °C	2 min
2: 98 °C	15 s
3: 60 °C	30 s
4: 68 °C	30 s
5: Repeat **steps 2~4** 40 times	
6: 68 °C	1 min
7: 12 °C	Hold

3.4.3 CAPS Assay

When the expected cleavage site of FnCpf1 is overlapped with recognition site of an appropriate restriction enzyme, a CAPS assay is performed to detect the mutations.

1. Digest the PCR product from Subheading 3.4.2 in a reaction as follows

Restriction enzyme reaction	
PCR product	3 μL
10× buffer	2 μL
Restriction enzyme	0.5 μL
H_2O	14.5 μL
Total	20 μL

   Incubate the reaction at the appropriate temperature for 2 h or longer to ensure complete digestion of the PCR product amplified from a DNA sample of untransformed tobacco.

2. Resolve the digestion reaction on an 3% agarose gel and stain the gel with ethidium bromide to visualize DNA. If the PCR product harbors mutations at the target site, uncleaved PCR product will be observed.
3. To estimate the frequency of mutations, quantify the fluorescence intensities of the PCR amplicon and cleaved bands with a gel quantification software. Mutation (%) = $A/(A+B+C) \times 100$ (A, fluorescent intensity of the uncleaved PCR product; B and C, fluorescent intensity of the digested PCR product).

3.4.4 Heteroduplex Mobility Assay (HMA)

HMA is the method to detect mutations in PCR products by using the difference of mobility of PCR products containing heteroduplex and homoduplex. When mismatched pairs of nucleotides exist in PCR product, the heterogeneous mixture of PCR product contains homo- and heteroduplexes, which have different mobility in electrophoresis due to difference in the secondary structure. By using polyacrylamide gel electrophoresis (PAGE), heteroduplexes of PCR products can be distinguished from homoduplexes because heteroduplexes migrate more slowly because the mismatched region in the heteroduplex tends to form an opened single-strand structure [15]. Since PAGE is not appropriate to analyze a large number of samples, the microchip electrophoresis system is utilized to perform HMA in a high throughput manner. PCR products (200–300 bp) are resolved using a microchip electrophoresis system as described in previous reports [10, 16, 17].

3.4.5 Analysis of Mutation Patterns by Sequencing

1. Purify PCR products from Subheading 3.4.2 using a PCR purification kit or purify the undigested bands (from Subheading 3.4.3) using a gel purification kit in accordance with the manufacturer's guides. Clone the purified DNA fragments into pCR-Blunt using a Zero Blunt TOPO PCR cloning kit. Perform colony PCR with M13 forward and M13 reverse primers to identify clones with successful inserts. The example colony PCR mixture and program are as follows.

Mixture of colony PCR	
2× GoTaq Green Master Mix	5 μL
10 μM M13 forward primer	0.5 μL
10 μM M13 reverse primer	0.5 μL
Sterilized H_2O	4 μL
Total	10 μL

PCR program	
1: 95 °C	2 min
2: 95 °C	15 s
3: 55 °C	30 s
4: 72 °C	30 s
5: Repeat **steps 2–4** 25 times	
6: 12 °C	Hold

2. Identify the mutations by sequencing of the colony PCR products using M13 forward or M13 reverse primers. Dilute the PCR mixture 1/10 and use as template in a sequencing reaction shown below.

Mixture of PCR for sequence	
Template (1/10 diluted PCR product)	1 μL
5× sequencing reaction buffer	1 μL
Big Dye terminator v3.1	1 μL
5 μM M13 reverse or forward primer	1 μL
Sterilized H_2O	6 μL
Total	10 μL

3. Remove excess dye-labeled nucleotides from the reaction mixture using a BigDye XTerminator Purification kit according to the manufacturer's instruction. Analyze the sequences using Genetic Analyzer, 3500xL.

4 Notes

1. We recommend the use of multiple crRNAs for your gene of interest. In many cases, some crRNAs did not work to induce mutations. It may be due to the short crRNA scaffold (19 nt) of Cpf1 as compared to sgRNA scaffold of Cas9 (approximately 80 nt). While one paper reported that addition of extra sequence to 19 nt crRNA could improve the genome editing efficiency [18], the other one demonstrated high editing efficiency of Cpf1 based on precise processing of crRNA by ribozymes [14].
2. When the unexpanded young leaf is used to prepare leaf disks, leaf disks will expand and curl during the selection. Since this growth can decrease contact of leaf disks to the selection media, escapers will frequently emerge under this condition.
3. A sterile condition is necessary for this step. We use the clean bench for aseptic manipulation.
4. The transformation efficiency is largely affected by *Agrobacterium* vigor. Freshly transformed *Agrobacterium* is preferred for tobacco transformation experiments.
5. Before transferring leaf disks to the selection medium, the surface of the medium is briefly mashed up with tweezers to embed the disks. The rim of the leaf disks should be in full contact with the medium to subject leaf cells to antibiotics

within the medium. When the contact is not enough, non-transgenic escapers will regenerate from the disks.

6. When roots do not emerge from shoots, the stems of plants will be recut by razor blades. They are taken back to the MS medium for re-culture.
7. Regenerated tobacco plants mostly possess various mutations. This fact indicates mutation events constitutively occur independently in cells in tobacco plants during their growth. We fail to isolate homogenous biallelic mutants at T_0 generation in tobacco. When regenerated T_0 plants are highly mutated (more than 50%), genetical transmission of mutations is observed in T_1 generation.

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Part VII

Precise Gene Editing

Chapter 21

Gene Replacement by Intron Targeting with CRISPR-Cas9

Jun Li, Xiangbing Meng, Jiayang Li, and Caixia Gao

Abstract

The CRISPR-Cas9 system has become the most widely adopted genome editing platform and is used in an expanding number of organisms, mainly by creating targeted knockouts through non-homologous end joining (NHEJ) of DNA double-strand breaks (DSBs). It would also be highly desirable to be able to use homology-directed repair (HDR) to perform precise gene editing, for example, by replacing a small section of DNA to substitute one amino acid for another in a given gene product. However, this remains a serious challenge in plants. Here, we describe a recently developed intron-mediated site-specific gene replacement method acting through the NHEJ pathway in which Cas9 simultaneously introduces DSBs in adjacent introns and the donor template. This approach is of general use for replacing targeted gene fragments at specific genomic sites in plants.

Key words Gene replacement, CRISPR-Cas9, NHEJ, Intron, DSB

1 Introduction

The type II prokaryotic clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein9 (Cas) system exists in bacteria and archaea as an adaptive immune defense system [1, 2], and it has been adapted as a genome editing tool in eukaryotes [3, 4]. Due to the high efficiency, low cost, simplicity, and versatility of this system, it has become the most widely used editing platform for precisely modifying eukaryotic genomes [5–8]. Using a single guide RNA (sgRNA) recognizing target DNA sequences through Watson-Crick base pairing, Cas9 can be precisely directed to target sites where it generates targeted DNA double-strand breaks (DSBs). The DSBs can be repaired by non-homologous end joining (NHEJ) and homology-directed repair (HDR) pathways [9]. The dominant process is the NHEJ pathway, in which the two ends simply rejoin [10], accompanying small insertions and/or deletions (indels) at the break point. Imprecise

Jun Li and Xiangbing Meng contributed equally to this work

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NHEJ repair has been exploited to create targeted gene knockouts in multiple cell types and organisms [11]. HDR is a high-fidelity process, with which a genomic sequence can be altered in a predefined way by providing a homologous DNA template [12, 13].

Most eukaryotic open reading frames (ORFs) contain untranslated introns, which are removed during splicing of precursor-mRNAs (pre-mRNAs) [14]. The intron-defining splicing signals are conserved, including GU and AG dinucleotides constituting the 5′ and 3′ splice sites, respectively, and the branch point sequence [15]. Minor alterations of an intronic sequence outside the signal sequences may not affect the alternative splicing of the target gene, and transcription is not greatly affected.

Point mutations and gene replacements are of great value for functional genomics studies in plants and may help create agronomically valuable traits. However, generating precise genome modifications by HDR remains a serious challenge in most plants [16]. Since NHEJ is the dominant DNA repair process and Cas9 usually creates 1-bp deletion or insertion right upstream of the DSB, at the fourth base from the protospacer-adjacent motif (PAM) site [17], and small changes in introns are tolerated, we chose to target adjacent introns with Cas9 to generate gene replacement events in rice [18]. The efficiency of the gene replacement mainly depends on two factors: the efficiency of DSB induction and the amount of the available donor fragment. In this approach, pHUN411 vector containing a pair of sgRNAs targeting adjacent introns of an exon with high efficiency and a donor vector containing a donor fragment with the sgRNA target sequences at either end are introduced into rice callus cells by bombardment. Upon expression of Cas9 and the sgRNAs in the plant cells, simultaneous cleavage of the sgRNA sites in the genomic sequence and the donor plasmid would produce four DSBs (DSB1 and DSB2 in the donor plasmid releasing the donor sequence, and DSB3 and DSB4 in the endogenous locus), and appropriate end joining leads to insertion of the donor sequence in place of the corresponding segment of the host gene, generating the desired gene replacement event (Fig. 1). Unlike the HDR pathway, this replacement method does not need additional homology arms on the donor fragment and uses the dominant NHEJ DNA repair machinery. It is error-prone and often results in indels at the target sites, as well as deletions of genomic fragments, insertions of donor fragment, inversions of the genomic fragment, and inversions of the inserted donor fragment, in addition to the desired replacement (Fig. 1). Although indels are often generated in the targeted introns, minor alterations are tolerated, provided that the splicing signal is not affected, and transcription is not greatly influenced. A rice callus with the site-specific gene replacement is regenerated into a plantlet carrying the gene replacement, and the latter is inherited faithfully (Fig. 2). Following transcription and splicing, new protein molecules

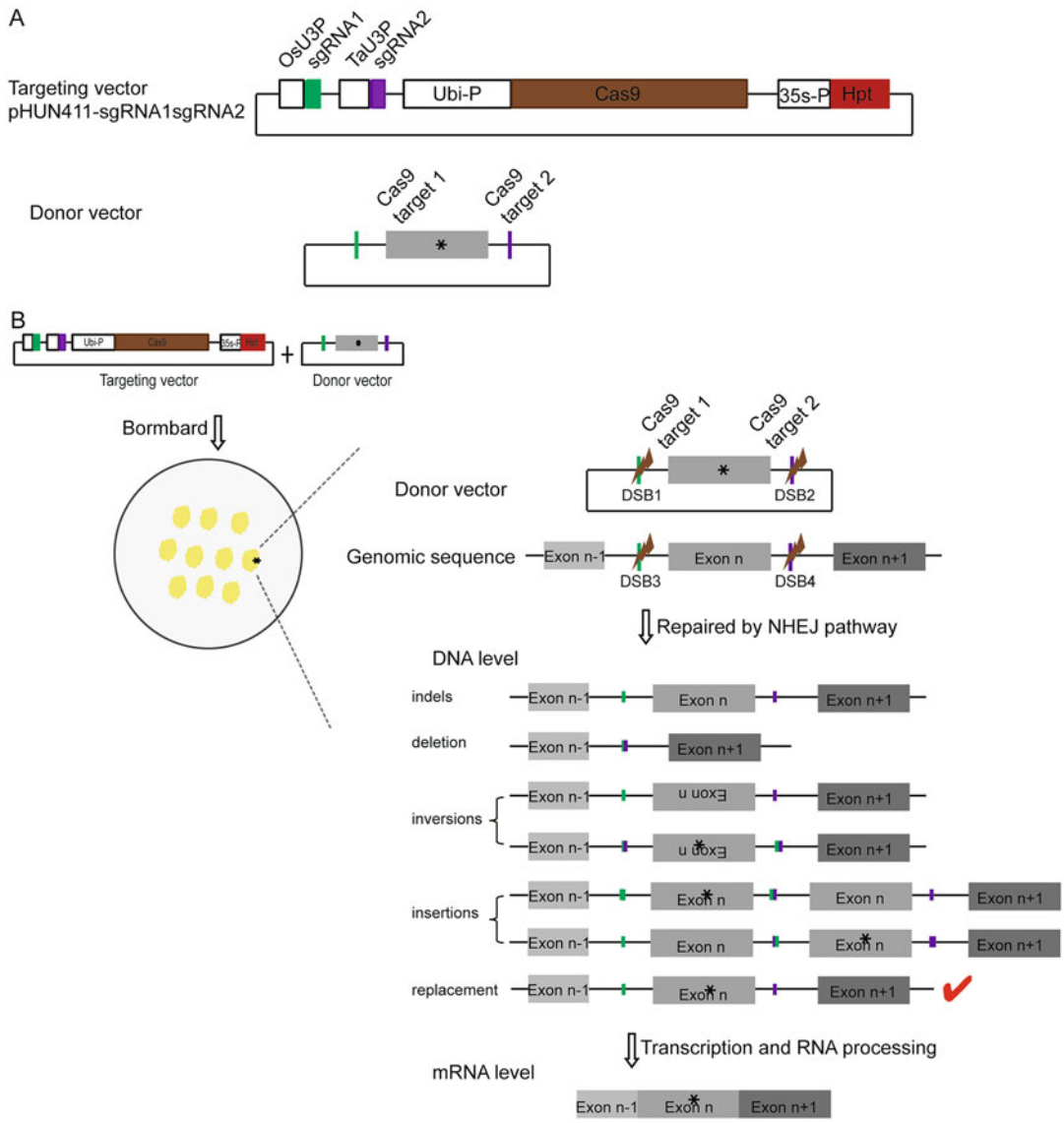

Fig. 1 Outline of intron-targeted site-specific gene replacement. (**a**) Schematic of the targeting vector and donor vector. The targeting vector contains a Cas9 cassette driven by a constitutive ubiquitin promoter, an Hpt cassette driven by a constitutive 35S promoter, and two sgRNA cassettes, driven by the OsU3 promoter and TaU3 promoter, respectively. The donor vector contains the replacement fragment and two sgRNAs including the PAM at the 5′ and 3′ ends of the fragment. The "*" indicates the predesigned mutation. Alternatively, the exon may be replaced by a predesigned fragment flanked by the intron sequences required by splicing. The two target sites in the donor vector are marked by green and purple lines, respectively. (**b**) Schematic of the intron-targeted gene replacement procedure via the NHEJ pathway. The targeting vector and donor vector are co-delivered into rice callus cells. Upon expression of Cas9 and sgRNAs, DSB1 and DSB2 are created in the donor plasmid, releasing the donor sequence, and DSB3 and DSB4 are created in the endogenous locus. The simultaneous cleavage of the two sgRNAs could generate indels in the target sites, genomic fragment deletions, genomic fragment inversions, donor fragment inversions, donor fragment insertions, and gene replacement via the NHEJ pathway. Indels in the introns are indicated by short green and purple lines. The red tick indicates the gene replacement event. After transcription and RNA processing, the mRNA contains the predefined sequence alteration

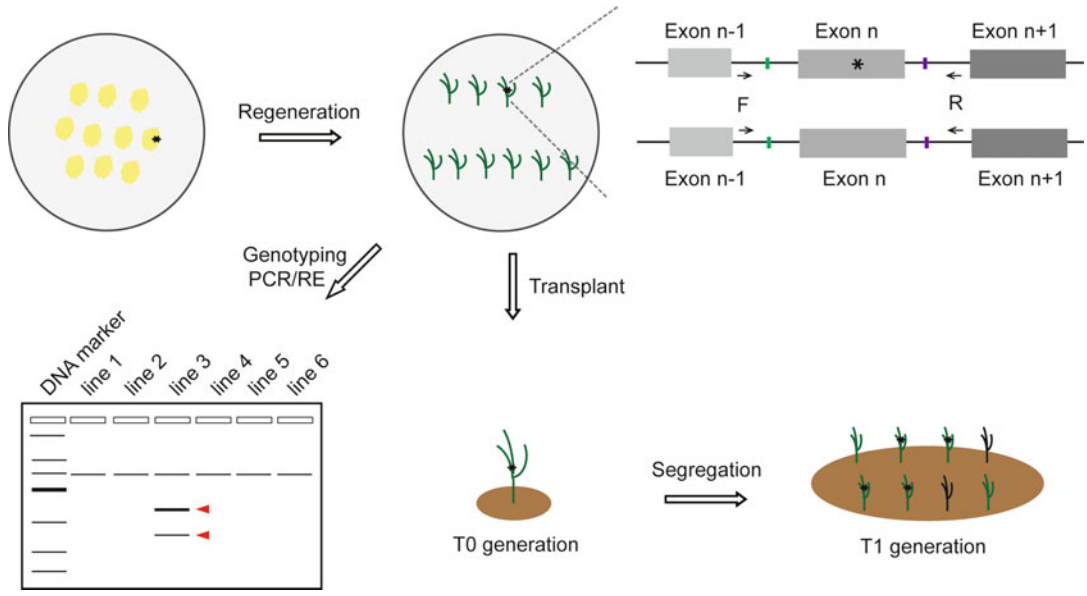

Fig. 2 Identification of gene replacement events. Transgenic rice plantlets are regenerated from hygromycin-resistant calli. The "*" on the third plantlet indicates that the desired gene replacement event has occurred on one of the two homologous chromosomes. The genotyping primers F1 and R1 for detecting gene replacements are outside the two target sites. Replacement events are identified in the regenerated plants by PCR/RE assays. Gene replacement results in two cleaved bands (indicated by red arrowheads) in an agarose gel because the donor fragment contains the designed restriction site. Plantlets with gene replacements are transferred to soil to produce progeny seeds. Homozygous T1 mutants are identified by PCR/RE assays

resulting from the gene replacement will be made in such edited plants. Here, we describe a protocol for obtaining intron-targeted site-specific gene replacements in plants via the NHEJ pathway using the CRISPR-Cas9 system (Fig. 3), replacing a fragment at the desired locus to create heritable amino acid substitutions.

2 Materials

2.1 Plasmids

Plasmids pHUN411, pJIT163-Ubi-GFP, and pCBC-MT1T2 are available directly from the authors upon request [19, 20].

2.2 Organisms

1. *Escherichia coli* (e.g., DH5α) competent cells for all cloning steps (*see* **Note 1**).
2. Rice cultivar: Nipponbare.

2.3 Reagents

1. Forward and reverse oligonucleotides for sgRNA cloning into the targeting vector.
2. Restriction enzymes: *Bsa*I and *Pvu*I.
3. Easytaq DNA polymerase for *E. coli* colony PCR and for identifying plants with gene replacement.

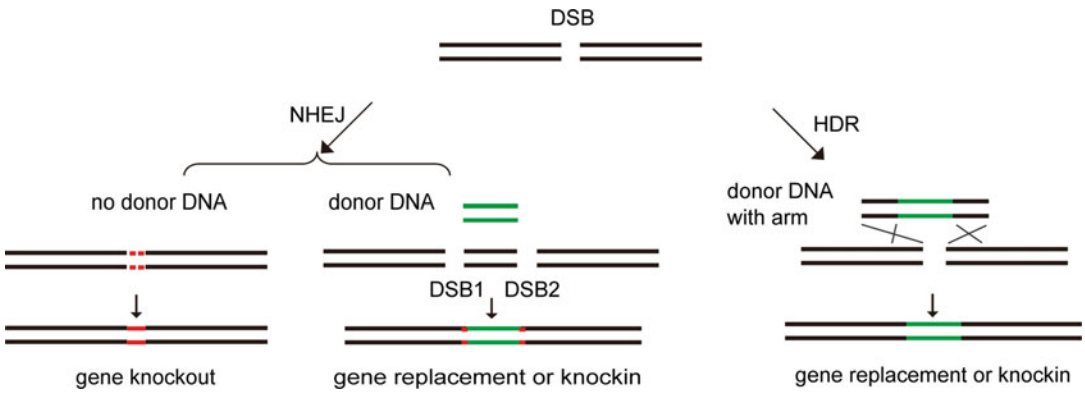

Fig. 3 DSB repair induces editing of targeted gene. DSBs activate the NHEJ and HDR DNA repair pathways. The NHEJ pathway is dominant and imprecise, often introducing small indels at breaks. When there is no donor DNA, it generates knockout mutations. However, in the presence of a donor DNA, it can result in gene replacements and knock-in mutations. Alternatively, when the donor DNA has homologous arms spanning the DSB, the HDR pathway may generate gene replacements and knock-in mutations

4. FastPfu DNA polymerase for generating the donor sequences.
5. pEASY-Blunt cloning vector for donor construction.
6. T4 DNA ligase for conventional cloning steps.
7. Wizard Plus midiprep kit for extraction of plasmids used in protoplast transformation and biolistic transformation.
8. LB medium (for *E. coli*): 10 g/L peptone, 5 g/L yeast extract, and 10 g/L NaCl. Solid medium: 15 g/L agar.
9. 1/2 MS solid medium: 2.215 g/L Murashige Skoog (MS) salt, 15 g/L sucrose (pH 5.8), and 3 g/L phytage.
10. Enzyme solution (for dissolving the cell wall of rice): 20 mM/L MES (pH 5.7), 1.5% (wt/vol) cellulase R10, 0.75% (wt/vol) macerozyme R10, 0.6 M/L mannitol, 10 mM/L KCl, 10 mM/L $CaCl_2$, and 0.1% (wt/vol) BSA (*see* **Note 2**).
11. W5 solution (for washing protoplasts): 2 mM/L MES (pH 5.7), 154 mM/L NaCl, 125 mM/L $CaCl_2$, and 5 mM/L KCl.
12. MMG solution (for resuspending protoplasts): 4 mM/L MES (pH 5.7), 0.4 M/L mannitol, and 15 mM/L $MgCl_2$ (*see* **Note 3**).
13. PEG solution (for transforming protoplasts): 40% (wt/vol) PEG4000, 0.2 M/L mannitol, and 100 mM/L $CaCl_2$ (*see* **Note 3**).
14. WI solution (for incubating protoplasts): 4 mM/L MES (pH 5.7), 0.5 M/L mannitol, and 20 mM/L KCl.
15. Osmotic medium: 4.43 g/L MS salt, 5 mg/L 2,4-D, 90 g/L mannitol, 30 g/L sucrose (pH 6.0), and 3.5 g/L phytagel.

16. Recovery medium: 4.1 g/L N6B5 salt, 2 mg/L 2,4-D, 0.5 g/L glutamine, 0.1 g/L inositol, 2.8 g/L proline, 0.5 g/L N-Z-Amine A, 30 g/L sucrose (pH 5.8), and 4 g/L phytagel.
17. Selection medium: 4.1 g/L N6B5 salt, 2 mg/L 2,4-D, 0.5 g/L glutamine, 0.1 g/L inositol, 2.8 g/L proline, 0.5 g/L N-Z-amine a, 30 g/L sucrose (pH 5.72), and 4 g/L phytagel. After autoclave, cool and add 1 ml of 50 mg/mL hygromycin.
18. Regeneration medium: 4.1 g/L N6B5 salt, 2 mg/L 6-BA, 0.2 mg/L kinetin, 0.1 g/L inositol, 20 g/L sorbitol, 0.5 g/L N-Z-Amine A, 30 g/L sucrose (pH 5.72), and 4.6 g/L phytagel. After autoclave, cool and add 1 mL of 50 mg/mL hygromycin.
19. RNA extraction and *q*RT-PCR: TRIzol reagent, RNase-free DNase I, oligo (dT) primer, AMV reverse transcriptase, and SYBR Premix Ex Taq.

3 Methods

3.1 Construction of Cas9 and sgRNA Expression Vectors

1. Identify potential Cas9 target sites in introns adjacent to the location of the target fragment (*see* **Note 4**).
2. Predict the specificity of the potential target sites using the online tool Cas-OFFinder [21], and select sgRNAs in introns with high specificity (*see* **Note 5**).
3. Synthesize oligonucleotides (oligos) for the target sites (*see* **Note 6**).
4. Anneal the forward and reverse oligonucleotides and clone them into *Bsa*I-digested pHUN411 vector using T4 DNA ligase (*see* **Note 7**). Transform the ligation products into DH5α bacteria and plate the cells on kanamycin-containing LB plates.
5. Perform colony PCR using Easytaq DNA polymerase to identify positive colonies using OsU3F (5′-CATCCAGGTCACCAAGTTCTAG-3′) and target-specific reverse oligos, which generate a 450 bp band. Confirm the positive colonies by sequencing using OsU3F.
6. Extract plasmid pJIT163-Ubi-GFP and plasmids (targeting vector containing single sgRNAs) from 100 mL cultures of positive clones using a Wizard Plus midiprep kit (*see* **Note 8**).
7. Rice protoplasts isolation and transformation. 1/2 MS solid medium is used to grow Nipponbare seeds. The seedlings of 14 days are used to dissolve the cell wall using enzyme solution, release and wash the protoplasts using W5 solution, and then resuspend the protoplasts in MMG solution. Use the PEG-mediated method to transform the plasmids into rice

protoplasts, and then incubate the transformed protoplasts in WI solution for DNA extraction.

8. Validate and assess the targeting vectors for activity at the target sites and likely off-target sites by transient expression in protoplasts. Two sgRNAs with the highest activity and specificity in the adjacent introns are chosen (*see* **Note 9**). Integrate the paired sgRNAs into *Bsa*I-digested pHUN411 vector, to yield the final targeting vector pHUN411-sgRNA1sgRNA2 (*see* **Note 10**) (Fig. 1).
9. Extract this targeting vector from 100 mL cultures of a positive clone with a midiprep kit (*see* **Note 8**).

3.2 Construction of Donor Vectors

1. Besides the desired base changes in the exon of the donor sequence, it is necessary to introduce a synonymous mutation, creating a restriction enzyme site (*Pvu*I) (*see* **Note 11**).
2. To produce the replacement sequence, add the two sgRNAs including the PAM to the 5′ and 3′ ends of the donor fragment, respectively (*see* **Note 12**) (Fig. 1). The donor sequence is amplified using FastPfu DNA polymerase.
3. Clone the donor sequence into pEASY-Blunt cloning vector to generate the donor vector (*see* **Note 13**).
4. Perform colony PCR to identify positive colonies using M13F and M13R. Confirm that the donor sequence contains the desired base changes and the two sgRNAs including the PAM by sequencing using M13F and M13R.
5. Extract the plasmid (donor vector) from 100 mL cultures of a positive clone using a midiprep kit (*see* **Note 8**).

3.3 Biolistic Transformation of Rice Calli

1. For stable rice transformation, prepare 1-month-old embryogenic calli (80–100 pieces) of rice cultivar Nipponbare on an osmotic medium before bombardment.
2. Mix the targeting vector (pHUN411-sgRNA1sgRNA2) and donor vector in a 1:1 molar ratio prior to bombardment (*see* **Note 14**).
3. Perform the biolistic transformation using a PDS1000/He particle bombardment system with a target distance of 6.0 cm from the stopping plate at helium pressure 1100 psi. Incubate the bombarded calli in the dark overnight.
4. Transfer the calli to the recovery medium and incubate in the dark for 7 days.
5. Transfer the calli to the selection medium and incubate in the dark for 5–6 weeks.
6. Transfer the hygromycin-resistant calli to the regeneration medium and incubate with a photoperiod of 16 h light and 8 h dark for 4–5 weeks. The protocol for rice regeneration after

biolistic transformation is based on a previously reported protocol [22].

7. After 10–12 weeks selection on 50 mg/L hygromycin, T0 transgenic rice plantlets regenerate from hygromycin-resistant calli (*see* **Note 15**) (Fig. 2).

3.4 Screening of Intron-Mediated Replacements

1. Cut 2 cm segments from individual hygromycin-resistant lines in the culture dishes, and extract genomic DNA using a DNA quick plant system (*see* **Note 16**).
2. Optional: Detect mutations generated by the sgRNAs. The activity of the two sgRNAs can be detected by PCR restriction enzyme digestion (PCR/RE) assays.
3. Perform PCR as depicted in Fig. 2. Sequences containing the two sgRNA sites and gene replacement fragments are amplified from the genomic DNA of the transgenic seedlings. The restriction enzyme site (*Pvu*I) designed in the donor sequence is used to detect gene replacement events. Only the PCR products of gene replacements yield two bands (*see* **Note 17**) (Fig. 2).
4. Transfer the plantlets harboring gene replacements into soil and grow in a greenhouse to produce progeny seeds (Fig. 2).
5. Extract total RNA from these plantlets. Perform RT–PCR to examine the mRNA splicing in the plantlets, and perform quantitative PCR to compare the expression level of the gene in the wild-type and edited plants (*see* **Note 18**).
6. Test T1 lines to see whether the gene replacement events are transmitted to the next generation by sowing a few progeny seeds (~50–100) from each gene replacement line, and confirm segregation patterns by PCR/RE assays.

4 Notes

1. We use competent DH5α bacteria for cloning. However, other comparable competent bacterial strains can also be used.
2. Warm the enzyme solution at 55 °C for 10 min to inactivate DNases and proteases and to enhance enzyme solubility. The enzyme solution should be freshly prepared before use.
3. The MMG and PEG solutions should be freshly prepared before use.
4. When the DSBs created by CRISPR-Cas9 are repaired through the NHEJ pathway, small indels can be created at the break sites. So target sites should be chosen in introns, and they should be far from the 5′ and 3′ splice sites to avoid interference with the splicing machinery. Target sites can be selected

manually by looking for an NGG as a PAM, and for an enzyme restriction site (checked in the website http://nc2.neb.com/NEBcutter2/index.php) at the cutting site. Software-assisted selection is also useful with CRISPR-P [23]. In rice, the NAG PAM works as well as the canonical NGG PAM [24, 25]. To identify the intron sgRNAs with the highest activity and specificity, one should also consider the NAG PAM.

5. Off-target effects are the greatest concern when using the CRISPR-Cas9 system, and these should be minimized by choosing specific sites. PAM-distal mismatches can be tolerated by the system, so choose the sites for which off-target sties contain mismatches in the PAM-proximal region.
6. To identify the highest activity sgRNAs in adjacent introns, choose at least 3 target sites in each intron. The forward oligo should contain the 19 nt upstream of the NGG with GGCG added to the 5′ end. The reverse oligo should contain the reverse complement of the target sequence with AAAC added to the 5′ end.
7. Anneal the forward oligo and reverse oligo for 5 min at 95 °C, then cool to room temperature naturally.
8. The final concentrations of midiprep plasmids should exceed 1 μg μL^{-1} with an *A*260/280 ratio in the range of 1.7–1.9. Other comparable plasmid extraction methods can also be used.
9. The efficiencies of the two sgRNAs have a great influence on the efficiency of gene replacement. You should transiently express targeting vectors containing single sgRNA in rice protoplasts to identify those giving the highest mutation frequencies. After 48 hours, extract genomic DNA to identify mutations via PCR/RE assays. Estimate mutation frequencies from the intensities of the uncleaved band with UVP VisionWorks LS Image Acquisition Analysis Software 7.0 [26]. The potential off-target sites are examined in rice protoplasts by PCR/RE assays. Two sgRNAs with the highest activity and specificity in the adjacent introns are chosen for the gene replacement.
10. Amplify and purify a PCR fragment containing the paired sgRNAs from the vector pCBC-MT1T2 and insert it into *Bsa*I-digested pHUN411 by the Golden Gate method [20]. The two sgRNAs are driven by the rice U3 promoter and wheat U3 promoter, respectively.
11. The designed nucleotide substitutions provide the desired amino acid substitutions, as well as a synonymous mutation to create a restriction enzyme site in the replacement sequence that is used to detect the gene replacement events by PCR/RE assays. The restriction enzyme site created should differ from those already present in the donor fragment.

12. The remaining introns adjacent to the exon should be added to the donor fragment to provide the GU and AG dinucleotide splicing signals required for splicing of the target gene. The sgRNA sites on either side of the donor sequence are recognized by the targeting vector and cleaved, thus releasing the replacement fragment, which can then replace the genomic region between the two sgRNA sites via the NHEJ pathway.
13. The donor fragment includes the exons with the desired nucleotide changes, the rest of adjacent introns, and the two sgRNA target sites. The donor sequence can be obtained by overlap PCR, Gibson assembly, or gene synthesis. The donor vector is an individual vector, and there is no restriction on what types of backbone should be chosen to harbor the donor DNA. The amount of the donor can be increased when transforming rice calli, and this may improve the efficiency of gene replacement.
14. The concentrations of targeting vector and donor vector should each exceed 1 μg μL^{-1}, and they should be separate vectors. When bombarding rice calli, the ratio of donor vector to targeting vector can be increased to improve the availability of the DNA donor template, achieving the desired DNA sequence modification.
15. The targeting vector pHUN411 contains the *hygromycin B phosphotransferase* (*hpt*) expression cassette, conferring the transformed calli with resistance to hygromycin. *HPT* is the most efficient selectable marker for rice transformation [22], and hygromycin should be present in all subsequent tissue culture procedures.
16. Gene replacement is relatively inefficient, so it is better to detect replacement events before transferring plantlets into soil.
17. Since the donor plasmid and the host chromosome contain the same sgRNA target sites, simultaneous cleavage of these sites can result in the fragment between the two sgRNA sites in the donor plasmid replacing the region between the two sgRNA sites in the chromosome via the NHEJ pathway, leaving indels in the sgRNAs in the introns. Consequently, genomic DNA from transgenic plantlets is amplified by PCR and the amplicons are digested with restriction enzymes that recognize the donor sequence. Only the PCR products of gene replacement events can be cut and form two bands in agarose gels. The restriction enzyme site designed in the donor fragment is convenient for detecting gene replacement events in this way. Moreover, indels in the target sites, as well as genomic fragment deletions, genomic fragment inversions, donor fragment inversions, and donor fragment insertions events, are also generated by the two sgRNAs via the NHEJ pathway.

18. Gene replacement is produced by intron targeting via the NHEJ pathway using CRISPR-Cas9 here. Minor modifications are produced in the introns that could generate alternative 5′ or 3′ splicing signals and affect mRNA splicing and the level of expression of the gene. It is therefore necessary to examine mRNA splicing and the expression level of the target gene in the plants with successful gene replacement.

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Chapter 22

Targeted Base Editing with CRISPR-Deaminase in Tomato

Zenpei Shimatani, Tohru Ariizumi, Ushio Fujikura, Akihiko Kondo, Hiroshi Ezura, and Keiji Nishida

Abstract

The Target-AID system, consisting of a complex of cytidine deaminase and deficient CRISPR/Cas9, enables highly specific genomic nucleotide substitutions without the need for template DNA. The Cas9-fused cytidine deaminase is guided by sgRNAs and catalyzes the conversion of cytosine to uracil. The resulting U-G DNA mismatches trigger nucleotide substitutions (C to T or G to A) through DNA replication and repair pathways. Target-AID also retains the benefits of conventional CRISPR/Cas9 including robustness in various organisms, high targeting efficiency, and multiplex simultaneous gene editing. Our research group recently developed plant-optimized Target-AID system and demonstrated targeted base editing in tomato and rice. In this chapter, we introduce methods for Target-AID application in tomato.

Key words Targeted nucleotide substitution, Activation-induced cytidine deaminase (AID), Target-AID, CRISPR/Cas9, Tomato

Abbreviations

AID	Activation-induced cytidine deaminase
AP endonuclease	Apurinic/apyrimidinic endonuclease
bp	Base pair
Cas9	CRISPR-associated protein9
CRISPR	Clustered regularly interspaced short palindromic repeats
dCas9	Deactivated Cas9
nCas9	Nickase-Cas9
NHEJ	Non-homologous end joining
PAM	Protospacer adjacent motif
sgRNA	Single guide RNA

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1 Introduction

The remarkable progress of technologies in genomic research has greatly accelerated plant science. Accumulation of genome sequence information and transcriptional profiles of plant species has led to rapid and detailed identification of genetic factors for elite traits conferring improved palatability, nutrient density, disease resistance, and tolerance for abiotic stresses. Technological innovation of plant breeding is spurring based on such knowledge as well as genome editing-based techniques to alter genetic and epigenetic factors.

Tomato (*Solanum lycopersicum*) is one of the most important commercial horticultural crops grown throughout the world and is a model system, particularly for studies of fresh fruit development in the family of Solanaceae. As tomatoes contain many functional materials and nutrients, improvement of yield and quality is a major breeding objective. However, tomato productivity and quality are generally sensitive to abiotic stresses such as salt, drought, and heat, which are on the rise due to climate change. Therefore, a rapid plant breeding process based on functional genomics is essential for the creation of improved genetic varieties with stress tolerance or other desired traits. Moreover, genome editing technologies utilizing programmable nucleases are promising strategies to accelerate plant breeding and reverse genetics in tomato.

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein9 (CRISPR/Cas9) is a popular genome editing tool due to efficiency, robustness, and applicability to virtually any organism [1]. CRISPR/Cas9 can be applied as a molecular scissor (Fig. 1a), and as a homing device when combined with a nuclease-deficient CRISPR system together with various functional effectors. Homing applications include sgRNA-directed transcriptional modulation, epigenetic control, and visualization of specific loci in chromosomal structures [2].

Activation-induced cytidine deaminase (AID) is an essential factor for diversification of adaptive immune responses in vertebrates by altering endogenous genetic information. AID triggers somatic hypermutation by catalyzing transcription-dependent deamination of cytosine to uracil in DNA. The resulting AID-mediated U-G mismatch in genomic DNA is then a possible cause of nucleotide substitution through DNA replication and repair pathways.

Target-AID is a CRISPR-based targeted nucleotide editing technology consisting of an activation-induced cytidine deaminase (AID) from *Petromyzon marinus* (PmCDA1), fused to nuclease-deficient CRISPR/Cas9 variants (Fig. 1b) [3]. Successful targeted nucleotide substitutions by Target-AID at desired loci have been demonstrated in yeast, mammalian cells, and *Escherichia coli* [3–5].

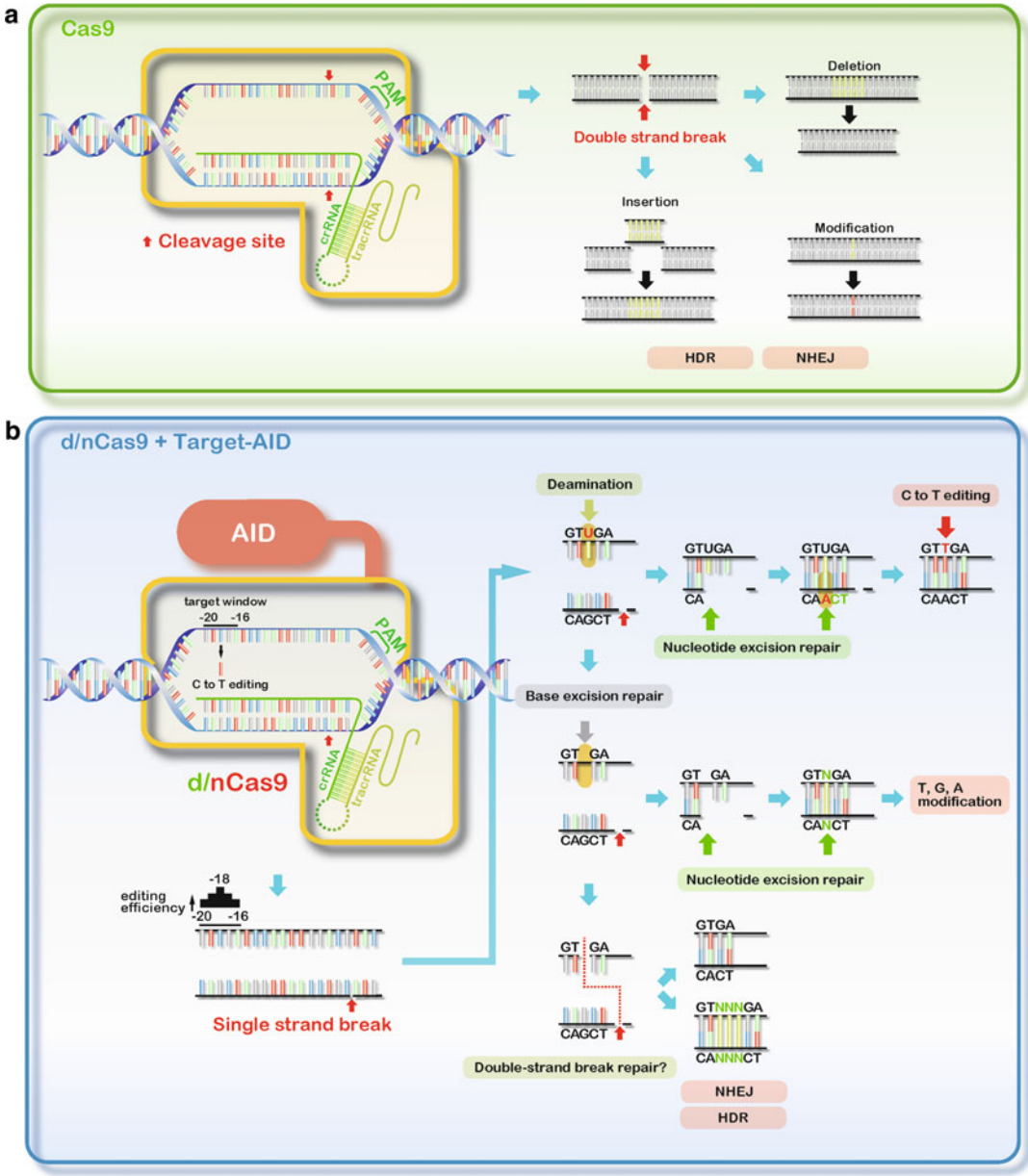

Fig. 1 Schematic illustration for CRISPR-Cas9 and d/nCas9–deaminase fusion. (**a**) Target binding of the Cas9-sgRNA complex induces double strand breaks and leads to homology-directed repair or non-homologous end joining (NHEJ). (**b**) The d/nCas9–sgRNA complex provides the single-stranded part of the noncomplementary DNA strand, which is subject to DNA deamination by the cytidine deaminase. Approximate rates at each base position of mutable cytosine for Target-AID are indicated by bar graphs. Theoretical mutagenesis and repair events which can be induced by deamination are shown. Cytosine deamination generates uracil, which is subjected to base excision repair processed by uracil DNA glycosylase and apurinic/apyrimidinic (AP) endonuclease. Nickase Cas9 (D10A) induces a nick on the complementary strand, which leads to misrepair because uracil can be recognized as thymine as a template for repair polymerase (C to T editing). An abasic site (apurinic/apyrimidinic site) generated by uracil DNA glycosylase provides no reference template for the complementary strand and allows random nucleotide insertion, depending on the preference of the polymerase (T, G, A modification). AP endonuclease incises the abasic site, which may result in DNA double strand break to induce indels via the NHEJ pathway in animals and plants

Plant-optimized Target-AID was also demonstrated to generate missense or nonsense amino acid mutations through targeted base editing in rice and tomato [6]. Here, we describe a procedure to conduct targeted nucleotide substitutions using Target-AID in tomato.

2 Materials

2.1 Construction of Dicot-Optimized Target-AID Vector

1. Plasmids

   pDicAID: a dicot-optimized Target-AID vector (Fig. 2a) [6].
   pChimera: a vector carrying sgRNA expression unit for dicot plants (Fig. 2b) [7].
2. Primers

   The position and direction of the respective primers are shown in Fig. 2b.

   AtU6_F (5′-CTTTGTACAAAAAAGCAGGCG-3′)

   AtU6_R (5′-CAATCACTACTTCGACTCTAG-3′)

   Target_F (5′-CTAGAGTCGAAGTAGTGATTG-(20 nucleotides for spacer sequence)-GTTTTAGAGCTAGAAATAGCAAG-3′)

   gRNA_R (5′-GCCATAGAAAAGTTGGGTG-3′)
3. High-fidelity DNA polymerase [e.g., PrimeSTAR GXL DNA polymerase, Takara Bio].
4. DNA purification kit [e.g., QIAquick PCR purification kit, QIAGEN].

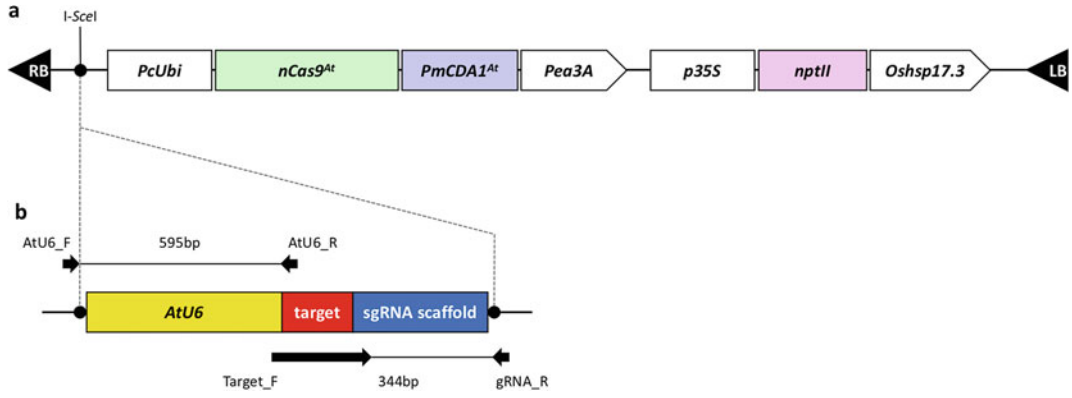

Fig. 2 Diagram for the Target-AID vector. (**a**) pDicAID, a Target-AID vector optimized for dicot plants. Engineered Cas9-PmCDA1 fusion (*nCas9*^{At}- *PmCDA1*^{At}) is under the control of the *PcUbi* promoter, and transcription is terminated by the *Pea3A* terminator from *Pisum sativum*. *p35S*, cauliflower mosaic virus (CaMV) *35S* promoter; *nptII*, *neomycin phosphotransferase II*; *Oshsp17.3*, transcriptional terminator from *Oryza sativa*; LB/RB, left/right border of T-DNA. (**b**) The sgRNA expression unit for dicot plants. *AtU6*, *Arabidopsis U6* promoter

5. Homing endonuclease: I-*Sce*I.
6. Ligation kit [e.g., Ligation high, Toyobo].
7. Alkaline phosphatase [e.g., Alkaline Phosphatase (Shrimp), Takara Bio].
8. Competent *Escherichia coli* cell [e.g., XL10 gold, Agilent].
9. Plasmid extraction kit [e.g., QIAprep Miniprep, QIAGEN].
10. Agarose gel equipment and supplies, DNA ladders.

2.2 Tomato Transformation

1. Tomato cv., i.e., Micro-Tom, Ailsa Craig, Moneymaker.
2. Germination medium: Murashige and Skoog basal medium (MS medium, Sigma-Aldrich) with 1.5% sucrose, 0.3% Gerlite.
3. Infection medium: MS medium with 100 μM Acetosyringone and 10 μM 2-mercaptethanol.
4. Cocultivation medium: MS medium with 3% sucrose, 0.3% Gerlite, 1.5 mg/L zeatin.
5. Selection medium: MS medium with 3% sucrose, 0.3% Gerlite, 1.5 mg/L zeatin, 100 mg/L kanamycin, 375 mg/L Augmentin.
6. Shoot induction medium: MS medium with 3% sucrose, 0.3% Gerlite, 1 mg/L zeatin, 100 mg/L kanamycin, 375 mg/L Augmentin.
7. Root induction medium: MS medium with 1.5% sucrose, 0.3% Gerlite, 50 mg/L kanamycin, 375 mg/L Augmentin.
8. *Agrobacterium* preculture medium: LB medium (Thermo-Fisher Scientific), 50 mg/L kanamycin.
9. *Agrobacterium tumefaciens*; strain GV2260.
10. Plant DNA isolation kit [e.g., DNeasy plant mini kit, QIAGEN].

2.3 Analysis of Tomato Plants

1. DNA polymerase [e.g., Tks Gflex DNA polymerase, Takara Bio].
2. Gel extraction kit [e.g., QIAquick Gel Extraction kit, QIAGEN].
3. PCR Cloning Kits [e.g., Zero Blunt TOPO PCR cloning kit, ThermoFisher Scientific].
4. Competent *E. coli* cells [e.g., XL10 gold, Agilent].
5. Plasmid extraction kit [e.g., QIAprep Miniprep, QIAGEN].
6. Next Multiplex Oligos for Illumina sequencing (New England Biolabs).

3 Methods

3.1 Construction of Target-AID Vectors

1. Choose target genes for study. To design the target sequences, computational prediction algorithms such as CRISPR-P program (crispr.hzau.edu.cn/) are used to avoid off-target effects (unexpected DNA modification outside the region specified by the sgRNA sequence). *See* **Notes 1** and **2** for detailed descriptions.
2. Perform overlap extension PCR to synthesize the transcription templates of the sgRNA for the target sequence. The sgRNA comprises a scaffold sequence essential for Cas-binding and a 20 nucleotide spacer sequence that specifies the genomic target to be modified. To construct the sgRNA expression unit for tomato, a spacer sequence is integrated between the *AtU6* promoter and chimeric gRNA scaffold. In the first round PCR, two DNA fragments are amplified from pChimera using high-fidelity DNA polymerase with appropriate primes (Fig. 2b). One fragment is the *AtU6* promoter region that will be amplified using the primers AtU6_F and R. The other is a fusion of the spacer and scaffold sequences amplified using a pair of primers including the target-specific nucleotide sequence (Target-F) and gRNA_R. Check the PCR products by agarose gel electrophoresis. Purify the DNA fragments using DNA purification kit.
3. Perform the second round PCR to connect the *AtU6* promoter and spacer-scaffold sequence. Each DNA fragment amplified in the first round PCR is used as template for PCR using the primers AtU6_F and gRNA_R. Check the PCR products by agarose gel electrophoresis. Purify the DNA fragment using a DNA purification kit.
4. Digest the pDicAID and the fragment of sgRNA expression unit using I-*Sce*I for 2–4 h at 37 °C. Purify the digestion products with a DNA purification kit.
5. Dephosphorylate the 5′ ends of the restricted fragment of pDicAID vector using alkaline phosphatase for 30 min at 37 °C. Then purify with DNA purification kit.
6. Ligate the digested sgRNA expression unit from **step 4** and linearized pDicAID from **step 5** using a ligation kit.
7. Transform *E. coli* competent cells with the ligation product. Plate transformed cells on solidified LB medium with spectinomycin (at 100 mg/L). Incubate the plates overnight at 37 °C.
8. Pick several spectinomycin-resistant clones and culture overnight in liquid LB media with spectinomycin (at 100 mg/L), then extract the plasmids using a plasmid extraction kit.

9. Confirm the inserted sgRNA expression cassette in each Target-AID vectors by Sanger sequencing with primers AtU6_F and gRNA_R. The restriction enzyme digestion analysis can be combined in this process if necessary.
10. Introduce the Target-AID vectors to *A. tumefaciens* strain GV2260 by electroporation.

3.2 Introduction of Target-AID Vector into Tomato

Details have been reported previously by Sun et al., 2006 [8]. All plant manipulations should be performed under aseptic conditions.

1. Sterilize tomato seeds by imbibing with 10% bleach solution for 15 min and washing with distilled water five times. Incubate the sterilized seeds with distilled water for 3 days, and leave on a rocker or rotator so that seeds are gently agitated.
2. Place the sterilized tomato seeds on germination medium for 4 days at 25 °C under a 16 h light/8 h dark cycle to stimulate germination until cotyledon expands. Avoid using tomato shoots that already start to produce main leaflet in addition to fully expanding cotyledon. Use of such old cotyledon may reduce transformation efficiency (based on our experiences).
3. Incubate *Agrobacterium* carrying Target-AID vector plasmid for 20–24 h at 28 °C in LB liquid medium supplemented with 100 mg/L spectinomycin and 100 mg/L ampicillin if *Agrobacterium* strain GV2260 is used.
4. Centrifuge the liquid medium to collect *Agrobacterium* as pellet, then resuspend with 50 mL of infection medium.
5. Prepare the explants by sectioning cotyledons from **step 2** into two halves at the mid-vein region. Size of each explants is about 10–15 mm^2 (3–5 mm pieces).
6. Submerge the explants in the *Agrobacterium* suspension for 15 min and blot on a sterilized paper towel to remove infection medium.
7. Carefully place the explants on the cocultivation medium with the adaxial surface of the leaflets in contact with the medium and incubate at 25 °C in dark for up to 3–4 days. Avoid placing the explants with the abaxial surface of the leaflets on the medium. Abaxial side contains more stomata than the other side and blocking respiration may reduce transformation efficiency.
8. Transfer the explants onto the shoot induction medium for 10 days. Transfer the explants onto the freshly prepared shoot induction medium and continue subculture for another 10 days. Callus formation is usually observed at this time point. If so, go to the next step. If the callus has not yet appeared at this time point, continue subculturing the explants on the shoot induction medium until callus formation occurs,

while replacing the media every 10 days. Take note that longer subculture times will increase the risk of production of tetraploid transgenic plants that show complete infertility.

9. Transfer the explants with callus formation to shoot induction medium and continue subculture until shoot emergence is observed. Replace the shoot induction medium every 2 weeks. When shoots with the adventitious bud develop from the calli, cut them off with a sterile razor blade from the callus-forming explants and transfer to the root induction medium. Place the remaining explants on newly prepared shoot induction medium and continue subculture until another shoot formation is observed. When new shoots emerged, cut them off for transferring onto root induction medium.
10. Wait until regenerated shoots develop leaves and roots with laterally developing roots. Select these plants for the next step.
11. Examine the rooted plants for ploidy level by ploidy analyzer or flow cytometer and select only diploid transformants, which are regarded as putative transgenic plants available for molecular analysis. Avoid using transgenic plants that do show altered ploidy levels.

3.3 Analysis of Transgenic Tomato Plants

1. Extract genomic DNA of the regenerated T_0 plants using plant DNA isolation kit according to the manufacturer's instructions.
2. Perform PCR with the appropriate primers for the target loci.
3. Check the PCR amplicons by gel electrophoresis.
4. Subclone the amplified DNA fragments into pGEM-T Easy vectors according to manufacturer's instructions and transform *E. coli*, and streaked onto LB agar plates containing 100 mg/L ampicillin.
5. Pick several colonies and perform colony PCR using appropriate primers.
6. Purify the amplified DNA fragment using QIAquick DNA purification kit according to manufacturer's instructions.
7. Analyze the DNA sequence using 3130XL Genetic Analyzer to screen for the T_0 plants carrying the desired mutation in targeted loci (*see* **Note 3**).
8. Select the T_0 plants with desired mutations for the ones carrying a single copy of Target-AID vector by Southern blot analysis (*see* **Note 4**).
9. Propagate the progenies of each T_0 plant via self-pollination.
10. Sterilize the obtained T_1 seeds, place onto germinating medium, and incubate at 25 °C under a 16 h light/8 h dark cycle.

11. Extract genomic DNA of the T_1 plants, and analyze DNA sequences to confirm the inheritance of desired mutations.

    <Optional>

12. Analyze segregation of the *nptII* gene in T_1 and T_2 plants to screen for selectable marker-free (SMF) plants.

3.4 Next-Generation Sequencing (NGS) Analysis (Optional)

Minor mutations and comprehensive mutational spectrum are analyzed by NGS.

1. Extract genomic DNA using plant DNA isolation kit according to manufacturer's instructions.
2. Perform first round PCR with a pair of primers flanking a region of approximately 500 bp with the target site at the center.
3. Check the first PCR products by agarose gel electrophoresis. Purify the amplified DNA fragments using a DNA purification kit according to manufacturer's instructions.
4. Using the first PCR product as a template, perform a second nested PCR with primers containing an adaptor sequence (Fw, 5′-TCTTTCCCTACACCGACGCTCTTCCGATCT-(forward target specific sequence)-3′; Rev., 5-′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-(reverse target specific sequence)-3′), to amplify the adaptor added-amplicon (~300 bp) fragment including the target site at the center.
5. Label the fragments with index sequences by using NEBNext Multiplex Oligos for Illumina sequencing according to the manufacturer's instructions.
6. Using the MiSeq system, perform deep-sequencing analysis to obtain paired 300 bp length and >100,000 reads per sample on average, according to the manufacturer's instructions.
7. Analyze mutation frequency and profile for each sample based on NGS data.

4 Notes

1. Design of the appropriate target sequences is the most important process of this technique. Because Target-AID is based on the CRISPR/Cas9 system of *Streptococcus pyogenes*, DNA target sequences should be designed according to the following recommendations. The 3′ ends of DNA target sequences should be nearby the protospacer adjacent motif (PAM) sequence (5′-NGG-3′). The 20 nucleotides upstream of the PAM sequence can be the target sequence (Fig. 1b). Any PAM sequence and/or polyT tracks should not be included

in the target sequence. In addition, properties of Target-AID should be considered. The mutational spectrum of Target-AID is highly specific, with point mutations dominantly induced at genomic cytosine in a window from position −20 to −16 upstream of the PAM sequence on the strand noncomplementary to sgRNA (Fig. 1b). The targeted base substitution of cytosines in coding or noncoding strands can generate amino-acid changing missense mutations as well as nonsense mutations.

2. The efficiency of genome editing by Target-AID likely varies depending on target DNA sequences for unknown reasons. When making Target-AID vectors, it is recommended to design multiple vectors with different target sequences for the gene of interest. Alternatively, multiple target sequences can be aligned as multiplex sgRNAs for co-expression. Using many different sgRNA sequences will increase the chance to successfully edit the genes of interest.
3. When using a constitutive promoter (like cauliflower mosaic virus (CaMV) 35S promoter) for inducing transcripts of Cas9 or sgRNA, it is difficult to precisely recognize the timing or sites of mutations induced by CRISPR/Cas9 and related systems. It is possible that first branches do not contain mutations, whereas secondary or later branches contain mutations. Therefore, transgenic plants are most likely in a chimeric state for mutations. It is recommended to keep T_0 transgenic plants for longer periods to search for new branches or axillary (lateral) buds in which mutations are induced. Further, because of unpredictable mutagenesis, mutation frequency varies depending on tissue type and plant age. When examining mutation frequency by NGS or Sanger sequencing, it is better to extract DNA from many plant organs/tissues under different developmental stages to increase the chance of identifying transgenic plants that contain mutations.
4. Careful analyses should be done with DNA-edited transgenic plants without T-DNA integration, since CRISPR/Cas9 or related systems may also induce off-target mutations. T-DNA-free plants can be obtained through self-pollination of transgenic plants or from F_1 and following offspring plants derived from a cross between transgenic plants with WT. Therefore, selection of the transgenic plants with a single copy of T-DNA integration simplifies the procedure for obtaining T-DNA-free plants due to the monogenic inheriting pattern (with or without T-DNA = 3:1). Multicopy T-DNA insertions make it more difficult to produce offspring without T-DNA integration. For example, if the T_0 plant carried two or three copies of T-DNA, the segregation ratio of T-DNA-free plants in T_1 generation are expected to be 15:1 or 63:1,

respectively. Therefore, we recommend performing Southern blot analysis with primary transgenic plants (T_0 generations) to select those with single T-DNA copy insertions.

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Part VIII

Non-Agrobacterium Based CRISPR Delivery Systems

Chapter 23

Virus-Mediated Genome Editing in Plants Using the CRISPR/Cas9 System

Ahmed Mahas, Zahir Ali, Manal Tashkandi, and Magdy M. Mahfouz

Abstract

Targeted modification of plant genomes is a powerful strategy for investigating and engineering cellular systems, paving the way for the discovery and development of important, novel agricultural traits. Cas9, an RNA-guided DNA endonuclease from the type II adaptive immune CRISPR system of the prokaryote *Streptococcus pyogenes*, has gained widespread popularity as a genome-editing tool for use in a wide array of cells and organisms, including model and crop plants. Effective genome engineering requires the delivery of the Cas9 protein and guide RNAs into target cells. However, *in planta* genome modification faces many hurdles, including the difficulty in efficiently delivering genome engineering reagents to the desired tissues. We recently developed a *Tobacco rattle virus* (TRV)-mediated genome engineering system for *Nicotiana benthamiana*. Using this platform, genome engineering reagents can be delivered into all plant parts in a simple, efficient manner, facilitating the recovery of progeny plants with the desired genomic modifications, thus bypassing the need for transformation and tissue culture. This platform expands the utility of the CRISPR/Cas9 system for *in planta*, targeted genome modification. Here, we provide a detailed protocol explaining the methodologies used to develop and implement TRV-mediated genome engineering in *N. benthamiana*. The protocol described here can be extended to any other plant species susceptible to systemic infection by TRV. However, this approach is not limited to vectors derived from TRV, as other RNA viruses could be used to develop similar delivery platforms.

Key words CRISPR/Cas9, TRV, *Nicotiana benthamiana*, Genome editing, Targeted modification, Genome engineering, RNA viruses

1 Introduction

Precise genetic manipulation of living cells via the introduction of controlled, targeted alterations in specific genomic sequences is an important goal with implications for fields ranging from functional biology to biotechnology and medicine [1, 2]. In plants, targeted genome editing has enormous potential for facilitating the analysis of gene function and uncovering and developing novel traits for crop improvement and sustainable agriculture [3]. Precise genome

Ahmed Mahas and Zahir Ali contributed equally to this work

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modification relies on the induction of DNA double-strand breaks (DSBs) at a targeted genomic locus and the subsequent repair mechanism, which is mediated by the two major repair pathways, the error-prone non-homologous end joining (NHEJ) mechanism and the precise homology-directed repair (HDR) mechanism, both of which can be harnessed to achieve the desired genome engineering outcome [4]. The use of various programmable, site-specific nucleases capable of precisely generating DNA DSBs has accelerated targeted genome engineering in a wide range of cell types and organisms, including plants [5].

The recent development of the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats [CRISPR]/CRISPR-associated endonuclease 9 [Cas9]) system as a genome-editing platform has dramatically simplified the field of genome engineering [1, 6]. The CRISPR/Cas9 system, which is based on the adaptive immune system of the prokaryote *Streptococcus pyogenes*, is composed of two components: the RNA-guided DNA endonuclease Cas9 and an engineered single guide RNA (gRNA) capable of guiding the Cas9 endonuclease to the genomic sequence of interest, which is complementary to the user-defined 20-nucleotide targeting or "spacer" sequence within the gRNA. In addition to the spacer sequence, which confers specificity to Cas9, target recognition and cleavage require the presence of a DNA protospacer-adjacent motif (PAM) immediately adjacent to the 3′ end of the 20-nt target sequence [7]. The simplicity and robustness of this system have led to its extensive use for genome engineering and efficient genetic manipulation across a wide array of plants, including model plants such as Arabidopsis [8–10] and *Nicotiana benthamiana* [9, 11] and crop plants such as rice [12, 13], wheat [14, 15], maize [16, 17], tomato [18], and sweet orange [19]. Furthermore, other CRISPR/Cas variants have been developed by engineering of catalytically inactivated Cas variants (nuclease-deficient or nuclease-deactivated (dCas) in combination with various nucleases, transcriptional repressors, activators, or epigenetic modifiers, resulting in enhanced targeting efficiency and specificity and enabling sequence-specific regulation of gene expression and chromatin state [20–22]. In addition, the CRISPR/Cas9 system has been successfully harnessed to engineer plants with increased resistance to a wide range of plant pathogenic viruses [23–30], highlighting its potential to fundamentally transform agriculture.

To achieve effective CRISPR/Cas9-mediated targeted genome modifications for crop trait discovery and improvement, Cas9 and its cognate gRNA must be efficiently expressed in the targeted cells, and the genome modification events must be heritable, as they should occur in the germline cells and allow for subsequent recovery of progeny with the desired DNA modification. Therefore, the efficient delivery of genome engineering reagents into plant cells is crucial for the effective use of this technology. *In planta* delivery of

these editing components for heritable modification is often accomplished by generating transgenic lines that stably express Cas9 and gRNAs. This goal can be achieved by stably integrating Cas9 and gRNA cassettes into the plant genome via *Agrobacterium*-mediated transformation or using biolistic-based methods, which should result in the expression of the CRISPR/Cas9 components in all cells of the transgenic plant, including germline cells, thus allowing the targeted modifications to be transmitted to subsequent generations. While such delivery approaches can result in the efficient expression of genome engineering reagents and heritable genome modifications, the production of transgenic lines is expensive and time consuming, as tissue culture and repeated transformation are needed to produce every new, targeted modification. In addition, the use of these genetically modified plants can raise public concerns, as well as regulatory hurdles.

Autonomously replicating DNA and RNA virus-based vectors offer an alternative means for efficiently delivering genome engineering reagents into plant cells [31]. The advantage of RNA virus-based vectors versus their DNA viruses-based counterparts is that they do not integrate into the plant genome, thus avoiding unintended genome integration. Therefore, plants modified using RNA viruses are considered to be transgene-free edited plants. One such virus-based vector is *Tobacco rattle virus* (TRV), which is widely used for efficient virus-induced gene silencing (VIGS) in functional genomics studies in diverse plant species [32, 33]. TRV viruses have bipartite genomes comprising two positive-sense ssRNAs: TRV1 (RNA1) and TRV2 (RNA2). TRV1 is essential for virus replication and movement, and TRV2 harbors genes encoding nonstructural proteins that can be replaced by multiple cloning sites, into which different exogenous fragments of the target gene to be silenced can be inserted [34]. When the recombinant TRV vector is introduced into plant cells via *Agrobacterium*-mediated infiltration, the virus expression system mediates the synthesis of the recombinant viral RNA inside the infected plant cells. These initially infected cells presumably serve as a source for further replication of viral RNAs and their systemic infection and spread into a variety of tissues and cells, such as developing and meristematic tissues, including germline cells [35, 36]. The small size of the TRV genome facilitates cloning, multiplexing, and agroinfection, pointing to its great potential for use as a vector for delivering genome engineering reagents.

We recently developed TRV as a vehicle for systemic delivery of gRNAs for targeted genome modification in *N. benthamiana* plants overexpressing *Cas9* [37, 38]. To construct this TRV-mediated genome editing system, we generated *Cas9*-overexpressing (*Cas9*-OE) *N. benthamiana* transgenic lines. We then generated a TRV RNA2 genome-derived vector for systemic gRNA delivery. In the TRV RNA2 vector, the gRNA was expressed under the control of

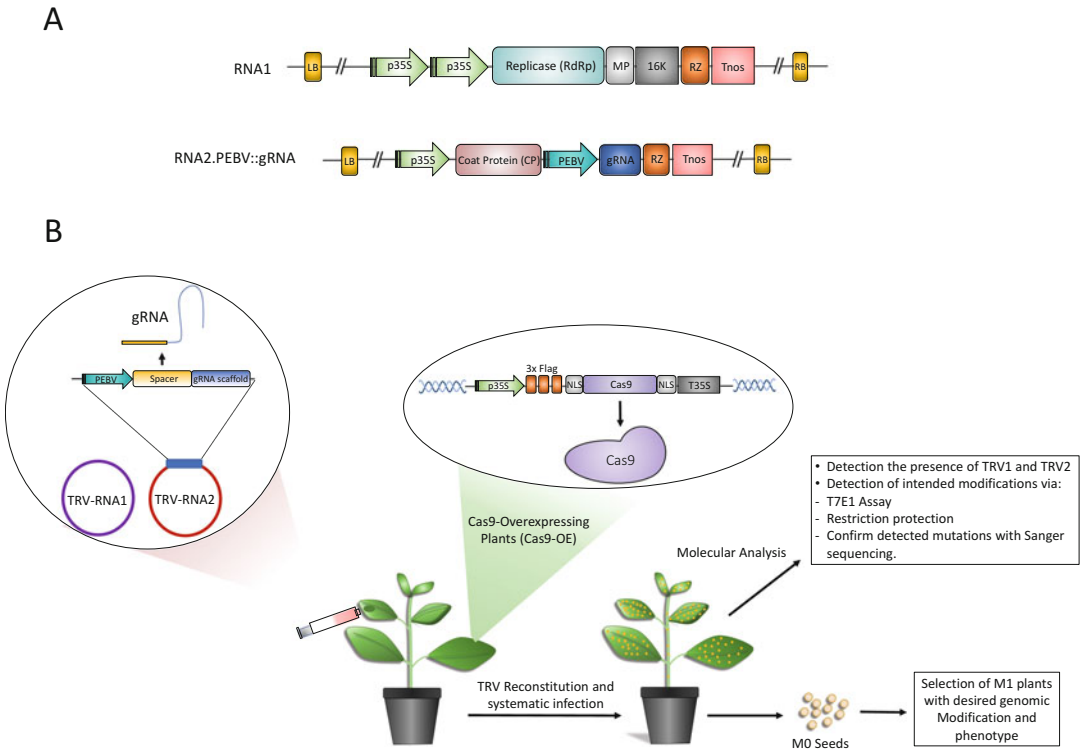

Fig. 1 TRV-mediated genome editing in *N. benthamiana*. (**a**) Schematic representation of the genome organization of TRV RNA1 and RNA2. RNA1 in the *Agrobacterium* binary vector system: LB (left border), 2Xp35S (2X CaMV 35S promoter), RdRNAP (134/194 kDa RNA-dependent RNA polymerase, RdRp), MP (movement protein), 16k (cysteine rich protein), Rz (self-cleaving ribozyme), Tnos (nopaline synthase terminator), RB (right border). RNA2 in the *Agrobacterium* binary vector system: LB, p35S, CP (coat protein), Rz, Tnos, and RB. In RNA2, the gRNA is under the control of the *Pea early browning virus* (PEBV) promoter (pPEBV::gRNA). (**b**) Experimental scheme for TRV-mediated genome editing. A 20-nucleotide target sequence (shown in yellow) preceding the PAM sequence is cloned into the gRNA backbone (shown in blue) under the control of the PEBV promoter in the RNA2 genome. *Agrobacterium* cultures carrying the engineered TRV RNA2 genome (conferring user-selected sequence specificity) and the RNA1 genome are co-infiltrated into the leaves of *N. benthamiana* overexpressing *Cas9* (*Cas9*-OE) via agroinfection. After agroinfection, the plants are analyzed for the presence of the targeted modification. Leaf disks carrying modified genomes can be regenerated to recover mutant plants, or the seed progeny can be screened for the presence of the modification, thereby bypassing the need for tissue culture

the *Pea early browning virus* (PEBV) promoter (PEBV::gRNA), permitting the expression of the gRNA from the virus expression system. We then reconstituted the TRV virus in *N. benthamiana* leaves via agroinfiltration of mixed *Agrobacterium* cultures harboring the RNA1 genome in combination with different RNA2 vectors, in which gRNAs with binding specificity for a single target (the phytoene desaturase [*PDS*] gene) or multiplex targets (*PCNA* and *PDS)* were driven by the PEBV promoter (pRNA2.PEBV::PDS/PCNA.gRNA) (Fig. 1a). This TRV-mediated genome engineering resulted in highly efficient targeted genome modification in both

the inoculated and systemic leaves of *Cas9*-OE *N. benthamiana*. In addition, the ability of the TRV to infect germline cells resulted in the detection of targeted genome modifications in the seeds of the agro-infiltrated plants, indicating that we successfully recovered the desired modification in the progeny [38]. Moreover, the TRV-mediated CRISPR/Cas9 activity persisted for up to 30 days post-agroinfiltration, and the genetic modification was specific, as no off-target activity was detected [37].

TRV-mediated CRISPR/Cas9 is a simple, versatile genome-editing platform for *in planta* targeted genome modification that eliminates the need for transformation and tissue culture to produce targeted modifications. This system meets several important requirements for highly efficient, multiplexed editing. For example, TRV can be used to systemically infect many different plant species, both naturally and under laboratory conditions. In addition, the virus is easily introduced into plants via *Agrobacterium* and systemic delivery into growing points of the plant. Moreover, the small genome size of TRV facilitates cloning, multiplexing, library construction, and agroinfection. Finally, the viral RNA genome does not integrate into the plant genome, overcoming the regulatory hurdles that might impede the commercialization of engineered plants. Thus, our work expands the utility of the CRISPR/Cas9 system for functional genomic studies in plants and for agricultural biotechnological applications. Here, we present our stepwise method for TRV-mediated genome engineering in plants.

2 Materials

2.1 Reagents

1. Enzymes: Phusion high-fidelity DNA polymerase, T7 endonuclease 1 (T7E1), *Nco*1, *Xba*1, and *Xma*1 (or other restriction enzymes as required, preferably high-fidelity) (New England Biolabs), SuperScript 3 reverse transcriptase (Invitrogen), T4 DNA ligase (Promega).
2. Growth media: Luria Bertani (LB) medium for bacterial culture and Murashige and Skoog (MS) basal salt mixture for plant tissue culture (Sigma-Aldrich).
3. Antibiotics: Spectinomycin, gentamicin, rifampicin, kanamycin, and Timentin (Sigma-Aldrich).
4. Primers, as detailed in Table 1.
5. MES buffer (Sigma-Aldrich).
6. Growth hormones: 1-naphthaleneacetic acid (NAA), 6-benzylaminopurine (BA) (Sigma-Aldrich).
7. Acetosyringone (Sigma-Aldrich).
8. Anti-FLAG antibody (Sigma-Aldrich).

Table 1
Primers used in this protocol

Primer name	Sequence (5′----3′)	Use
Cas9-gw-Fw	CACCATGGACTATAAGGACCACG	To clone *Cas9*
Cas9-R	TT ACTTTTTCTTTTTTGCCTGGC	To clone *Cas9* and as a reverse primer for *Cas9* detection via RT-PCR
CAS9-SEQ-F6	GCCCTCCAAATATGTGACTTCC	Forward primer for *Cas9* detection via RT-PCR
Cas9-R-No-Stop	CTTTTTCTTTTTTGCCTGGC	To construct *Cas9-GFP*
NB-ACTIN1-RT-F NB-ACTIN1QRT-LIU-R	TGAAGA TCCTCACAGAGCGTGG TTGT A TGTGGTCTCGTGGA TTC	RT-PCR normalization control
TRV1-Replicase-RT-F TRV1-Replicase-RT-R	CT ACTGGGAGAGCAGCAACC CTGAGCGCAAAAGT ACACCA	To detect systemic movement of TRV-RNA1
TRV2-CP-RT-F TRV2-CP-RT-R	TTGGGTGGAATCAGTTTCGT TCTTCCAAAGTCGAGCCAGT	To detect systemic movement of TRV-RNA2
NB-PDS3-TR1,2-gDNA-F2 NB-PDS3-TR1,2-gDNA-R	GAAACACATCACCTAGGCGG GGGCGTGAGGAAGT ACGAAA	For PCR of the region flanking the *PDS* target
NBPDS3-gDNA-404 bp-F NBPDS3-gDNA-404 bp-R	GTAAAATGCCCCAAATTGGACTTGT CGTGAGGAAGTACGAAATGATGATGA	To amplify the 404-bp target-flanking region around *PDS*
NB.PCNA gDNA F1 NB.PCNA gDNA R1	CCTAACCCTAATTTCCCCAG TCACTGTCAATGTCCATCAG	For PCR of the region flanking the *PCNA* target

9. Goat anti-mouse IgG-HRP secondary antibody (Santa Cruz).
10. Enhanced Chemiluminescence (ECL)-detection reagent (Thermo Scientific).
11. Skim milk powder, Tween 20, Sodium dodecyl sulfate (SDS), HEPES, Tris buffer, Glycerol, Agarose powder, Ethidium bromide, Lithium chloride, Bleach (Sodium hypochlorite).
12. Polyacrylamide gels (Precise Tris-HEPES Gels, Thermo Scientific).
13. TBS-T: Tris-buffered saline with Tween 20, pH 8.0.
14. Infiltration medium: 10 mM MES, 10 mM $CaCl_2$, 200 μM acetosyringone, pH 5.7.
15. Regeneration and selection medium: 4.4 g/L MS salts with vitamins, 1 mg/L BA, 0.1 mg/L NAA, 30 g sucrose, 50 mg/L kanamycin, 200 mg/L Timentin, pH 5.8.
16. Root-inducing medium: 2.2 g/L MS salts with vitamins, 50 mg/L kanamycin.
17. DNA extraction buffer: 100 mM Tris–Cl, 1 mM EDTA, 100 mM NaCl, 100 mM LiCl, 100 mM β-mercaptoethanol, 0.4% RNase.
18. PCI solution: Phenol:chloroform:isoamyl alcohol.
19. CDP-Star Chemiluminescent Substrate (Roche).
20. PVDF membrane.

2.2 Plasmids and Vectors

1. *Tobacco rattle virus* (TRV) RNA1 (pYL192) and RNA2 (pYL156 modified with the PEBV promoter), provided by Savithramma Dinesh-Kumar, University of California at Davis.
2. pK2GW7 binary vector, which is Gateway compatible [39].
3. pX330 plasmid [40].
4. pEarleyGate 103.
5. pENTR/D-TOPO (Life Technologies).
6. LR Clonase (Life Technologies, Invitrogen).
7. TOPO TA cloning vector (Thermo Scientific).

2.3 Kits

1. RNeasy Mini kit.
2. PCR purification.
3. Plasmid Miniprep kit.

2.4 Organisms

1. *Nicotiana benthamiana*.
2. *Agrobacterium tumefaciens* strain GV3101.

2.5 Software and Programs

1. CRISPR-P/CCTop [41, 42].
2. ImageJ (http//rsb.info.nih.gov/ij).

3 Methods

3.1 Designing and Cloning of sgRNA

1. Select the genomic region of the plant genome to be targeted. In this example, we selected the phytoene desaturase (*PDS*) gene for single targeting and the proliferating cell nuclear antigen gene (*PCNA*) for multiplex targeting (*PCNA* and *PDS,* simultaneously), as mutations at these genes can result in obvious visual phenotypes. Target site selection can be performed manually by looking for an "NGG" as the protospacer-adjacent motif (PAM); use the 20 bp upstream sequence, avoiding five or more thymines (T) in a row. Software-assisted selection can be performed using CRISPR-P or CCTop [41, 42] (*see* **Note 1**). Manual selection is preferable for targeting a specific structure, motif, or domain.
2. Use a PCR-based restriction ligation procedure to clone sgRNAs targeting the *PDS* and/or *PCNA* genes into the TRV RNA2 vector under the control of the *Pea early browning virus* (PEBV) promoter (*see* **Note 2**).
3. Use a forward primer containing an *Xba*1 recognition site, a 20-nucleotide target sequence, and an 84-nucleotide Cas9-binding sgRNA scaffold with a reverse primer containing complementary sequence to the sgRNA end, a 7-T repeat (terminator), and an *Xma*1 recognition site to amplify a 116-bp PCR fragment.
4. PCR amplify the fragment containing the 20-nucleotide target sequence, the 84-bp Cas9 binding loop for sgRNA, and a 7-T repeat (as a terminator), using a backbone containing the 84 bp sgRNA Cas9 binding scaffold as template.
5. Digest both the PCR product and the RNA2 vector with *Xba*I and *Xma*I and gel purify.
6. Using T4 ligase, clone the 116-bp PCR fragment of the sgRNA for each target into the TRV RNA2 vector under the control of the PEBV promoter (Fig. 1a).
7. Perform Sanger sequencing to confirm all cloned sequences.

3.2 Cloning of Cas9

1. PCR amplify the complete 3XFlag–NLS-Cas9-NLS cassette with Cas9-GW-F and Cas9-R primers using the pX330 plasmid as template [40].
2. Clone the gel-purified PCR product into pENTR/D-TOPO.
3. Transfer *Cas9* into the pK2GW7 binary vector via the LR Clonase reaction to generate the *35S::Cas9-T35S* construct. Use this construct to produce the *N. benthamiana Cas9*-OE line (*see* **Note 3**).

4. Subject the clones to restriction digestion and Sanger sequencing to confirm the authenticity of the clones and the in-frame translational fusions.

3.3 Cloning the Cas9: GFP Reporter

1. PCR amplify the complete 3XFlag-NLS-Cas9-NLS cassette with Cas9-GW-F and Cas9-R-no-stop primers (to remove the stop codon) using the pX330 plasmid as template [40].
2. Clone the gel-purified PCR product into pENTR/D-TOPO.
3. Transfer *Cas9* into the pEarleyGate 103 binary vector via the LR Clonase reaction to generate the *35S::Cas9:GFP-T35S* construct. Use this construct to transiently express the GFP-fusion variant in *N. benthamiana* leaves for subcellular localization analysis of Cas9 (*see* **Note 4**).
4. Subject the clones to restriction digestion and Sanger sequencing to confirm the authenticity of the clones and the in-frame translational fusions.

3.4 Generation of N. benthamiana Plants Overexpressing Cas9 (Cas9-OE)

1. Introduce the pK2GW7.Cas9 binary vector obtained in Subheading 3.2 into *Agrobacterium tumefaciens* strain GV3101 by electroporation.
2. Grow single colonies carrying the T-DNA vector overnight at 28 °C in 5 mL *Agrobacterium* culturing medium.
3. Inoculate the overnight culture in 50 mL of LB containing the selective antibiotics in 5 mM MES buffer.
4. Grow the cells to OD_{600} of 1.0, resuspend them in transformation medium to OD_{600} of 0.4, and pour onto a Petri dish.
5. Use a surgical blade to cut leaf tissues from aseptically grown 2-week-old *N. benthamiana* plants into small (approximately 1 cm) leaf disks. Make small incisions on the leaf surface with the scalpel to maximize contact of the *Agrobacteria* with the plant cells.
6. Incubate leaf disks in *Agrobacterium* culture for 30 min with occasional shaking.
7. Remove excess *Agrobacterium* cells with sterile filter paper and cocultivate the leaf disks in *Agrobacterium* on MS medium in a growth chamber at ~25 °C for 2 days.
8. Transfer the leaf disks onto the regeneration and selection medium.
9. Excise shoot tissues after 3–4 weeks and place them onto root-inducing medium.
10. After proper roots have formed (2–3 weeks), acclimate the plantlets in soil under plastic domes.

3.5 Confirmation of Cas9 Expression at RNA and Protein Levels

3.5.1 RNA Confirmation by Semiquantitative RT-PCR

1. Extract total RNA from the leaves using an RNeasy Mini kit.
2. Synthesize first-strand cDNA using SuperScript 3 reverse transcriptase.
3. PCR amplify a fragment corresponding to the C-terminus of Cas9 with Phusion Polymerase.
4. Perform RT-PCR under the following conditions: 98 °C for 30 s, 30 cycles of 98 °C for 15 s, 60 °C for 30 s, and 72 °C for 15 s, followed by 72 °C for 5 min.
5. Confirm the presence of the Cas9 by electrophoresis.

3.5.2 Protein Confirmation by Western Blot

1. Extract total proteins from 100 μg leaf tissue and separate the proteins by SDS polyacrylamide gel electrophoresis.
2. Perform immunoblot analysis using primary mouse anti-FLAG antibody (1:1000) in 5% skim milk in Tris-buffered saline with Tween 20 (TBS-T) and secondary goat anti-mouse (1:1000; Santa Cruz) in 1% skim milk in TBS-T.
3. Detect reacting proteins based on chemiluminescence using ECL detection reagent.

3.6 TRV-Mediated Delivery of Cas9/sgRNA

The complete strategy for TRV-mediated genome editing is depicted in Fig. 1b and is described as follows.

1. Grow *N. benthamiana Cas9*-OE plants in soil in a greenhouse to the 6–8 leaf stage (2–3 weeks).
2. Introduce vectors containing the TRV RNA1 and recombinant TRV RNA2 genomes separately into *A. tumefaciens* strain GV3101 and spread the cells on LB agar plates containing kanamycin (50 mg/L), gentamicin (30 mg/L), and rifampicin (25 mg/L).
3. Grow the transformed single colonies overnight in selective medium to OD_{600} of 1.2.
4. Collect *Agrobacterium* cells by centrifugation and resuspend in the infiltration medium to an OD_{600} of 0.3.
5. Incubate the cultures at ambient temperature in the dark for 2–4 h.
6. Prior to infiltration, combine bacterial cultures (at OD_{600} 0.1) harboring TRV-RNA1 and TRV-RNA2::PDS at a 1:1 ratio (for single targeting) or TRV-RNA1, TRV-RNA2::PDS, PCNA at a 1:1:1 ratio (for multiplexed targeting; *see* **Note 5**).
7. Infiltrate the bacterial culture mix into the abaxial sides of 3–4-week-old fully extended leaves of *Cas9*-OE *N. benthamiana* plants using a needleless 1 mL syringe.
8. Collect leaf disk samples from inoculated and systemic leaves at 5, 10, 15, and 30 days post-infiltration and subject them to targeted genome modification analysis (*see* Subheading 3.8)

to determine and quantify mutations induced by Cas9 (*see* **Note 6**).

3.7 Confirmation of TRV RNA1 and TRV RNA2 in Systemic Leaves by Semiquantitative RT-PCR

1. Extract total RNA from systemic leaves of interest using an RNeasy Mini kit.
2. Synthesize first-strand cDNA using SuperScript 3 reverse transcriptase.
3. To detect the presence of TRV RNA1, perform PCR with primers TRV1-Replicase-RT-F and R (Table 1) to amplify a fragment corresponding to part of the Replicase (RNA-dependent RNA polymerase) gene in TRV RNA1.
4. To detect the presence of recombinant TRV RNA2, perform PCR with primers TRV2-CP-RT-F and R (Table 1) to amplify a fragment corresponding to part of the Coat protein (CP) gene in TRV RNA2.
5. Perform RT-PCR using the following conditions: 98 °C for 30 s, 30 cycles of 98 °C for 15 s, 60 °C for 30 s, and 72 °C for 15 s, followed by 72 °C for 5 min.
6. Confirm the presence of the TRV RNA1 and RNA2 by electrophoresis.

3.8 Mutation Detection by the T7EI Assay, Loss of Restriction Enzyme Recognition Site Assay, and Sanger Sequencing

1. Extract genomic DNA from samples collected at 5, 10, and 30 days post-infiltration using DNA extraction buffer and PCI solution, followed by ethanol precipitation.
2. PCR amplify the fragment encompassing the target sequence using the appropriate primers (*see* Table 1 for the PDS and PCNA primers used in this study) and High-Fidelity Phusion Polymerase (*see* **Note 7**).

3.8.1 Mutation Detection by the T7EI Assay and Sanger Sequencing

1. In a total reaction volume of 20 μL, denature and reanneal 200 ng PCR products in New England Biolabs Buffer 2 (1.8 μL) in a thermal cycler to allow for hetero-duplex formation using the following cycling program: 95 °C for 10 min, 85 °C for 2 min, 75 °C for 3 min, 65 °C for 3 min, 55 °C for 3 min, 45 °C for 3 min, 35 °C for 3 min, 25 °C for 3 min, and 4 °C on hold.
2. Treat the hybrid PCR products with T7EI by adding 0.5 μL of T7EI and 0.2 μL Buffer 2 and incubate at 37 °C (see example results in Fig. 2a).
3. Calculate the mutation rates using ImageJ software.
4. To validate the mutations detected by the T7E1 assay, clone the PCR products into the TOPO TA cloning vector and subject the clones to Sanger sequencing.

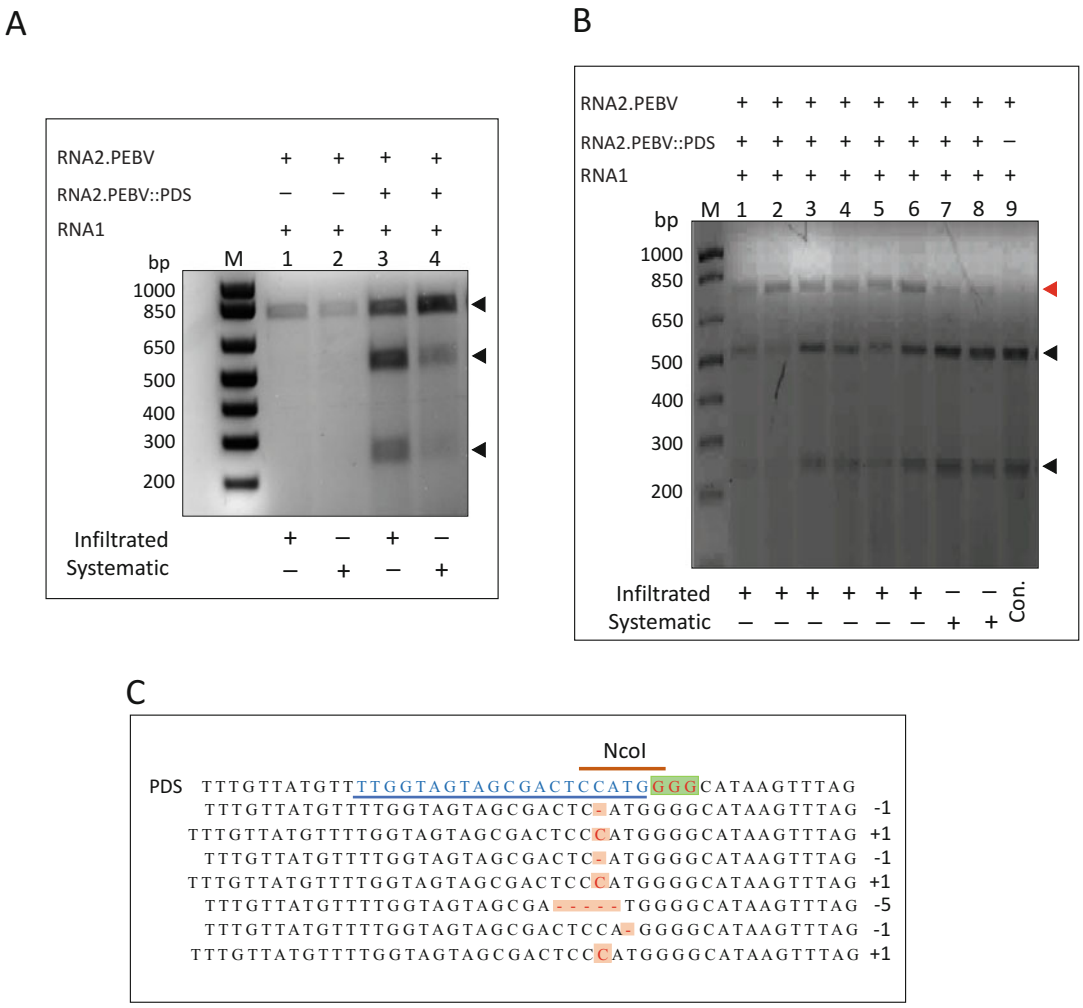

Fig. 2 Analysis of TRV-mediated CRISPR/Cas9 targeted mutagenesis of *PDS3*. (**a**) T7EI-based mutation detection in systemic leaves. Mutagenesis was detected in inoculated (lane 3) and systemic (lane 4) leaves co-infiltrated with RNA1 and RNA2 carrying pPEBV::PDS.gRNA compared to the vector control (lanes 1 and 2). Arrowheads indicate the restriction digestion products. (**b**) RFLP-based mutation detection in systemic leaves. DNA was extracted from *Cas9*-OE plants and PCR was performed with a primer set to amplify a fragment encompassing the target site. Purified PCR product (300 ng) was treated with *Nco*I and separated on a 2% agarose gel. Inoculated plants clearly showed an *Nco*I-resistant (uncut) DNA fragment of 802 bp (red arrowhead), indicating the occurrence of targeted mutagenesis. Mutations were detected in inoculated leaves (lanes 1, 2, 3, 4, 5, and 6) and systemic leaves (lanes 7 and 8) in plants co-infiltrated with RNA1 and RNA2. PEBV::PDS-gRNA compared to the control (lane 9). Arrowheads indicate the restriction products. (**c**) Sanger sequencing of indels at the *PDS* target site from inoculated plants. (**b**) The wild-type (WT) sequence is shown at the top (the target sequence is shown in blue and underlined, the *NcoI* site is indicated by a line, and the protospacer-associated motif (PAM) is shown in red and highlighted in green). Different indel mutations are shown ("–" indicates deletion and "+" indicates insertion)

3.8.2 Mutation Detection by Restriction Fragment Length Polymorphism (RFLP) Assay and Sanger Sequencing

The target sequence in the *PDS* gene contains an *NcoI* restriction enzyme recognition site that overlaps with the Cas9 cleavage site (about 3 bp ahead of the NGG PAM). Cas9 induced mutations are likely to destroy this *NcoI* recognition site, allowing for mutation detection based on an RFLP assay.

1. In a 20-μL reaction volume, add *NcoI* to 300 ng of PCR product containing the region flanking the *PDS* target sequence.
2. To ensure complete digestion, the reaction should be performed for 8 h at 37 °C.
3. Confirm mutations based on uncut bands (*see* Fig. 2b).
4. Gel purify and clone the undigested PCR product into the TOPO TA cloning vector and confirm that mutations via Sanger sequencing (*see* Fig. 2c).

3.9 Screening Progeny Plants for Heritable Genome Modifications

1. Collect seed capsules (a total of 100–200 seeds) from plants successfully infected with recombinant TRV-RNA2 carrying the desired gRNA at various stages of plant growth (Fig. 1b).
2. Grow seeds in soil, 10 seeds in per pot, for 10–15 days.
3. Collect cotyledonary leaves from all 10 seedlings (per pot) as one pool.
4. From each seedling pool, extract genomic DNA as one pool. Follow the procedure in Subheading 3.8 for genomic DNA extraction.
5. PCR amplify the fragment encompassing the target sequence.
6. Detect mutations using the T7E1 assay (as described in Subheading 3.8.1) or RFLP assay (as described in Subheading 3.8.2).
7. Clone positive PCR products into the TOPO TA cloning vector and subject the clones to Sanger sequencing.
8. If mutations are detected, screen each plant from the positive pool separately to identify plants carrying the intended genomic modifications.
9. Remove any negative plant from the pot and allow the selected mutants to set seed (*see* **Note 8**).

4 Notes

1. We highly recommend checking the off-targeting activity of the designed sgRNA using an online tool such as CRISPR-P or CCTop [41, 42].
2. Alternatively, the sgRNA clones can be custom synthesized in the pUC19 (-MCS) plasmid using the BlueHeronBio gene synthesis service (BlueHeronBio, Bothell, WA USA). Each

sgRNA (flanked by *Xba*1 and *Xma*1 restriction sites) should consist of a 116-bp fragment containing the 20-nucleotide target sequence and an 84 bp Cas9 binding loop, followed by a tandem repeat sequence of seven thymines (T) as the transcriptional terminator. The 116 bp sgRNA fragment of each target can be subcloned into the TRV RNA2 vector under the control of the PEBV viral promoter by restriction ligation cloning using the restriction enzymes *Xba*1 and *Xma*1.

3. In this protocol, we describe TRV-mediated genome editing in *N. benthamiana*, but any plant species where CRISPR/Cas9 and TRV has been experimentally demonstrated can be used.
4. This experiment is performed to investigate and confirm the subcellular localization of Cas9 in plants.
5. The OD_{600} of the combined TRV1 and TRV2(n) cultures should not exceed 0.5, as higher concentrations might be toxic to the infiltrated leaves. For multiplexed editing on more than four targets, an OD_{600} as low as 0.05 can be used efficiently.
6. Photograph the plants at each stage and compare the molecular data with phenotypic data.
7. Perform PCR using undigested genomic DNA or genomic DNA predigested with restriction enzymes to enrich for the modification of interest.
8. Seeds collected from late flowers typically show lower mutation frequencies than seeds from early flowers.

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Chapter 24

Biolistic Delivery of CRISPR/Cas9 with Ribonucleoprotein Complex in Wheat

Zhen Liang, Kunling Chen, and Caixia Gao

Abstract

The great advances in exploiting the CRISPR/Cas9 system are paving the way for targeted genome engineering in plants. Genome editing by direct delivery of CRISPR/Cas9 ribonucleoprotein complexes (RNPs) into plant cells reduces off-target mutations and avoids the integration of foreign DNA fragments, thus providing an efficient and accurate method for precision crop breeding. Here we describe an RNP-based genome editing protocol for wheat. The protocol covers the in vitro transcription of sgRNA, purification of Cas9 protein, biolistic delivery of CRISPR/Cas9 RNPs, and tissue culture procedures for regenerating testable seedlings.

Key words CRISPR/Cas9, Ribonucleoprotein, Biolistic delivery, Wheat

1 Introduction

The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) system has been developed as a powerful tool for targeted genome engineering and has been widely used in plants including wheat [1, 2]. The CRISPR/Cas9 system has two components: Cas9 protein and a single guide RNA (sgRNA). To create genome modifications, three types of CRISPR/Cas9 forms, expression plasmids (DNA), in vitro transcripts (IVTs), and preassembled ribonucleoprotein complexes (RNPs), can be delivered into host cells [3]. Conventional plant genome editing mainly relies on the expression of CRISPR/Cas9 DNA delivered in the form of a vector carrying a CRISPR/Cas9 cassette by *Agrobacterium* transformation or particle bombardment [4]. In this method the integrated CRISPR/Cas9 cassette needs to be segregated out by crossing or backcrossing to obtain genome-edited plants without foreign DNA sequences. The

Zhen Liang and Kunling Chen contributed equally to this work

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method may also result in unwanted off-target mutations, mainly due to continuous expression of the integrated CRISPR/Cas9. Moreover, partial DNA fragments of CRIPSR/Cas9 DNA are sometime randomly inserted into the plant genome. Therefore, plants edited by this procedure may be challenged by the regulatory authorities, due to the presence, or possible presence, of foreign DNA sequences [5]. Previously, we had established transgene-free editing methods by transient expression of CRISPR/Cas9 DNA or in vitro transcripts (IVTs) in wheat by biolistic delivery, which however still face the problems of off-target effects and foreign DNA insertion [6].

The CRISPR/Cas9 RNP method involves delivery of preassembled ribonucleoprotein complexes of Cas9 and in vitro transcribed sgRNA into host cells, and it is a DNA-free genome editing method. It had been adopted in many organisms such as mouse [7], *Caenorhabditis elegans* [8], *Chlamydomonas reinhardtii* [9], and human cell lines [10, 11]. In 2015, Woo et al. described targeted mutagenesis in rice, *Arabidopsis*, tobacco, and lettuce by delivery of RNPs into protoplasts via PEG-mediated transfection, and the regeneration of edited lettuce plants from mutated protoplasts [5]. Later, the use of this method was reported in petunia [12], grapevine, and apple [13], but no mutant plants were regenerated. A CRISPR/Cpf1 RNP editing method has been recently developed for soybean and wild tobacco protoplasts [14]. However, this approach remains challenging for major cereal crops including wheat since they cannot be regenerated from protoplasts. In 2017, we demonstrated that RNPs could be delivered into wheat embryos, rather than into protoplasts, to obtain edited mutants. Using this method, we obtained *gw2* gene knockout mutants in wheat varieties KN199 and YZ814 [15]. A similar DNA-free editing method involving biolistic delivery of CRISPR/Cas9 RNP complexes has also been reported in maize [16]. All these studies show that editing by RNPs is a simple, specific, efficient DNA-free genome editing method in plants that may knock out the consumer concerns and regulators' rules [17]. Here, we present our RNP-based genome editing protocol for wheat. The protocol comprises in vitro transcription of sgRNA, purification of Cas9 protein, biolistic delivery of CRISPR/Cas9 RNPs, and regeneration of testable seedlings.

2 Materials

2.1 Preparation of sgRNA

1. Primers for amplifying the sgRNA DNA template: forward primer (5′-TAATACGACTCACTATAGGN_{19}-3′), (N19 indicates the protospacer sequence for each sgRNA) and reverse primer (5′- GCACCGACTCGGTGCCACTT-3′).
2. HiScribe™ T7 High Yield RNA Synthesis Kit (NEB).

3. DNase I (NEB).
4. Ethanol: prepare 70% ethanol with absolute ethanol and RNase-free H_2O.
5. NanoDrop 2000c spectrophotometer (Thermo).
6. Standard equipment and reagents for agarose gel electrophoresis (Bio-Rad).

2.2 Preparation of Cas9 Protein

1. Plasmid: the pET28a-Cas9-His plasmid can be obtained from the authors on request.
2. 50 mg mL^{-1} kanamycin (10 mL): dissolve 500 mg kanamycin in 10 mL ddH_2O, sterilize with a 0.22 μm filter, and store at −20 °C.
3. 0.5 M IPTG (10 mL): add 1.19 g IPTG to 10 mL ddH_2O, filter sterilize, and store at −20 °C.
4. 1× PBS buffer (1 L): dissolve 8 g NaCl, 0.2 g KCl, 3.58 g Na_2HPO_4 $12H_2O$, and 0.24 g KH_2PO_4 to ddH_2O. Adjust the pH to 7.4 with HCl and add ddH_2O to 1 L. Sterilize by autoclaving at 121 °C for 20 min and store at room temperature for up to 6 months.
5. Buffer I (1 L): add 25 mL 1 M Tris (pH 8.0) and 29.22 g NaCl to ddH_2O. Adjust volume to 1 L. Store at 4 °C for up to 6 months.
6. Buffer E (500 mL): add 12.5 mL 1 M Tris (pH 8.0), 50 mL 5 M NaCl, and 34.04 g imidazole to ddH_2O. Adjust volume to 500 mL. Store at 4 °C for up to 6 months.
7. Lysis buffer (100 mL): add 2.5 mL Buffer E to 97.5 mL Buffer I. Store at 4 °C for up to 6 months.
8. Wash buffer (100 mL): add 5 mL Buffer E to 95 mL Buffer I. Store at 4 °C for up to 6 months.
9. Elution buffer (100 mL): add 25 mL Buffer E to 75 mL Buffer I. Store at 4 °C for up to 6 months.
10. Cas9 storage buffer (100 mL): add 2 mL 1 M HEPES, 5 mL 3 M KCl, 100 μL 1 M DTT, and 3 mL glycerol to autoclaved ddH_2O. Adjust volume to 100 mL. Store at 4 °C for up to 6 months.
11. Ni-Sepharose 6 Fast Flow (GE Healthcare).
12. Quick Start Bradford 1× Dye Reagent (Bio-Rad).
13. 30 kDa Amicon Ultra-15 centrifugal filter units (Millipore).
14. High-speed centrifuge (Beckman Coulter).
15. Sonic Dismembrator (Fisher Scientific).
16. Standard equipment and reagents for SDS-PAGE gel electrophoresis (Bio-Rad).

2.3 Particle Bombardment and Tissue Culture

1. Gold nanoparticles (1.5 mL): weigh 0.06 g gold nanoparticles (60-μm) in a 1.5 mL RNase-free microfuge tube and sterilize by washing three times with 75% ethanol. Discard the supernatant by centrifuging and resuspend in 1.5 mL RNase-free H_2O.
2. 10× Cas9 Reaction Buffer (10 mL): add 2 mL 1 M HEPES (pH 7.5), 1 mL 1 M $MgCl_2$, 50 μL 1 M DTT, and 5 mL 3 M KCl to RNase-free H_2O. Adjust volume to 10 mL. Prepare 1-mL aliquots and store at −20 °C.
3. Osmotic medium (1 L): dissolve 4.4 g Murashige and Skoog (MS) medium basal salt mixture, 5 mL 2,4-D (1 mg/mL), and 72.86 g mannitol in 800 mL ddH_2O. Adjust pH to 5.8 with 1 M KOH and volume to 1 L and add 3.2 g phytagel. Autoclave at 121 °C for 20 min. After cooling to 50 °C, pour ~20 mL into sterile Petri dishes (6-cm). Store the dishes at 4 °C in dark containers.
4. Recovery medium (1 L): dissolve 4.4 g MS salts including vitamins, 30 g sucrose, 2 mL 2,4-D (1 mg/mL), 0.5 g N-Z-Amine A, and 600 μL $CuSO_4$ (1 mg/mL) in 800 mL ddH_2O. Adjust pH to 5.8 with 1 M KOH and add ddH_2O to 1 L, and then add 3.2 g phytagel. Autoclave at 121 °C for 20 min. Pour ~30 mL into sterile Petri dishes (9-cm) after cooling to 50 °C. Store dishes at 4 °C in dark containers.
5. Regeneration medium (1 L): dissolve 4.4 g MS salts including vitamins, 30 g sucrose, and 200 μL kinetin (1 mg/mL) in 800 mL of ddH_2O. Adjust pH to 5.8 with 1 M KOH and volume to 1 L and add 3.2 g of phytagel. Autoclave at 121 °C for 20 min. Pour ~30 mL of medium into sterile Petri dishes (9-cm) after cooling to 50 °C. Store the dishes at 4 °C in dark containers.
6. Rooting medium (1 L): dissolve 2.2 g MS salts including vitamins and 30 g sucrose in 800 mL of ddH_2O. Adjust pH to 5.8 with 1 M KOH and add ddH_2O to 1 L, and then add 3.2 g phytagel. Autoclave at 121 °C for 20 min. After cooling to about 50 °C, add 100 μL NAA (0.5 mg/mL). Pour ~30 mL of medium into sterile Petri dishes (9-cm). Store at 4 °C in dark containers.
7. Optical Microscope (Olympus).
8. PDS1000/He particle bombardment system (Bio-Rad).

3 Methods

3.1 In Vitro Transcription of sgRNA

1. PCR amplify the dsDNA template (containing the T7 promoter, protospacer and sgRNA scaffold sequence) for in vitro transcription. Then purify the PCR products and determine their concentration with a NanoDrop spectrophotometer (*see* **Note 1**).

2. In vitro transcription: Set up a 20 μL reaction using a HiScribe™ T7 In Vitro Transcription Kit (NEB) according to the manufacturer's instructions. Mix gently and thoroughly by pipetting and incubate at 37 °C in the PCR machine for at least 3 h (*see* **Note 2**).
3. Digest the template dsDNA by adding 70 μL RNase-free H_2O, 10 μL 1× DNase I buffer, and 1 μL DNase I and incubate at 37 °C for 1 h.
4. Transfer the mixture into a fresh RNase-free 1.5 mL microfuge tube. Add 300 μL absolute ethanol and store at −20 °C for 3–4 h to precipitate the sgRNA.
5. Centrifuge the tube at 12,000 rpm for 10 min at 4 °C and discard the supernatant.
6. Wash the precipitate by adding 600 μL ice-cold 70% ethanol. Centrifuge at 12,000 rpm for 5 min at 4 °C and discard the supernatant.
7. Centrifuge again at 12,000 *g* for 2 min at 4 °C and remove residual supernatant with a pipette. Air-dry the pellet for 2 min at room temperature.
8. Add 50 μL RNase-free H_2O and determine the sgRNA concentration with the NanoDrop spectrophotometer. Sub-package the sgRNA for single use (10 μL each) and store at −80 °C (*see* **Note 3**).

3.2 Cas9 Protein Purification

1. Transfect the Cas9 bacterial expression (pET28a-Cas9-His) plasmid into *Rosetta* (DE3) competent cells. Plate the transfected cells onto LB agar plates containing 50 μg/mL kanamycin and incubate at 37 °C overnight (*see* **Note 4**).
2. Pick one single colony into 10 mL LB medium containing 50 μg/mL kanamycin and incubate at 37 °C with shaking at 200 rpm for 6–8 h. Transfer the culture into 1 L of LB medium containing 50 μg/mL kanamycin and incubate as above for another 2 h until the OD600 reached to 0.6–0.8 (*see* **Note 5**).
3. Induce Cas9 protein expression with 0.5 mM IPTG and incubate at 18 °C with shaking at 200 rpm for about 16 h.
4. Harvest the bacterial cells by centrifugation at 4000 rpm for 10 min at 4 °C and discard the supernatant. Wash the pellet with precooled 1× PBS buffer. Centrifuge at 4000 rpm for 10 min at 4 °C and discard the supernatant.
5. Resuspend the cell pellet with 30 mL lysis buffer and transfer to a fresh 50 mL centrifuge tube. Lyse the cells by sonication using a 10 min process time with cycles of 3 s on /6 s off (*see* **Note 6**).
6. Centrifuge the lysate at 11,000 rpm for 50 min at 4 °C. Transfer the supernatant into a fresh 50 mL collection tube.

7. Add the supernatant to the gravity-flow purification column containing 1 mL nickel-nitrilotriacetic acid (Ni-NTA) agarose beads. Collect the flow-through and repeat this step three times for maximum binding of the protein.
8. Wash the column with about 40 mL wash buffer to remove nonspecific binding proteins (*see* **Note 7**).
9. Elute the Cas9 protein with about 15 mL elution buffer.
10. Exchange the buffer of the purified protein with Cas9 storage buffer and concentrate the proteins to about 2 mL using a 30-kDa MWCO column. Determine the Cas9 protein concentration and its purity with the Bradford assay and by SDS-PAGE, respectively.
11. Sub-package the Cas9 protein tube for single use (30–50 μg each). Flash-freeze in liquid nitrogen and store at −80 °C (*see* **Note 8**).

3.3 Biolistic Delivery of CRISPR/Cas9 RNPs

1. Plant wheat varieties in either a greenhouse or a field. A disease-free greenhouse is recommended. Supplementary lighting is needed to maintain light intensity at about 400 μmol m^{-2} s^{-1} and maintain a long-day photoperiod (LD 16:8) during the entire procedure. Air-conditioning is also needed to maintain a suitable temperature for the various developmental stages.
2. Between 12 and 14 days after pollination, harvest ears at the right developmental stage. Immature embryos of about 1 mm in length are suitable for transformation (*see* **Note 9**).
3. Manually detach the kernels from the ears without damaging the embryos. To sterilize, wash the kernels with 75% ethanol for 1 min, followed by 2.5% sodium hypochlorite (NaClO) for 20 min, and rinse six times with autoclaved ddH_2O.
4. Isolate the immature embryos from the detached kernels with a sharp scalpel under an optical microscope on a clean bench.
5. Place about 80 embryos each in individual 6-cm diameter Petri dishes containing high-osmotic medium. Distribute the embryos as a circular monolayer of 1.5-cm diameter in the middle of each Petri dish (*see* **Note 10**).
6. Incubate the Petri dishes at room temperature for 3–4 h before bombardment.
7. For each shot, set up a 10 μL reaction containing 2 μg Cas9 protein, 2 μg sgRNA, 1 μL 10× Cas9 Reaction Buffer made up to 10 μL with RNase-free H_2O. Incubate the mixture at 25 °C for 10 min to form CRISPR/Cas9 RNP complexes.
8. Add 5 μL of gold nanoparticles (60-μm; 40 mg mL^{-1}) to the mixture and mix gently and thoroughly by pipetting.
9. Assemble the macro-carrier onto the macro-carrier holder and spread a 15 μL mixture onto the central region of the macro-

carrier. Air-dry on the bench top at room temperature to coat the RNP onto the gold nanoparticles (*see* **Note 11**).

10. After drying, perform the bombardment immediately using a PDS1000/He particle bombardment system according to the manufacturer's instructions. Set up the equipment with a target distance (between target cell and stopping screen) of 6.0 cm and a helium gas pressure of 1100 p.s.i.

3.4 Tissue Culture to Regenerate Testable Seedlings

1. Incubate the bombarded embryos on the high-osmotic medium at 23 °C overnight in the dark.
2. Transfer the bombarded embryos into 9-cm Petri dishes containing recovery medium (about 30 embryos per plate) to induce callus formation. Culture at 23 °C in the dark for 2 weeks, and about 4–6 mm calli will form.
3. Transfer all the calli into 9-cm Petri dishes containing regeneration medium and incubate at 23 °C with a long-day photoperiod of 16 h light (150 μmol m^{-2} s^{-1}) and 8 h dark for about 2 weeks.
4. After 2 weeks, many green tips will have appeared on the surfaces of the calli. Usually more than half of the calli can form 2~4 green tips. Detach the green tips from the calli with forceps and transfer them into 9-cm Petri dishes containing regeneration medium and incubate at 23 °C with a long-day photoperiod as specified above for another 2 weeks.
5. Transfer the green plantlets that have regenerated into 9-cm Petri dishes containing rooting medium. After culturing at 23 °C with a long-day photoperiod for 7–10 days, seedlings of about 5–6 cm will have formed and can be screened for mutants (*see* **Note 12**).

4 Notes

1. The sgRNA can be designed and validated as previously described [4]. The T7 promoter sequence of the PCR product is added by the forward primer containing the promoter sequence (5′-TAATACGACTCACTATAGG-3′) upstream of the protospacer sequence. Elute the PCR products using RNase-free H_2O to minimize RNase contamination.
2. Assemble the transcription reaction at room temperature. Keep all the reagents on ice except for the 10× T7 reaction buffer which should be kept at room temperature since otherwise the spermidine in the reaction buffer will precipitate the DNA templates.
3. After adding RNase-free H_2O, the transcribed sgRNA is dissolved; the remaining pellet may consist of impurities and can be removed by centrifuging at 12,000 *g* for 2 min at 4 °C.

4. BL21 (DE3) bacterial cells can also be used for Cas9 protein expression with a yield a little lower than that of *Rosetta* (DE3).
5. To prevent the OD600 exceeding 0.8, we recommend monitoring the optical density at 600 nm every 15 min after incubating the 1 L bacterial cells for 1.5 h.
6. Keep the bacterial cells on ice during the sonication to avoid overheating which may cause misfolding of Cas9 in the lysate.
7. The washing process can be monitored by adding a drop of the flow-through to 200 μL Quick Start Bradford 1× Dye Reagent until the color does not change.
8. Purified Cas9 protein together with in vitro transcribed sgRNA can be determined by cleaving PCR products containing the target site in vitro. Transient protoplast assay can also be used to determine the nuclease activities of RNPs.
9. In case there are not enough suitable ears on a particular day, the harvested ears can be dipped in 75% ethanol for 1 min, washed twice with water for sterilization, then wrapped in wet paper and stored at 4 °C for up to 5 days.
10. Wheat varieties on average formed scutellum callus at a higher frequency than epiblast callus. Keep the epiblast in contact with the medium, with the scutellum upward.
11. Make sure the proportion of glycerol in the Cas9 storage buffer is less than 5%, because too much glycerol in the buffer may result in failure to air-dry to coat the RNPs.
12. Tissue culture without selection will give rise to large numbers of testable seedlings in the T0 generation. To save labor, we combine groups of 3–4 seedlings as pools for mutant screening [6].

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Chapter 25

DNA-Free Genome Editing via Ribonucleoprotein (RNP) Delivery of CRISPR/Cas in Lettuce

Jongjin Park, Sunmee Choi, Slki Park, Jiyoung Yoon, Aiden Y. Park, and Sunghwa Choe

Abstract

CRISPR/Cas9 nuclease system is getting popular in precise genome editing of both eukaryotic and prokaryotic systems due to its accuracy, programmability, and relative ease of use. CRISPR/Cas systems can be delivered into live cells via plasmid DNA, RNA, and ribonucleoprotein (RNP). Of these, the RNP method is of special interest due to enzymatic action in shorter time and controllability over their activity. In addition, because RNP does not involve DNA, none of unwanted DNA footprints are left in the host genome. Previously, we demonstrated that plant protoplasts can be transfected with functional RNPs and the whole plants can be regenerated from an engineered protoplast. Relative to the published methods, the revised protocols described here should help increase the success rate of whole plant regeneration by reducing damages to the naked protoplast cells.

Key words CRISPR/Cas, Ribonucleoprotein (RNP), Genome editing, Lettuce, Protoplast, Tissue culture

1 Introduction

The class 2 and type II in Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)—CRISPR associated (CRISPR/Cas) system has been developed into a robust RNA-guided genome editing tool. Different from multicomponent Class 1 effector system, SpyCas9 effector protein from *Streptococcus pyogenes* is composed of a single polypeptide of 1368 amino acid long and consists of three subdomains: two endonuclease domains (HNH and RuvC-like) and a DNA-binding domain. The Cas effector protein in complex with dual-component guide RNAs consisting of CRISPR RNA (crRNA) and trans-acting CRISPR RNA (tracrRNA) becomes a fully functional nuclease. The two RNA components can be physically fused to form a single guide RNA (sgRNA). The CRISPR/Cas9 nuclease systems were discovered in bacteria and archaebacteria as an adaptive immune system against infecting foreign mobile genetic elements such as

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phages or plasmids [1, 2]. The HNH domain cleaves the DNA strand complementary to the guide RNA (gRNA) sequence, while the RuvC-like domain cuts the other noncomplementary DNA strand [3, 4]. The action of ribonucleoprotein (RNP) complex of Cas9 and a sgRNA results in double-stranded breaks (DSBs) at 3 bp upstream of the 5′-NGG-3′ protospacer-adjacent motif (PAM) site in the target DNA [1, 4]. Such DSBs are repaired by non-homologous end joining (NHEJ) or homology-directed recombination (HDR) pathways. NHEJ is often error-prone such that it results in small insertion, deletions, or substitutions. These mutations may result in loss of function for the gene of interest.

Cas12a, formally known as Cpf1, is a class 2 type V CRISPR system found in *Prevotella* and *Francisella* [5]. Compared to Cas9, Cas12a systems possess distinct features such as single component of crRNA, recognition of 5′-TTTV-3′ PAMs, and generation of staggered DNA DSBs [5, 6]. Together, CRISPR/Cas9 and Cas12a genome editing systems shed light on various fields of biotechnology, crop breeding, and medicine.

Plants have the remarkable ability to drive cellular dedifferentiation and regeneration, which are induced from various mature somatic tissues, and whole plants can be regenerated from single protoplasts through de novo organogenesis or somatic embryogenesis [7]. Development of protoplasts into plants is cumbersome and time-consuming. However, gene editing within a single cell is the most certain way to produce edited homozygous plants in T0 generation [8]. In addition, because RNP does not contain any deoxynucleotide, theoretically, none of unwanted DNA footprints are left in the host genome.

We and others have demonstrated RNP delivery of CRISPR/Cas9 and CRISPR/Cas12a into plant cells for genome editing [8, 9]. This DNA-free RNP delivery approach is promising for plant breeding since the resulting edited crops are likely falling outside of GMO regulation. In this chapter, we describe a detailed protocol on practicing RNP-based CRISPR genome editing using lettuce as an example. It involved four major steps: (1) purification of the Cas effector proteins and guide RNA, (2) preparation of protoplasts, (3) transfection of preassembled RNPs into protoplasts, and (4) regeneration of whole plants from engineered protoplasts.

2 Materials

2.1 Plant and Other Materials

1. 20–30 seeds of lettuce (*Lactuca sativa* var. Chungchima).
2. MS salt with vitamins (M0222, Duchefa, RV Haarlem, Netherlands).

3. Razor blades (No. 10, FEATHER SAFETY RAZOR, Osaka, Japan).
4. Forceps (Cat. 3-SA, Jonostick by Regine Switzerland Standard, China).
5. Cell strainer (Cat. 93,100, SPL, Korea).
6. 1000 μL wide-bore tip (T-205-WB-C-R-S, Axygen, NY).
7. Controlled environment growth chamber 24 °C (HB103M, HanBaek Scientific Co., Korea).
8. pH meter (STARA2115, ThermoFisher Scientific, Waltham, MA, USA).
9. Sterilizer (Cat. BF-60 AC, BioFree, Korea).

2.2 PEG Transfection

2.2.1 Enzyme Solution

1. Mannitol (M0803, Duchefa, RV Haarlem, Netherlands).
2. KCl (P5405, Sigma-Aldrich, USA).
3. MES (M1503, Duchefa, RV Haarlem, Netherlands).
4. $CaCl_2$ (C3881, Sigma-Aldrich, Japan).
5. BSA (A9056, Sigma-Aldrich, USA).
6. Cellulase R-10 (Yakurt Pharmaceutical Inc., Tokyo, Japan).
7. Macerozyme R-10 (Yakurt Pharmaceutical Inc., Tokyo, Japan).

2.2.2 PEG Solution

1. PEG 4000 (81240, Sigma-Aldrich, Germany).
2. $CaCl_2$ (C3881, Sigma-Aldrich, Japan).
3. Mannitol (M0803, Duchefa, RV Haarlem, Netherlands).

2.2.3 W5 Solution

1. NaCl (7548-4405, Daejung chemicals and metals, Korea).
2. KCl (P5405, Sigma-Aldrich, USA).
3. $CaCl_2$ (C3881, Sigma-Aldrich, Japan).
4. MES (M1503, Duchefa, RV Haarlem, Netherlands).

2.2.4 MMG Solution

1. Mannitol (M0803, Duchefa, RV Haarlem, Netherlands).
2. $MgCl_2$ (M0533, Duchefa, RV Haarlem, Netherlands).
3. MES (M1503, Duchefa, RV Haarlem, Netherlands).

2.2.5 Transfection Reagent

1. Lipofectamine™ 3000 (L3000008, Invitrogen™, Carlsbad, CA, USA).
2. Plus™ reagent (11514015, Invitrogen™, Carlsbad, CA, USA).

2.3 CRISPR/Cas9 Protein Purification

1. LB agar (204010, BD, USA).
2. LB liquid (LB-05, LPS SOLUTION, Korea).

3. Kanamycin (MB-K4390, MBcell, USA).
4. BL21 Rosetta2™ (DE3) pLysS cells (Novagen, Madison, WI) and BL21 cells (Cat. 230280, Agilent Technologies, USA).
5. pET28a-SpyCas9 plasmid (#98158, AddGene, Cambridge, Massachusetts, USA).
6. Tris–HCl (TRI05, LPS solution, Korea).
7. HEPES (PHG0001-100G-KC, Sigma-Aldrich, USA).
8. PMSF (P7626, Sigma-Aldrich, Germany).
9. Imidazol (288-32-4, Merk KGaA, Germany).
10. IPTG (IPTG025, LPS solution, Korea).
11. DTT (D0632, Sigma-Aldrich, Canada).
12. Sonicator (CPX5800H-E, EMERSON, USA).
13. Histrap-HP column (GE Healthcare Life Sciences, Marlborough, MA).
14. HiPrep desalt column (GE Healthcare Life Sciences, Marlborough, MA).
15. FPLC (AKTA™ Avant 150, GE Healthcare Bio-Sciences AB, Sweden).
16. Amicon centrifugal concentrator (VIVASPIN TURBO 15, VS15T21, Sartorius, UK).
17. Centrifuge (COMBI-514R, Hanil Science Industrial, Korea).
18. Sterilizer (Cat. BF-60 AC, BioFree, Korea).
19. Incubator for 37 °C (HB-201SL, HanBaek Scientific Co., Korea).
20. Low temp shaking incubator (HB-201SL, HanBaek Scientific Co., Korea).

2.4 In Vitro sgRNA Transcription

1. A 60 mer forward oligonucleotide (Macrogen, Korea).
2. An 80 mer reverse oligonucleotide (Macrogen, Korea).
3. Q5 DNA polymerase (M0491, NEB, Ipswich, MA, USA).
4. T4 DNA polymerase (M4211, Promega, Madison, WI, USA).
5. T7 RNA polymerase (MEGAshortscript kit, AM1354, Ambion, Invitrogen, Vilnius, Lithuania).
6. MEGAclean-up (MEGAclean-up kit, AM1908, Ambion, Invitrogen, Vilnius, Lithuania).
7. HiScribe™ T7 High Yield RNA Synthesis Kit (NEB, Ipswich, MA).
8. A thermocycler (SimpliAmp Thermal Cycler, ThermoFisher Scientific, Waltham, MA, USA).

2.5 In Vitro Cleavage Assay

1. Double-stranded DNA template.
2. sgRNA.
3. SpyCas9 protein (Seoul National University, Korea).
4. Agarose (Cat.32033, iNtRON Biotechnology, Korea).
5. RedSafe (Cat.21141, iNtRON Biotechnology, Korea).
6. 6× Loading dye (B7024S, NEB, Ipswich, Massachusetts, USA).
7. Incubator at 37 °C (HB-201SL, HanBaek Scientific Co., Korea).
8. Gel electrophoresis system (MINI HD9, UVItec Cambridge, LA Abcoude, Netherlands).

2.6 Plant Regeneration

1. B5 salt (G0209, Duchefa, RV Haarlem, Netherlands).
2. MS salt (M0221, Duchefa, RV Haarlem, Netherlands).
3. Sucrose (S0809, Duchefa, RV Haarlem, Netherlands).
4. 2.4-D (D0911, Duchefa, RV Haarlem, Netherlands).
5. BAP (B0904, Duchefa, RV Haarlem, Netherlands).
6. MES (M1503, Duchefa, RV Haarlem, Netherlands).
7. $CaCl_2$ (C3881, Sigma-Aldrich, Japan).
8. Sodium succinate (S9637, Sigma-Aldrich, China).
9. NaFe-EDTA (E6760, Sigma-Aldrich, USA).
10. Low-melting agarose (A9045, Sigma-Aldrich, USA).
11. Plant Agar (P1001, Duchefa, RV Haarlem, Netherlands).
12. pH meter (STARA2115, ThermoFisher Scientific, Waltham, MA, USA).
13. Growth chamber (HB103M, HanBaek Scientific Co., Korea).
14. Sterilizer (Cat. BF-60 AC, BioFree, Korea).

3 Methods

3.1 SpCas9 or FnCpf1 Protein Purification

Plasmid vectors, pET28a-*S. pyogenes* Cas9 (SpCas9) and pET28a-*Francisella novicida* Cpf1 (FnCpf1), are transformed into the *E. coli* strain BL21 DE3. The expressible fusion protein vector contains an N-terminal His 6-tag and the SpCas9 sequence spanning amino acid residues 1-1368. The procedure can be useful for the expression and purification of SpCas9, SpCas9 variants from other bacterial species, SpCas9-fused moieties proteins, FnCpf1, FnCpf1 variants from other bacterial species, and FnCpf1-fused moieties proteins.

1. Day 1—Transform pET28a-SpCas9-BPNLS or pET28a-FnCpf1-BPNLS chemically into competent BL21 RosettaTM2 (DE3) pLysS cells: Add 10 ng of plasmid DNA to 50 μL of freshly thawed competent cells and incubate on ice for 30 min. Heat-shock cells by incubation at 42 °C for 1 min, then add 600 μL of SOC medium to the cells and incubate the culture at 37 °C for 1 h in a shaking incubator. Plate 50 μL of culture out on LB agar containing 50 μg mL/L kanamycin. Incubate the plate overnight at 37 °C.
2. Day 2—Cell culture: Grow three 25-mL seed cultures with a serial dilution (original, 1000×, 100,000× dilutions) in baffled flasks overnight. Pick one colony from the agar plate to inoculate 25 mL LB medium containing 50 μg mL/L kanamycin (original). To make 1000× dilution, transfer 25 μL into a new 25 mL LB medium containing 50 μg mL/L kanamycin. Then, to make 100,000× dilution, transfer 250 μL of the 1000× diluted medium into a new 25 mL LB medium containing 50 μg mL/L kanamycin. Incubate the preculture at 30 °C or 37 °C in a shaking incubator (250 rpm) for overnight.
3. Day 3—SpCas9 or FnCpf1 protein induction: Use 10 mL of the preculture to inoculate 500 mL prewarmed LB medium supplemented with 50 μg mL/L kanamycin in a 2 L baffled flask. The cells are cultured at 2 × 500 mL total volume at once. Incubate the cultures at 37 °C in a shaking incubator at 200 rpm while monitoring the cell growth every hour by measuring optical density at 600 nm (OD600). At an OD of 0.6~0.7, decrease the temperature to 18 °C and add 500 μL 0.5 M isopropyl-β-D-1-thiogalactopyranoside (IPTG) to each flask and continue shaking for 20 h.
4. Day 4—Cell Resuspension: Harvest cells by centrifugation at 4000 rpm for 30 min in a swing-out bucket rotor in 500 mL bottles. Decant the supernatant and resuspend the cell pellets using 25 mL ice-chilled lysis buffer (20 mM Tris–HCl (pH 8.0), 0.5 M NaCl, 5 mM imidazole, 1 mM 1,4-dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF)) per cell pellet from 1 L culture. The resuspended cell pellets can either be used directly for further purification or flash frozen in liquid nitrogen and stored at −80 °C for SpCas9 or FnCpf1 purification later.
5. Cell Lysis: Lyse the resuspended cell pellets using a probe sonicator. Pass the cell suspension through the homogenizer three to four times at 40% amplitude for 1 min to ensure complete lysis. The lysate should be cooled on ice between passes.
6. Debris removal: Clarify the lysate by centrifugation in 50 mL Nalgene Oak Ridge tubes at 15,000 rpm (~30,000 × *g*) for

60 min at 4 °C. collect the supernatant. After centrifugation, filtrate the lysate with two connected syringe filters, 1 μm and 0.45 μm, and collect the filtrate.

7. Preparation of Binding and Elution Buffers: Prepare 1 L of the binding buffer (20 mM Tris–HCl (pH 8.0), 0.5 M NaCl, 5 mM imidazole, and 1 mM DTT). Also, prepare 1 L of the elution buffer (20 mM Tris–HCl (pH 8.0), 0.5 M NaCl, 500 mM imidazole, and 1 mM DTT).
8. Purification by Histrap-HP Affinity Column: All chromatographic steps are better to be performed at 4 °C. Load 20 mL the cleared lysate on the superloop at a time. Attach the column with bound protein to an FPLC system equilibrated in binding buffer (20 mM Tris–HCl (pH 8.0), 0.5 M NaCl, 5 mM imidazole). Wash with 50 mL wash buffer at 5 mL/min until the absorbance nearly reaches the baseline again. Elute with 50 mL elution buffer (20 mM Tris–HCl (pH 8.0), 0.5 M NaCl, 500 mM Imidazole). Set the flow rate to 5 mL/min and the pressure limit to 0.3 MPa for further steps using the Histrap-HP column.
9. Collect in two 5 mL fractions: Connect a 50 mL syringe to Histrap-HP column. Wash Histrap-HP column with 10 column volumes of distilled water. Change to a new 50 mL syringe, which connects to Histrap-HP column. Equilibrate Histrap-HP column with 10 column volumes of binding buffer. Press a syringe piston to adjust the flow rate as well as FPLC flow speed (5 mL/min). Change to a new 50 mL syringe, which connects to Histrap-HP column. Load 10 mL of the filtrate into the 50 mL syringe. Press a syringe piston to adjust the flow rate as well as FPLC flow speed (5 mL/min). Harvest flow-through to observe His-protein loss. Change to a new 50 mL syringe, which connects to Histrap-HP column. Wash the column with 10 column volumes of binding buffer. Change to a new 50 mL syringe, which connects to Histrap-HP column. Add 5 column volumes of elution buffer. Fractionate every 5 mL elute. Change to a new 50 ml syringe, which connects to Histrap-HP column. Wash the column with 10 column volumes of binding buffer.
10. Desalting His-purified SpCas9 or FnCpf1 Protein: Desalt the 10 mL fractions with 10 mL of storage buffer (20 mM HEPES, 150 mM KCl, 1 mM DTT, pH 7.5, 10% (v/v) glycerol, 1 mM DTT) by 53 mL HiPrep desalt column. Fresh DTT should be added immediately prior to use. Analyze the peak fractions using SDS-PAGE.
11. Estimating protein concentration by Bradford assay: Concentrate the eluted SpCas9 or FnCpf1 protein using a 30 kDa Amicon centrifugal concentrator to a concentration required for further experiments. SpCas9 or FnCpf1 protein can be

concentrated up to 3–7 mg/mL without precipitation. The concentration is determined based on the assumption that 1 mg/mL has an absorbance at 280 nm of 0.76 (based on a calculated extinction coefficient of 120,450/M cm).

3.2 In Vitro Transcription of sgRNA or crRNA

1. Day 1—Dimerization of single-stranded sgDNA: SpCas9 can be programmed with chimeric sgRNAs, which combine the essential parts of the crRNA and tracrRNA molecules in a single oligonucleotide chain [10]. The resulting sgRNA contains a 20-mer target-specific sequence with the T7 polymerase-binding site to its upstream and the Cas9 protein-binding region to its downstream. Designing gene specific targeting sequences are done using a web tool CHOPCHOP (http://chopchop.cbu.uib.no). Our sgRNAs are designed to target within a coding region without any mismatches, and the sequences are preferably bearing GG at the 5′-end. The sequences are followed by NGG as their PAM motifs. When using dual-RNA guides, the crRNA guide is composed of a 5′-terminal 20-nt spacer sequence, followed by an invariant 76-nt guide RNA scaffold at the 3′ end (5′-XXXXXXXXXXXXXXXXXXX-GTTTTAGAGCTAGAAA-TAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTT-GAAAAAGTGGCACCGAGTCGGTGC-3′).
2. Preparation of transcription template for Cas9 sgRNA: The target-specific sgRNA sequences are synthesized with 17-mer T7 promoter region to their 5′-end, and 23-mer gRNA scaffold annealing region to their 3′-end, that the total length of the oligonucleotide to be 60-mer. For the transcribed sgRNA to have gRNA-binding region in its 3′-end, an 80-mer gRNA scaffold sequence is also synthesized separately. Then, the 60-mer and 80-mer oligonucleotides are annealed together using a thermocycler, and a complete dsDNA were synthesized using T4 DNA polymerases and the annealed dimerized oligonucleotides as the template. An alternative gRNA synthesis method is introduced (*see* **Note 1**).
3. Preparation of transcription template for FnCpf1 crRNA: A plasmid carrying T7 promoter and guide RNA scaffold is constructed. Only a target 20 bp double-stranded oligonucleotide is cloned into the end of guide RNA scaffold by two BsaI type IIS restriction enzyme from golden gate cloning method (*see* Fig. 1b). A forward single oligonucleotide should embody 5′-AGAT-3′ overhang in front of the target 20 nt, while a reverse single oligonucleotide gets initiated with 5′-AAAA-3′ in front of the reverse target 20 nt. Both one picomole of forward and reverse single oligonucleotides are mixed in 45 μL distilled water, which is transferred into 0.2 mL PCR tube, and anneal at 95 °C for 5 min and 55 °C for 10 min by a thermocycler, then place annealed double-stranded

Fig. 1 Schematic of cloning sites for guide RNAs of SpCas9 and FnCpf1. (**a**) For sgRNA of SpCas9, two *Bsa*I enzyme sites are placed between the T7 promoter and sgRNA scaffold sequences. (**b**) For FnCpf1 crRNA, two *Bsa*I enzyme sites are at downstream of crRNA scaffold sequence

oligonucleotides (dsODN) on ice. As a result, the dimerized oligonucleotides are employed to clone into a linear plasmid with two flanking sequences, 5′-ATCT-3′ and 5′-TTTT-3′. The completed construct is used to synthesize sgRNAs as templates. An alternative gRNA synthesis method is introduced (*see* **Note 2**).

4. Day 2—Amplification of dsDNA templates for sgRNAs by PCR amplification: Transcription templates for sgRNA synthesis can be PCR amplified from plasmid or synthetic oligonucleotide templates with appropriate PCR primers (A forward primer is 5′-AATTCTAATACGACTCACTATAGG-3′, which has additional five AATTC nt in front of T7 promoter sequence and a reverse primer is from end of sgRNA scaffold 5-′-GCACCGACTCGGTGCCACTT-3′). The high amount of dsDNA template is obtained simply by PCR performance. Q5® polymerase is used to amplify transcription templates. PCR products should be subjected to DNA electrophoresis to estimate concentration and to confirm amplicon size prior to its use as a template in the T7 RNA transcription synthesis. PCR mixture may be used directly if diluted at least 10× in the transcription reaction. However, better yields will be obtained with purified PCR products. PCR products can be purified according to the protocol for commercial clean-up kit instruction. Details of PCR are shown as follows.

PCR program		
95 °C	3 min	1×
95 °C	15 s	
60 °C	30 s	35×
72 °C	30 s	
72 °C	10 min	1×
17 °C	Hold	

PCR components		
Component	**Amount**	**Volume**
Template	Plasmid DNA or oligodimers (30 ng/μL)	1 μL
Forward Primer	5 mM	1 μL
Reverse Primer	5 mM	1 μL
dNTP	2.5 mM dNTP	1 μL
Reaction buffer	5× Q5® polymerase buffer	5 μL
Polymerase	Q5® polymerase	0.5 μL
Water	14.5 μL	
Final reaction volume	25 μL	

5. sgRNA transcription by T7 RNA polymerase: Generally, 1.4 μM (1 μg of a 120 bp PCR product or annealed dsODN) can be used in a 20 μL in vitro transcription reaction. Employing 1 μg templates is critically required to harvest 100 μg sgRNAs with above 1 μg/μl high concentration (*see* **Note 3**). Thaw MEGAshortscript T7 Transcription Kit or HiScribe™ T7 High Yield RNA Synthesis Kit components, mix and pulse-spin in microfuge to collect solutions to the bottoms of tubes. Keep on ice. Assemble the reaction at room temperature as follows.

Transcription components		
Name	**Component**	**Volume**
Template	PCR products or oligodimers (100 ng/μL)	8 μL
dATP	T7 ATP solution (75 mM)	2 μL
dCTP	T7 CTP solution (75 mM)	2 μL
dTTP	T7 TTP solution (75 mM)	2 μL
dGTP	T7 GTP solution (75 mM)	2 μL
Polymerase	T7 enzyme mix	2 μL
Polymerase buffer	10× T7 reaction buffer	2 μL
Final reaction volume 20 μL		20 μL

Mix thoroughly and pulse-spin in a microfuge. Incubate at 37 °C for 4 h or longer (O/N available) for maximum yield. It is safe to incubate the reaction for 16 h. Amount of sgRNA may be synthesized sufficiently in 4 h. It is recommended to incubate in a thermocycler to prevent evaporation of the sample. DNase is applied to remove DNA template. To remove template DNA, add 20 μL nuclease-free water to each 20 μL reaction, followed by 2 μL of DNase I (RNase-free), mix and incubate for 15 min at 37 °C.

6. Day3—Clean-up sgRNA: After 15 min, transcripts are cleaned up through MEGAclean-up kit. The products are transferred into a new 1.5 mL tube. Added 100 μL with Elution Solution. Mix and add 350 μL of Binding Solution Concentrate to the sample. Mix by pipetting, add 250 μL of 100% ethanol to the sample and mix by pipetting. Follow the manual of MEGAclean-up kit, the mixed samples are transferred into spin-down column/2 mL tube. Centrifuge at 12,000 rpm for 1 min. The flow-through is discarded. Add 500 μL of washing solution. Centrifuge at 12,000 rpm for 1 min. Discard the flow-through. Repeat one more time, add 500 μL of washing solution. Centrifuge at 12,000 rpm for 1 min. Discard the flow-through. The spin-column/2 mL tubes are centrifuged at 12,000 rpm for 1 min. The spin-column only is transferred into a new 1.5 mL tube. Add 50 μL of water into the spin-column/1.5 mL tube each. Put the spin-column/1.5 mL tubes on the heat-block at 70 °C for 10 min. After 10 min, the spin-column/1.5 mL tubes are centrifuged at 12,000 rpm for 1 min. Add additionally 50 μL of water into the spin-

column/1.5 mL tube each. The flow-through is measured the concentration of sgRNA.

7. <Alternative> Clean-up crRNA for FnCpf1: After 15 min, transcript products are also cleaned up through ethanol precipitation. The ethanol precipitation is recommended to precipitate sgRNAs with smaller size RNA than 100 nt. FnCpf1 crRNA size is 66 nt being much smaller than 100 nt, which is a preparation limit when using MEGAclean-up kit. Add 1/10 volume 3 M sodium acetate of PCR products to PCR products, and invert for mixing gently. Add ice-chilled 100% ethanol to each sample tube. Incubate the sample tubes in −20 °C for 30 min. Centrifuge the precipitated sgRNAs at 14,000 rpm (16,900 × *g*) for 10 min at 4 °C. Remove supernatant and wash the RNA pellet with 200 μL ice-chilled 70% ethanol. Centrifuge for 1 min, remove supernatant, and air-dry RNA pellets for 5 min. Dissolve the RNA pellet in 50 μL RNase-free water. RNA concentration can be determined by measuring the ultraviolet light absorbance at 260 nm.

3.3 Protoplast Transformation and Regeneration

3.3.1 Prepare Plants and Reagents for Protoplast Transformation

1. Sterilize lettuce seeds by 2% sodium hypochlorite (Clorox) for 10 min.
2. Wash seeds five times with sterile dH_2O.
3. Plant the sterile seeds on ½ MS media. Lettuce leaves can be harvested 5 days after germination for protoplast preparation.
5. Make 40 mL enzyme solution with ingredients as follows:

0.4 M Mannitol
20 mM KCl
20 mM MES (pH 5.7)
1.5% Cellulase R-10 (Yakurt)
0.3% Macerozyme R-10 (Yakurt)

6. Incubate at 55 °C for 10 min.
7. Add to make 10 mM $CaCl_2$ and 0.1% BSA.
8. Filter enzyme solution through a 0.45 μm syringe filter.

3.3.2 Protoplast Preparation

1. Cut 10–15 leaves from lettuce plantlets with a razor. Pile two or three leaves on a droplet of sterile water. Slice piled leaves together.
2. Pour a 20 mL enzyme solution into a 90 mm diameter plate. Transfer sliced 15 leaves in a 20 mL enzyme solution. Cover it with Aluminum foil.
3. Place the 90 mm plate at gyratory shaker with 50 rev/min. Incubate the plate for 4–5 h.

4. Pour the enzyme solution with protoplasts in a round tube. Add the same volume of W5 solution to the 20 mL enzyme solution (W5 ingredients shown as follows).

W5 solution
154 mM NaCl
125 mM $CaCl_2$
5 mM KCl
2 mM MES (pH 5.7)

5. Flow the 40 mL enzyme solution containing protoplasts through a 100 μm cell strainer into a 50 mL round tube.
6. Remove the cell strainer.
7. Centrifuge the 50 mL tube at 100 g (or 80 g in Hanil centrifuge, Korea) for 5 min.
8. Remove the supernatant using a 20 mL long pipette.
9. Add a 1 mL of MMG solution (ingredients shown below).

MMG solution	5 mL	10 mL
0.8 M Mannitol	2.5 mL	5 mL
300 mM $MgCl_2$	0.25 mL	0.5 mL
200 mM MES (pH 5.7)	0.1 mL	0.2 mL

10. Count protoplasts with a hematocytometer.
11. Adjust cell number up to 2×10^6/mL by adding MMG solution.
12. Aliquot 200 μL containing 2×10^5/mL protoplasts into a 1.5 mL tube (*see* Fig. 2a, b).

3.3.3 Protoplast Transformation and Regeneration

1. Set up a 20 μL transformation reaction in 1.5 mL tube as follows.

RNP	2×10^5/mL protoplasts
sgRNA	5 μg
Cas9 protein	10 μg
Plus reagent™	2 μL
Lipofectamine™ 3000	2 μL
NEB Buffer 3.1	2 μL
dH_2O up to	20 μL

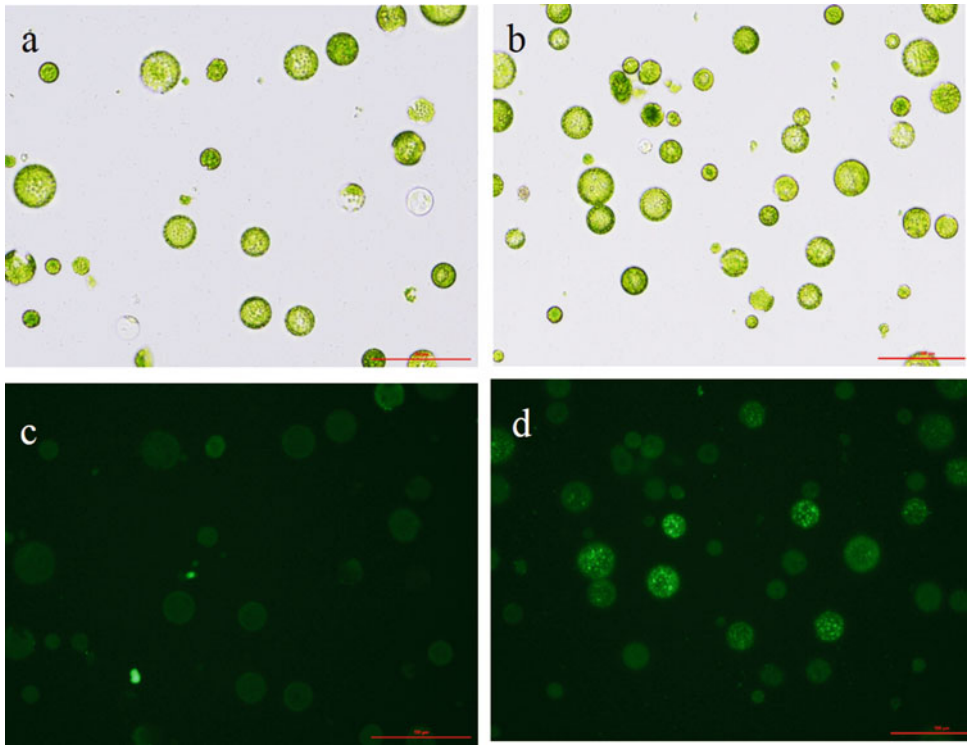

Fig. 2 Morphology of lettuce protoplasts after transfection with traceable GFP-labeled CRISPR/Cas9. (**a**, **c**) Protoplasts conventionally transfected with GFP-SpyCas9 RNPs and PEG 4000. Microscopic images are shown under bright field (**a**) and confocal laser scanning (**c**). (**b**, **d**) Protoplasts after transfection supplemented with Lipofectamine™ 3000 and the Plus™ reagent. Bright field (**b**) and confocal image (**d**)

Both Lipofectamine™ 3000 and Plus reagent™ transfection reagents are utilized for RNP delivery with PEG 4000. RNP combination can be replaced by Cpf1/other Cas proteins. GFP-Cas9 is employed to help to trace Cas9 localization instead of Cas9 in this study (*see* Fig. 2c, d).

2. Incubate RNP transformation mixture for 10 min at room temperature.
3. Aliquot 200 μL protoplast solution with a 1000 μL wide bore tip into a clean 1.5 mL tube.
4. Add the RNP mixture into the 200 μL protoplast solution, then mix gently.
5. Add the same volume (220 μL) of 40% PEG solution (shown below) into RNP-protoplast solution.

40% PEG solution	5 mL	10 mL
0.8 M Mannitol	1.25 mL	2.5 mL
1 M $CaCl_2$	0.5 mL	1 mL
PEG 4000	2 g	4 g
dH_2O up to	5 mL	10 mL

6. Pipette the RNP-protoplast-PEG solution 5–10 times gently.
7. Place the RNP-protoplast-PEG solution for 10 min at room temperature.
8. Add an 800 μL W5 solution into the RNP-protoplast-PEG solution, then invert four to five times.
9. Centrifuge at 100 g for 1 min in a large tabletop centrifuge, then discard the supernatant.
10. Add 400 μL W5 solution and sequentially, add 400 μL Plant Induction Medium (PIM) without sucrose into a protoplast pellet.
11. Centrifuge at 100 g for 1 min in a large tabletop centrifuge, then discard the supernatant.
12. Add a 500 μL Protoplast Induction Media (PIM) (shown below) into a protoplast pellet and resuspend the pellet.
13. Mix protoplasts with 2.4% low-melting gel in PIM.

PIM	1 L
1/2 B5 medium	1.58 g
Sucrose	103 g
2,4-D	0.2 mg
BAP	0.3 mg
MES	0.1 g
$CaCl_2 \cdot 2H_2O$	375 mg
NaFe-EDTA	18.35 mg
Sodium succinate	270 mg

14. Transfer protoplasts in 500 μL PIM (with sucrose) into a 6-well plate (3.5 cm diameter) with 1000 μL wide bore tips.
15. Add 500 μL PIM (with sucrose) containing 2.4% low-melting gel.
16. Plate the mixture, PIM and low-melting gel, using Bergmann's cell plating technique.
17. Change PIM solution every week.

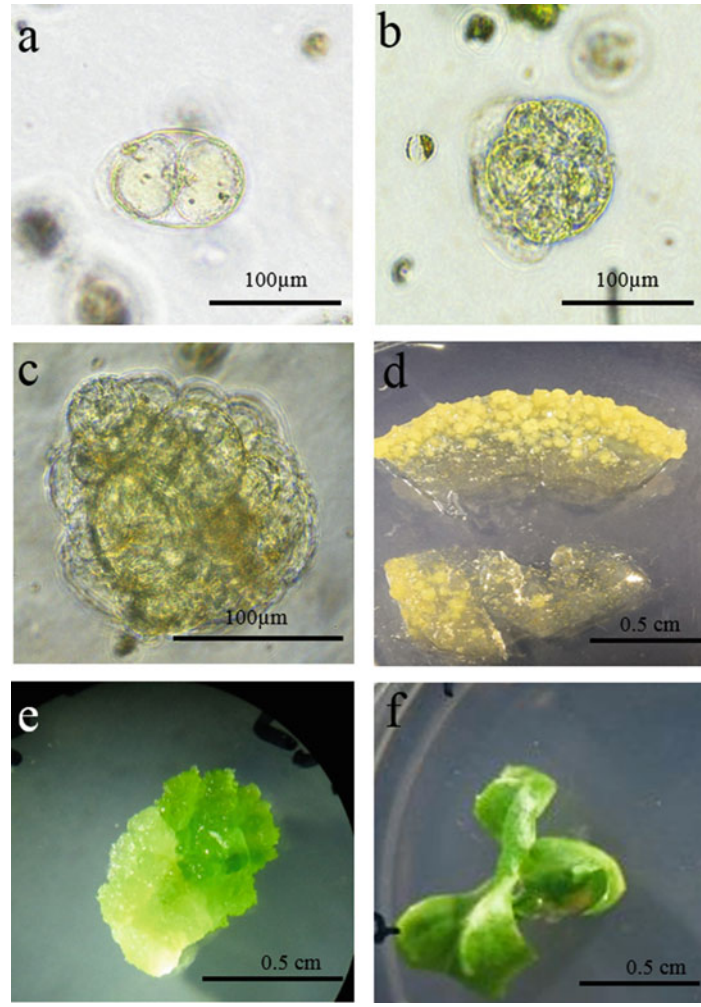

Fig. 3 Time course morphology of regenerating protoplasts. (**a**) Five-day-old protoplasts after transfection with RNPs; the protoplasts are doubled at 5 days. (**b**) The protoplasts form colonies at seventh day. (**c**) Microcalli. (**d**) Calli. (**e**) Calli turn green after 4 weeks under light. (**f**) Plantlets with shoots regenerated. Bars = 100 μm and 0.5 cm

18. After 4 weeks, transfer low-melting agar with microcalli to Shoot Induction Media (SIM) (*see* Fig. 3d).

SIM	1 L
MS powder	4.4 g
Sucrose	30 g
0.1 mg NAA	100 μL (1 mg/mL stock)
0.5 mg BAP	500 μL (0.1 mg/mL stock)
Plant agar	6 g

19. After 4 weeks, transfer calli from SIM to MS media and keep under light (*see* Fig. 3e).
20. When shoots emerge, transfer the tiny plantlets into MS media (*see* Fig. 3f).
21. Transfer rooted plants to soil.
22. Screen edited events among regenerated plants (*see* **Note 4**).

4 Notes

1. An alternative method for preparing transcription template for SpCas9 sgRNA: A plasmid carrying T7 promoter and guide RNA scaffold is constructed. Only a target 20 bp double-stranded oligonucleotide is cloned into the middle of two *Bsa*I sites (A↓TAGGTGAGACCGCAGGTCTCG↓GTTTT) placed between T7 promoter and guide RNA scaffold by two BsaI type IIS restriction enzyme from Golden Gate cloning method (*see* Fig. 1a). A forward single oligonucleotide should embody 5′-TAGG-3′ overhang in front of the target 20 nt, while a reverse single oligonucleotide gets initiated with 5′-CAAA-3′ in front of the reverse target 20 nt. Both one picomole of forward and reverse single oligonucleotides are mixed in 45 μL distilled water, which is transferred into 0.2 mL PCR tube, and anneal at 95 °C for 5 min and 55 °C for 10 min by a thermocycler, then place annealed oligonucleotides on ice. As a result, the dimerized oligonucleotides are employed to clone into a linear plasmid with two flanking sequences, 5′-CCTA-3′ and 5′-GTTT-3′. The completed construct is used to synthesize sgRNAs as templates.
2. An alternative method to prepare a transcription template for FnCpf1 crRNA: Synthesize two 63 nt single-stranded oligonucleotides, which compose of 5 nt overhang in front of T7 promoter, 19 nt T7 promoter, and 20 nt target spacer sequence. Both 10 μL of 200 nmol of forward and reverse single oligonucleotides are mixed, and the 20 μL mixture is transferred into a 0.2 mL PCR tube and annealed at 95 °C for 5 min and 55 °C for 10 min by a thermocycler, then place annealed dsODN on ice.
3. It is strongly recommended to wear gloves and use nuclease-free tubes and reagents to avoid RNase contamination. Reactions are typically 20 μL but can be scaled up as needed. Reactions should be assembled in nuclease-free micro centrifuge tubes or PCR strip tubes.
4. In the earlier chapters of this book, different genotyping methods are described for screening CRISPR-induced mutations. Readers can refer to these chapters for details.

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Chapter 26

An Automated Protoplast Transformation System

Scott C. Lenaghan and C. Neal Stewart Jr.

Abstract

Efficient plant protoplast production from cell suspension cultures, leaf, and stem tissue allows for single-cell plant biology. Since protoplasts do not have cell walls, they can be readily transformed to enable rapid assessment of regulatory elements, synthetic constructs, gene expression, and more recently genome-editing tools and approaches. Historically, enzymatic cell wall digestion has been both expensive and laborious. Protoplast production, transformation, and analysis of fluorescence have recently been automated using an integrated robotic system. Here we describe its use for bulk protoplast isolation, counting, transformation, and analysis at very low cost for high-throughput experiments.

Key words Tobacco, Protoplasts, Transformation, Enzymatic digestion, High-throughput screening, Automation, Robotics

1 Introduction

Significant effort has been placed on generating crops with advantageous traits, including disease resistance [1], herbicide resistance [2], drought [3, 4] and salt tolerance [5], increased biomass [6], and altered cell wall structure [7]. With the advent of a new generation of tools for molecular breeding, including CRISPR-Cas9 and TALENs [8], along with more traditional gene silencing tools, such as dsRNA [9], miRNA [10], and siRNA [11], a bottleneck has been created whereby more plants can be generated than could possibly be screened. In particular, the low cost of generating guide RNA targets (gRNAs) for CRISPR-Cas9 means that researchers can generate hundreds to thousands of constructs, and thus could theoretically rapidly generate similar number of transgenic plants. Unfortunately, while the technical and cost barriers for generation of the constructs are decreasing, the costs and space requirements to screen thousands of plants is extremely high. Further, many of these targets will lead to undesirable effects that ideally would be identified at an earlier stage. As such, plant protoplasts have emerged as plants' answer to single-cell biology. Protoplast

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platforms are especially useful for high-throughput gene expression assays, for genome-editing, gene silencing, and a variety of other molecular breeding approaches.

Protoplasts have several advantages compared to the use of intact plant tissue or cell suspension cultures. The primary advantage is the lack of a cell wall, which is a significant barrier that leads to the generally poor transformation efficiency of plant cells [12]. In addition, protoplasts represent a true single-cell culture, as opposed to plant cell suspension cultures and plant tissue, which are multicellular. The final advantage of protoplasts is the potential to extract protoplasts from nearly any organs and tissues from whole plants, thus representing the developmental and spatial features inherent in those organs and tissues. This enables the identification of tissue-specific expression, the assessment of functional chloroplasts, and the ability to look at gene expression from different tissues. In addition, protoplasts can also be isolated from homogeneous cell suspension cultures, which are primarily derived from callus [13]. Based on the utility of protoplasts for early screening of molecular targets, a protocol was developed for high-throughput, automated protoplast isolation, transformation, and screening [14]. In this work the widely used tobacco (*Nicotiana tabacum* L.) 'Bright Yellow' 2 (BY-2) suspension culture was used as a model to demonstrate the approach.

In this chapter, we will describe a general protocol for automated protoplast isolation, PEG-mediated transformation, and screening using BY-2 as the model system. While any automated protocol will be dependent on the equipment available to carry out the procedures, we will focus on the high-level equipment that would be required to carry out such a protocol.

2 Materials

As this chapter is focused on the development of an automated protocol for protoplast isolation and transformation, specialized equipment is required to carry out the procedure. To provide the reader with an idea of the required setup, a schematic of the robotic platform developed in our lab is shown in Fig. 1, which is based on microtiter plates. The essential components of the system include a plate mover (Thermo Scientific™ Orbitor™ RS microplate mover) to transfer the plates between equipment, a plate shaker (Thermo Scientific™ Teleshake magnetic microplate shaker), a plate heater/chiller (two InHeco peltier CPAC ultraflat HT 2-TEC microplate heater/chillers and one InHeco Multi-TEC controller), a large-volume liquid handler (Biotek MultiFlo FX multi-mode dispenser), a tip-based liquid handler (Agilent Bravo automated liquid handling system), and a plate reader (Biotek Synergy H1 hybrid multi-mode reader). In addition, the system must be housed in a

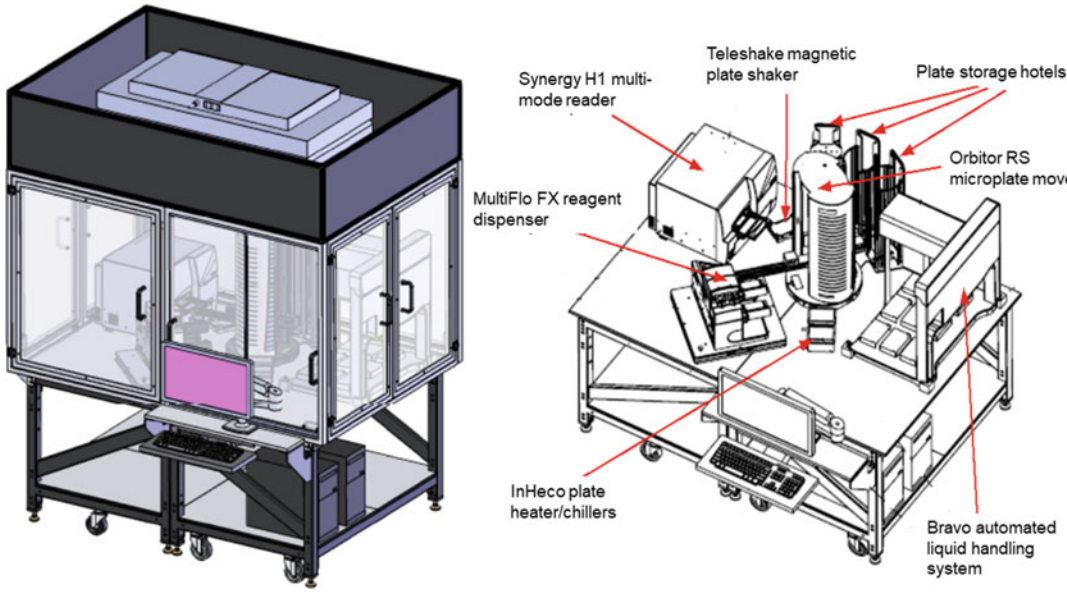

Fig. 1 Schematic of a robotic system for automated protoplast isolation, transformation, and screening. The central component is the Orbitor RS microplate mover, which can transfer plates to any of the pieces of equipment, including one nest on the Agilent Bravo. Protoplast isolation is primarily carried out using the Orbitor, MultiFlo FX reagent dispenser, the Teleshake plate shaker, and the InHeco peltier plate heater/chillers. Transformation is carried out using all pieces of equipment, with the exception of the Synergy H1 plate reader, which is used for screening. The Agilent Bravo is the primary piece of equipment used for transfer of protoplasts between the different containers, and also for dispensing reagents <70 μL. Upon completion of transformation and incubation for >18 h, plates are screened using the microplate reader to determine the efficiency of transformation

contained environment that ensures sterility throughout the entire procedure. Many system designs can be used to accomplish the methods described herein, thus the specific equipment will be annotated generally as described.

2.1 Cell Culture

1. BY-2 liquid culture media: Weigh 4.43 g Linsmaier and Skoog (LS) basal media (*see* **Note 1**), 30 g of sucrose, 200 mg, KH_2PO_4, and 200 μg of 2,4 dichlorophenoxyacetic acid (2,4-D) and add to a 2 L beaker. Add 900 mL of Milli-Q water and pH to 5.8 with 0.1 M KOH. Make up to 1 L with water and autoclave. Store at 4 °C for up to 2 weeks.
2. BY-2 solid culture media: Add 1% agar to BY-2 liquid media, mix, and autoclave. Pour plates prior to solidification of the agar.
3. BY-2 callus.
4. Wide bore 1.0 mL pipet tips.

2.2 Protoplast Isolation

1. Digestion buffer: 0.4 M mannitol, 5 mM $CaCl_2$, 12 mM sodium acetate, pH 5.7.
2. Protoplasting enzymes: Rohament CL (cellulase), Rohapect UF (pectinase), and Rohapect 10 L (pectinase/arabinase) (*see* **Note 2**).
3. Complete protoplast isolation solution: Add 160 μL of Rohapect CL, 24 μL of Rohapect 10 L, and 7 μL of Rohapect UF to 20 mL of digestion buffer and vortex.
4. Propidium iodide.

2.3 PEG-Mediated Transformation

1. Mmg solution: 0.4 M mannitol, 100 mM $MgCl_2$, 4 mM MES, pH 5.7.
2. PEG solution: Dissolve 4 g of PEG 4000 in 6.5 mL of Mmg solution and vortex.
3. W5 solution: 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, and 2 mM MES, pH 5.7.
4. Wide bore 1.0 mL pipet tips.
5. Deep 96-well, 1.2 mL plates.
6. Plasmid DNA: 1 μg/μL, $A_{260/280} > 1.8$.

3 Methods

3.1 Cell Culture

1. Propagate BY-2 callus on BY-2 solid culture media prior to establishment of cell suspension culture (*see* **Note 3**).
2. Initiate liquid suspension culture by adding a single callus piece, >1 cm, to a 250 mL Erlenmeyer flask containing 100 mL of BY-2 liquid medium and seal with aluminum foil. Incubate the culture at 28–30 °C with constant shaking in the dark for 5 days.
3. Subculture 2.0 mL of BY-2 cell suspension culture into 98 mL of BY-2 liquid medium in a 250 mL Erlenmeyer flask. Incubate the culture at 28–30 °C with constant shaking in the dark for 5–7 days (*see* **Note 4**).
4. Collect cells for protoplast isolation by thoroughly mixing the flask prior to transfer of 6.0 mL of the culture into a 15 mL conical bottom tube. Allow the culture to settle for ~10 min. Adjust the packed cell volume to 3.0 mL through removal of the supernatant (*see* **Note 5**).
5. Vortex the 15 mL conical bottom tube to thoroughly mix the cell suspension, and transfer 500 μL to each well of a 6-well plate for protoplast isolation (*see* **Note 6**).

3.2 Protoplast Isolation

1. Load 6-well plate(s) containing the cell suspension cultures onto the plate mover (*see* **Note 7**).
2. Add 2000 μL of complete protoplast isolation solution to each well of the 6-well plate using a large-volume liquid handler.
3. Move 6-well plate to plate heating station and incubate at 37 °C for 5 min.
4. Move 6-well plate to plate shaker and shake at 500 rpm for 5 min.
5. Repeat **steps 3** and **4** for a total of 18 loops (*see* **Note 8**).
6. Move 6-well plate to plate chiller and incubate at 4 °C for 5 min (*see* **Note 9**).
7. Move 6-well plate to plate shaker and shake at 800 rpm for 5 min. Move 6-well plate to tip-based liquid handler and transfer 70 μL of protoplasts from the 6-well plate to each well of a 96-well plate.
8. Pipet 70 μL of ethanol into the same wells previously loaded with protoplasts to fix the cells for counting. Allow >10 min at room temperature to fix and permeabilize the protoplasts.
9. Add 14 μL of propidium iodide (PI) to each of the fixed wells to label the nuclei of protoplasts. The binding of PI to the nucleus enables the use of a plate reader to determine the number of protoplasts in each well when compared to a previously generated standard curve (*see* **Note 10**).
10. Move the 96-well plate to the plate reader and measure the fluorescence (536 nm excitation, 620 nm emission) of propidium iodide in the well. All wells are compared to blank wells containing BY-2 liquid medium and propidium iodide.
11. Compare the fluorescence reading with the previously generated standard curve to determine the concentration of protoplasts in each well (*see* **Note 11**).

3.3 PEG-Mediated Transformation

1. Pipet 10 μL of plasmid DNA into each well of a deep 96-well plate (*see* **Note 12**).
2. Move 6-well plate containing protoplasts to the plate shaker and shake at 800 rpm for 5 min. Move 6-well plate to tip-based liquid handler and transfer 70 μL of protoplasts from the 6-well plate to each well of a deep 96-well plate (*see* **Note 13**).
3. Transfer 70 μL of PEG solution into each well of the deep 96-well plate containing protoplasts (*see* **Note 14**). The final concentration of PEG in each well should be ~20%, depending on the volume of DNA added.
4. Move the deep 96-well plate to the plate shaker and shake at 1500 rpm for 30 s (*see* **Note 15**).

5. Incubate at room temperature without shaking for at least 20 min to allow DNA to be taken up by the protoplasts.
6. Move deep 96-well plate to large-volume liquid handler and add 300 μL of W5 solution to each well and mix. Add an additional 400 μL of W5 to each well (a 1:10 dilution of PEG in W5 is achieved in this protocol) (*see* **Note 16**).
7. Move deep 96-well plate to plate shaker and shake at 1500 rpm for an additional 1 min to ensure complete mixing.
8. Incubate protoplasts for >1 h to allow protoplasts to settle to the bottom of the well. Transfer 200 μL of transformed protoplasts to a 96-well fluorescent screening plate and incubate for 18–24 h (*see* **Note 17**).
9. Move 96-well fluorescent screening plate to plate reader and measure the expression level of the fluorescent reporter gene (*see* **Note 18**).

4 Notes

1. Powdered media described in this work is typically purchased from Phytotech Laboratories® to prevent batch-to-batch variation of complex plant media. In addition, while LS media is appropriate for culture of BY-2 tobacco cells, different media would be required for callus and cell suspension media from other plant species.
2. The use of low-cost food-grade enzymes is critical to high-throughput protoplast isolation [14, 15], as there is significant dead volume in the lines of liquid handlers, and typical lab-grade enzymes are costly. Best results have been achieved using enzymes purchased from AB Enzymes.
3. For long-term maintenance of BY-2 cultures, it is recommended that callus be grown on solid media, as liquid cultures grow more rapidly. It is also possible to cryopreserve BY-2 cultures and thaw as needed [16].
4. Typically cell cultures are maintained in the dark; however, it is possible to obtain "green" cell suspension cultures in a variety of species by adjusting the media components and growing the cultures in the light. While this is not possible for BY-2, it may be advantageous for other cell suspension cultures.
5. The packed cell volume used in this protocol has been validated to maximize digestion of BY-2 cells, and would need to be adjusted if using cell suspension cultures from another species or changing the enzymes used for digestion.
6. Wide-bore pipets or serological pipettes should be used to transfer the cells at this stage, as the cells are dense and will clog standard pipet tips.

7. Numerous plate movers exist, and in robotic systems accomplish the automated movement of plates between the various pieces of equipment. As this protocol is generally describing what would be required for automation, specific instrumentation will not be annotated.
8. It is possible to achieve both incubation and shaking on a single device; however, no such device is used in the current protocol. In the event that shaking and incubation is accomplished on a single device, the cells would be incubated for ~3 h.
9. In order to prevent over-digestion of the cells, it is necessary to inactivate the enzymes by decreasing the temperature of the reaction. If the enzymes are allowed to continuously digest the cells, the overall viability of the protoplasts will be significantly decreased.
10. A variety of fluorescent dyes could be used to count the number of protoplasts, and it is possible to determine the protoplast viability using a combination of metabolically active dyes, such as fluorescein diacetate, in combination with propidium iodide.
11. A standard curve should be generated manually using a fluorescent viability dye, in this case propidium iodide, to calibrate the plate reader. Briefly, protoplasts should be isolated and concentrated to 1×10^6 protoplasts/mL in a volume of 1 mL. Protoplasts can then be fixed, stained with propidium iodide, and serially diluted across a plate. By plotting the cell concentration vs. fluorescence, a standard curve is generated that can be used to determine the number of protoplasts isolated in the automated procedure.
12. In the current protocol, the plasmid DNA used is at a concentration of 1 μg/mL, thus a total of 10 μg of DNA is used per transformation. It is important to use at least this much DNA, but a smaller volume will not affect the transformation efficiency.
13. The volume of protoplasts transferred, 70 μL, is the maximum volume of the tip-based liquid handler used in this protocol. If another device is used, the volume could be adjusted accordingly.
14. The PEG solution is highly viscous, thus it is important to slowly pipet the solution so that the volume is accurate. This is especially important if using a liquid handler, where the pipetting speed should be adjusted to account for the increased viscosity.
15. The PEG solution rapidly settles on the bottom of the well and thus complete mixing of protoplasts, DNA, and PEG requires additional mixing.

16. The PEG solution at the concentration required for transformation, 20%, is toxic to protoplasts after prolonged exposure, thus it is necessary to dilute the PEG after the transformation procedure is been completed.
17. To increase the transformation efficiency, and to concentrate the protoplasts prior to screening, plates can be centrifuged at $100 \times g$ to pellet the protoplasts, followed by removal of the supernatant and resuspension in a smaller volume.
18. In the current protocol, expression of a fluorescent protein reporter is used as the output; however, the output is not limited to fluorescent protein expression.

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