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Evaluation of diploid potato germplasm for applications of genome editing and genetic engineering

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Abstract

Conventional breeding and genetics in a vegetatively propagated, autotetraploid, highly heterozygous recalcitrant crop species such as potato is challenging. Diploid inbred lines in potato will serve as an invaluable source of material for breeding and functional genetics. Most of the available diploid germplasm in potato is either self-incompatible and/or is not amenable to genetic engineering. Traits including self-compatibility, tuber quality and amenability to genetic transformation are critical for current potato breeding and genetics. This study evaluates selected diploid potato germplasm from various breeding programs in the United States including 1S1, DMRH-S5-28–5, M6, MSEE720-04, MSEE737-05, MSEE824-04, and UW-W4 for the above traits. Our results indicate that diploid lines 1S1, MSEE737-05 and UW-W4 have tissue culture regeneration efficiency of over 75%, are amenable to *Agrobacterium*-mediated genetic transformation, produce viable fruit upon selfing and form tubers with desirable appearances.

Resumen

La reproducción convencional y la genética en una especie de cultivo recalcitrante altamente heterocigota, autotetraploide y propagada vegetativamente, como la papa, es un desafío. Las líneas endogámicas diploides en la papa se convertirán en una fuente invaluable de material para mejoramiento y análisis funcionales. La mayor parte del germoplasma diploide disponible en la papa es autoincompatinable y / o no es susceptible a la transformación genética. Los rasgos que incluyen la autocompatibilidad, la calidad del tubérculo y la capacidad de transformación genética son críticos para el mejoramiento y la genética actuales de la papa. Este estudio evalúa germoplasma de papa diploide seleccionado de varios programas de mejoramiento en los Estados Unidos, incluidos 1S1, DMRH-S5-28–5, M6, MSEE720-04, MSEE737-05, MSEE824-04 y UW-W4 para los rasgos mencionados anteriormente. Nuestros resultados indican que las líneas diploides 1S1, MSEE737-05 y UW-W4, los cuales tienen una eficiencia de regeneración de cultivo de tejidos de más del 75%, son susceptibles de transformación genética mediada por *Agrobacterium*, producen frutos viables al ser autopolinizadas y forman tubérculos con apariencias deseables.

Keywords Regeneration · *Agrobacterium*-mediated transformation · Self-Compatibility · Diploid

Introduction

Advancements in breeding of cultivated potato (*Solanum tuberosum* L. 2n = 4x = 48) have been limited by autopolyploidy and the presence of pre- and post-zygotic barriers limiting the introgression of new traits into cultivated germplasm (Jansky et al., 2016; Lindhout et al. 2011). In addition, polyploid genotypes have limited use in functional analyses because of the prevalence of paralogs and orthologs with functional redundancy and dosage effects (The Potato Genome Sequencing Consortium 2011). Due to these limitations, there is still an abundance of economically

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important-associated genes in potatoes whose functions have yet to be understood. To address these limitations, breeding and genetic engineering approaches are being employed in potato to create diploid self-compatible inbred lines that are more accommodating for forward and reverse genetic strategies (Jansky et al. 2016; Enciso-Rodriguez et al. 2019; Ye et al. 2018). The advantages of a diploid breeding system include ease of mapping with structured populations, potential for heterosis through F1 hybrids, introgression of resistance/tolerance genes from wild relatives, among others. At the diploid level, genome editing, an innovative technique to modify DNA, can be readily employed to introduce advanced traits and study gene function. However, this requires germplasm that is amenable to regeneration and genetic transformation. Having improved diploid germplasm that is amenable to these techniques will aid in further advances in both basic and applied research in potato.

Genome editing is a powerful and versatile technique that can make targeted changes to a genome, which can aid forward and reverse genetic approaches (Ben-Amar et al. 2016). Genome editing by CRISPR/Cas9 is also a new breeding technique and has already proven to be powerful in the development of advanced lines as previously reported with approaches like de novo domestication, introduction of herbicide resistance, and improvement of quality traits (Zsögön et al. 2018; Butler et al. 2016; Nadakuduti et al. 2018). In addition, in the US, genome edited food crops are eligible to be exempt from regulation, given they are free of regulated recombinant DNA (Hoffman 2021). This may expedite various applications of genome editing in potato that have commercial potential.

Currently, the most widely used line for genetic engineering in potatoes is the tetraploid *S. tuberosum* L. cv. Desiree (Sevestre et al. 2020). Although this line is highly amenable to standard transformation methods, its genetic redundancy due to polyploidy makes studying gene function challenging. This redundancy has also led to limitations in the design and recovery of events in applications of genome editing (Nicolia et al. 2015; Clasen et al. 2016; Veillet et al. 2020; González et al. 2020). Applications of genome editing to study gene function or introduce advanced traits is much simpler at the diploid level because there are fewer allelic and copy number variants to consider. Although many factors control the efficiency of gene editing reagents, a solution is to utilize diploid lines to simplify the limitations that are inherent to gene editing of a polyploid.

The many benefits of working at the diploid level can then be further leveraged by identifying and developing a diploid line that can be a model organism for basic and applied research in potato. Characteristics of model organisms for genetic research include fertility, small genome size, amenability to regeneration and transformations, and the presence of morpho-agronomic and quality-related traits with economic value (Chang et al. 2016). The reduced cost and ease of genome sequencing, complemented by modern reverse genetic approaches like genome editing, has led to a broadened list of emerging model plant organisms (Chang et al. 2016). In Solanaceae, model species such as tomato (*Solanum lycopersicum*) and petunia (*Petunia hybrida*) have been used to study traits like fruit ripening, floral development, domestication and evolution (Tomato Genome Consortium 2012; Vandenbussche et al. 2016). However, in order to study traits that are unique to potato such as resistances against biotic and abiotic factors, tuber quality-related traits and self-fertility there must be germplasm that can be used as a model for functional analysis.

Currently, the most important bottleneck for genetic transformation and gene editing in a plant species is the ability to regenerate (Atkins and Voytas 2020). Regeneration is the tissue culture method of using plant hormones to stimulate differentiated tissue to first de-differentiate into calli, an undifferentiated cluster of cells, and then re-differentiate into new shoots. This process is necessary for most plant transformation methods, including the widely used *Agrobacterium*-mediated transformation. Regeneration is a complex process in plant development, which is time consuming and resource intensive to phenotype. Each genotype within a species responds uniquely to the hormones used to induce callus formation and shoot differentiation, and identifying the ideal conditions needed to regenerate a given genotype is challenging (Atkins and Voytas 2020). *Agrobacterium*-mediated transformation in potato is also a complex process that is influenced by several factors including genotype, source of explant, vector type, selection media and *Agrobacterium* strain (Lacroix and Citovsky 2019). Media and culture conditions have been optimized for several tetraploid potatoes, but there is still a need for diploid germplasm that can respond to these conditions as well (Ooms et al. 1985; Tavazza et al. 1989; Visser 1991; Haesaert et al. 2015; Craze et al. 2018).

Another limitation in diploid potatoes is gametophytic self-incompatibility (GSI) which causes an inability to produce viable fruit after self-pollination (McClure et al. 2011). Briefly, the system involves the degradation of RNA in self-pollen tubes by stylar factors (S-RNAses), leading to a cytotoxic effect, halting pollen tube elongation (Sijacic et al. 2004). This mechanism greatly hinders the development of potato inbred lines. Different approaches have been used to overcome GSI, including introgression of the *S*-locus inhibitor (*Sli*), which is a pollen expressed, non *S*-locus F-Box protein that can recognize many different S-RNAses (Ma et al. 2021; Eggers et al. 2021), as well as the deletion of primary GSI-related genes such as S-RNase (Hosaka and Hanneman 1998; Enciso-Rodriguez et al. 2019; Ye et al. 2018). Self-fertile lines can also be leveraged to remove T-DNA regions, which are most likely incorporated due to

Agrobacterium-mediated transformation and could be segregated out in the progeny (Ricroch et al. 2017). Furthermore, self-pollination can also fix the targeted mutations from gene editing in a homozygous condition. This can be further complemented by being able to backcross to remove any unintended mutations caused by gene editing or somaclonal variation.

To address these limitations, this study assesses and ranks the selected diploid germplasm for the traits valuable to applications of genome editing and genetic engineering, in order to identify and develop a new genetic model organism for potato. Diploid potato clones for this study were selected based on availability of genomic resources, self-fertility, desired tuber-related traits or previous use in tissue culture regeneration and genetic transformation studies (De Jong and Rowe 1971; Jansky et al. 2014; Hardigan et al. 2016; Peterson et al. 2016; Leisner et al. 2018; Ko et al. 2018; Alsahlany et al. 2021). A set of seven lines were evaluated for their regeneration efficiency using internode and leaf explants, tuber yield based on tuber number and weight, and self-fertility based on pollen viability, fruit and seed set from self-pollinations as well as pollen tube growth. Based on their performance metrics for the above traits, three lines 1S1, MSEE737-05 and UW-W4 were selected for further evaluation of their transformation efficiency using *Agrobacterium*-mediated transformation. Although all three lines were transformable, the transformation efficiencies were below 5%.

Material and Methods

Plant Material and Growth Conditions

The following seven diploid lines: 1S1, DMRH-S5-28–5, M6, MSEE720-04, MSEE737-05, MSEE824-04, and UW-W4 were selected from the Michigan State University (MSU), University of Wisconsin and Virginia Tech diploid potato breeding and genetics programs, (Fig. 1, Table 1.).

Plants were propagated in vitro using nodal cuttings in tissue culture, on Murashige and Skoog (MS) medium (MS basal salts plus vitamins, 3% sucrose, 0.7% plant agar, pH 5.8) (Murashige and Skoog 1962) and grown in culture tubes in growth chambers with 16-h-light/8-h-dark photoperiod at 22 °C and average light intensity of 200 µmoles m^{-2} s^{-1}.

Five clones per genotype of four-week-old in vitro plants were planted in 19 L plastic pots with a peat and perlite growth medium mixture (Bacto professional planting mix) and placed into a greenhouse with a light intensity of 250 µmoles m^{-2} s^{-1}, 16/8-h light/dark photoperiod and an average temperature of 25 °C. Plants were fertilized with Peters Professional® 20: 20: 20 fertilizer (The Scotts, Co., Marysville, OH, United States) at a rate of 500 mg/l once a week.

Pollen Viability Evaluation by Acetocarmine Staining

Pollen viability test was performed for plants grown in the greenhouse following the methodology described by Ordoñez et al. (2017). Pollen was bulked from three flowers of the same clone. Twenty µl of acetocarmine stain (TCI America #A0050) was mixed with pollen and placed on a glass slide with coverslip. Pollen was observed under a Leica DM750 microscope at 10X and images were captured using ICC50 HD Camera. Field of view representative of the entire slide was used for analysis. Pollen that was stained red, round and turgid were considered viable.

Assessment of Self-Fertility

Self-fertility was measured as fruit and seed count, by self-pollinating 20 flowers per clone at anthesis. Pollen from the batch evaluated for viability was used for self-pollinations. Fruits were harvested four to six weeks after

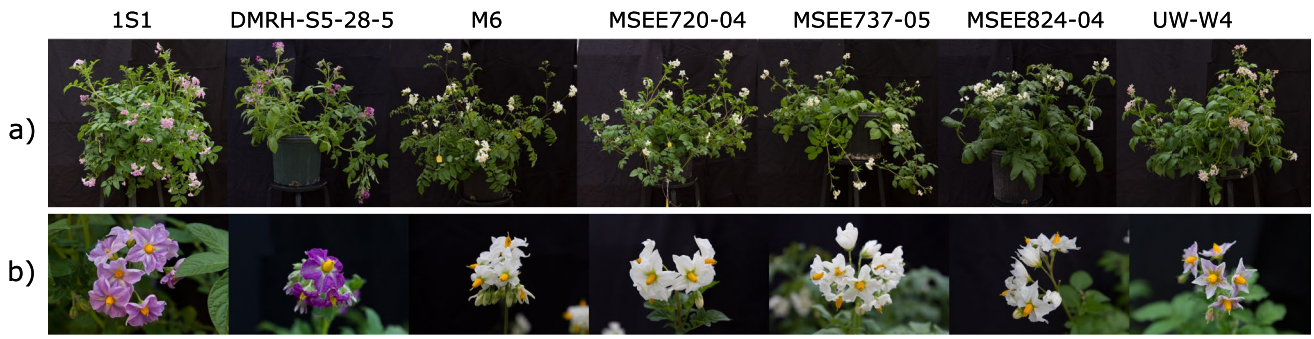

Fig. 1 Morphology of diploid potato germplasm used in this study. Representation of their (**a**) plant growth habit (**b**) and flower color patterns under greenhouse conditions

Table 1 Diploid germplasm selected for this study

Genotype	Source	Species	Pedigree	Citation
1S1	VT	*S. tuberosum* Group Phureja	DM 9–9 203/16 × PP5	Hardigan et al. 2016
DMRH-S5-28–5	VT	*S. tuberosum* Group Phureja	DM 1–3 516 R44 x RH89-039–16, selfed for five generations	Peterson et al. 2016
M6	UW-USDA	*S. chacoense*	*S. chaconese* clone, selfed for seven generations	Jansky et al. 2014; Leisner et al. 2018
MSEE720-04	MSU	*S. tuberosum*	DD837-02 × Bulk1[a] (MSU Recurrent Selection Cycle 3)	Alsahlany et al. 2021
MSEE737-05	MSU	*S. tuberosum*	DD853-04 × Bulk 1 (MSU Recurrent Selection Cycle 3)	Alsahlany et al. 2021
MSEE824-04	MSU	*S. tuberosum*	VT_SUP_96 H x CC863-25 (MSU Backcross cycle 1)	Alsahlany et al. 2021
UW-W4	UW-USDA	*S. tuberosum* Group Tuberosum	MN 20–20-34 dihaploid	De Jong and Rowe 1971

[a]pollen was bulked from SC individuals as described inAlsahlany et al. 2021

VT = Virginia Tech University

UW-USDA = University of Wisconsin-USDA ARS

MSU = Michigan State University

DM = Doubled Monoploid

self-pollination and kept at room temperature for two weeks prior to extracting seeds.

For the genotypes that could produce seeds, germination was induced by first incubating 30 seeds in 1500 ppm of gibberellic acid for 16 h. Seeds were then sterilized using 10% bleach and washed three times with sterile water. Sterilized seeds were then germinated on Petri dishes in the dark at room temperature. Viability of seeds was assessed by the germination rate, which was measured as the number of seeds that began to form seedlings out of the total number of seeds used after one week.

Aniline Blue Fluorochrome Staining of Pollen Tubes

Pollinations were performed using mature viable self-pollen and aniline blue fluorochrome staining of carpels was performed as described by Ye et al. (2018). Briefly, petals and anthers were removed from flowers and the remaining pistil, including the stigma, style, and ovary were collected 48 h post-pollination and immediately placed in 3:1 95% ethanol:glacial acetic acid. After fixation, the tissue was submerged in 5 M NaOH softening solution for 24 h. The tissue was washed with distilled water and then incubated in water for 2 h. The tissue was then incubated in 1 ml 0.1% aniline blue in 0.1 M K_3HPO_4 buffer for 1 h while gently shaking. Pistils were viewed at 4X under a Nikon Eclipse Ni-u upright compound fluorescence microscope with a SOLA light engine under a DAPI filter at 358 nm wavelength. Photographs were taken with an ANDOR Zyla sCMOS camera using the NIS-Elements BR software.

Assessment of Tuber Yield

The plants used for SC assessment were grown to maturity in the greenhouse for tuber assessment. At vine senescence (110 days after planting), tubers were harvested from the greenhouse pots. Total yield of tubers was recorded as the total tuber weight per pot, with five biological replications per genotype. Individual tuber weight was calculated as the average of the total tuber yield divided by the total number of tubers per pot.

Evaluation of Plant Tissue Culture Regeneration

Plant regeneration was performed as described by Li et al. (1999), using internode and leaf segments from four-week-old tissue culture plants. Briefly, explants were initially cultured on a Step I media (MS salts, 3% sucrose, 5 g/l phytoagar, 1 mg/l thiamine-HCl, 0.8 mg/l zeatin-riboside and 2 mg/l 2,4-D) for five days in dark conditions. The explants were then transferred to Step II media (MS salts, 3% sucrose, 5 g/l phytoagar, 1 mg/l thiamine-HCl, 0.8 mg/l zeatin- riboside, 2 mg/l gibberellic acid). Explants were transferred to fresh Step II media every week. Three independent experiments were conducted containing five explants per plate, and three plate replicates per genotype totaling to 45 explants per source tissue tested for each genotype.

After four weeks, data for callus induction rate and regeneration rate was collected. Callus induction rate was measured as the number of explants to produce calli out of the total number of explants used. Similarly, regeneration rate was measured as the number of explants to produce shoots

out of the total number of explants. The total number of shoots per regenerating explant was also collected.

Agrobacterium-Mediated Transformation and Event Characterization

Based on the results of SC, tuber traits and regeneration rate, the lines 1S1, MSEE737-05 and UW-W4 were selected to further explore their transformation ability. To evaluate transformation efficiency of the selected material, pTRANS_220d binary vector containing NPTII gene for kanamycin selection as described by Čermák et al. (2017) (a gift from Dr. Daniel Voytas; Addgene plasmid # 91,114) was electroporated into *Agrobacterium tumefaciens* strain GV3101 pMP90 (Koncz et al. 1994). *Agrobacterium*-mediated transformation was performed using leaf and internode explants from 4-week-old tissue culture plants as described by Li et al. (1999). Briefly, explants were pre-cultured on a step I media for three days then inoculated with *Agrobacterium*. Two days post inoculation, explants were rinsed with sterile distilled water containing 250 mg/l cefotaxime and 150 mg/l timentin and placed onto step II media containing 250 mg/l cefotaxime, 150 mg/l timentin and 50 mg/l kanamycin. Explants were transferred to fresh step II media every week. After 4 weeks, transformation events (T0 lines) were selected from step II media and transferred to MS medium supplemented with 250 mg/l cefotaxime, 150 mg/l timentin and 50 mg/l of kanamycin for rooting and selection.

Molecular Characterization of the Transgenic Lines

DNA from transformation events was isolated from young leaves using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). PCR for screening T-DNA insertion and *Agrobacterium* contamination was carried out using primers designed for the neomycin phosphotransferase gene (*nptII)* and *Agrobacterium* chromosomal serine hydroxymethyltransferase gene (*glyA)*, respectively, using the GoTaq DNA polymerase (Promega, Fitchburg, WI, United States) with the following thermocycler conditions: one cycle of initial denaturation for 4 min at 94 °C, followed by 34 cycles for 15 s at 94 °C, 45 s at 60 °C and 1 min at 72 °C and a final extension of 5 min at 72 °C. Amplicons were visualized on 1.5% (w/v) agarose gels stained with 1X Invitrogen Scientific SYBR-Safe.

Statistical Analysis

Regeneration rate, callus formation rate, percent fruit set, average tuber number and average individual tuber weight were analyzed with a Fisher's protected least significant difference (LSD) test. First, averages for each treatment were compared by conducting ANOVA using the "aov" procedure in R version 3.4.2 (R Core Team 2021) to confirm statistical differences between genotypes using an $\alpha = 0.05$. A least significant difference test for pairwise comparison of sample means was performed using the compact letter displays, "cld", procedure from the R package "emmeans", version 1.3.3, in R version 3.4.2.

Results

Morphological Characterization of the Diploid Germplasm Used in this Study

Diploid potato germplasm was obtained from potato breeding programs of Virginia Tech University, University of Wisconsin USDA-ARS and Michigan State University. A preliminary screen of regeneration ability for 16 diploid lines was conducted prior to this study (data not shown) in order to select only lines that could regenerate at least 40% of either internode or leaf explants (Table 1). M6 was an exception to this, as it exhibited recalcitrance to regeneration in its preliminary screen (data not shown), however it was included because of the genomic resources available for this line as well as to serve as negative control in the regeneration experiments.

All selected diploid clones produced flowers and tubers in greenhouse conditions (Fig. 1a). Clones exhibited a range of plant growth habits with MSEE824-04 having more compact growth and shorter vines (Fig. 1a). MSEE737-05 and 1S1 produced vigorous vines and flowered profusely. DMRH-S5-28–5 had a weaker growth probably associated with its inbred status. M6 formed tubers but long stolons were observed in the pots. Differences in plant flower color were also observed with 1S1 and UW-W4 being light purple, DMRH-S5-28–5 being dark purple, and the rest of the lines having similar white colored flowers (Fig. 1b).

Evaluating Self-Fertility Responses

A range of self-fertility responses was observed, with the percent of flowers setting viable fruit ranging from 0–88% (Table 2). DMRH-S5-28–5, MSEE720-04 and MSEE824-04 did not produce any fruit after self-pollination. 1S1 and UW-W4 did produce fruit, but the percent fruit set was less than 25%. M6 and MSEE737-05 produced significantly higher fruit sets than the rest of the lines, with MSEE737-05 having the highest percent fruit set with 88% of flowers setting viable fruit ($\alpha < 0.05$). Of the lines that produced fruit, all fruit contained seeds of varying amounts. The average number of seeds per fruit ranged from 4—47. Although 1S1 only had a fruit set of 8%, the fruit it produced contained on average 30 seeds per fruit, whereas UW-W4 which had a fruit set of 23% only had an

Table 2 Percent fruit set, average number of seeds per fruit and germination rate for the genotypes in this study

Genotype	Fruit Set %[a]	Seeds/Fruit	Germination Rate %[b]
1S1	8% d*	30	93%
DMRH-S5-28–5	0% d	N/A	N/A
M6	70% b	38	97%
MSE720-04	0% d	N/A	N/A
MSE737-05	88% a	47	63%
MSE824-04	0% d	N/A	N/A
UW-W4	23% c	4	60%

*The same letters are not significantly different according to the least significant difference test ($\alpha < 0.05$)

[a]number of fruit divided by twenty self-pollinated flowers × 100 for N = 5 plants

[b]number of seeds germinated divided by the total number of seeds used for the assay × 100 for N = 30 seeds

N/A = seed count and germination rate could not be collected for genotypes that did not produce fruit

average of 4 seeds per fruit (Table 2). For the genotypes that could produce seeds, at least 60% of the seeds could germinate and were considered viable, and in the cases of 1S1 and M6, over 90% of seeds were viable (Table 2).

To further dissect this trait, a pollen viability assay and pollen tube staining was performed to assess pollen germination and progressive growth after self-pollination. All genotypes in this study produced viable pollen based on acetocarmine staining, although DMRH-S5-28–5 produced the least amount of pollen overall (Fig. 2a). After selfing, pollen tubes from M6 and MSEE737-05 consistently reached the ovary (Fig. 2b). Pollen did germinate for 1S1, DMRH-S5-28–5, MSEE720-04, MSEE824-04 and UW-W4, but the majority of pollen tubes were arrested before reaching the ovaries (Fig. 2b). Although a few pollen tubes were observed to have reached the ovaries for the aforementioned genotypes, in DMRH-S5-28–5, MSEE720-04 and MSEE824-04, all pistils senesced within 4 days post pollinations, resulting in no viable fruit being formed (Table 2), suggesting the presence of a non-GSI-related postzygotic barrier. The low seed set in UW-W4 could be explained by the presence of a prezygotic barrier preventing the majority of the pollen tubes from reaching the ovaries.

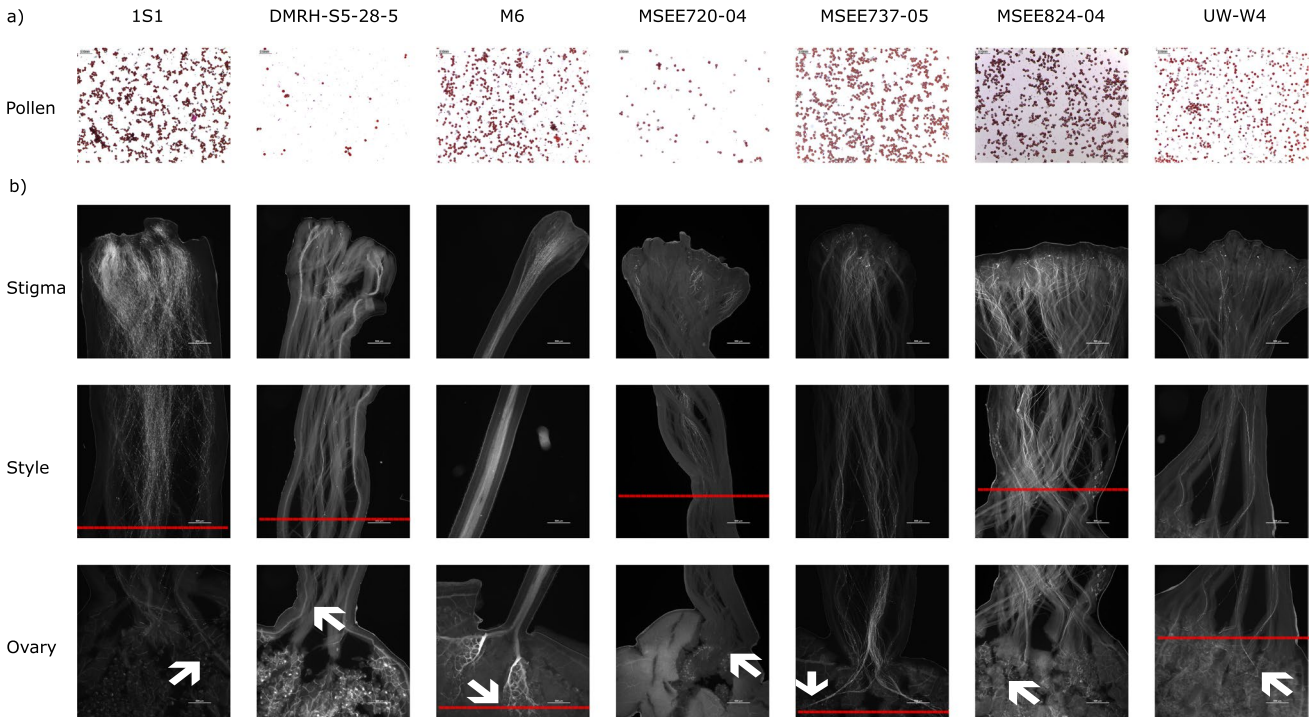

Fig. 2 Pollen and pistil staining for the seven selected diploid potatoes. **a** acetocarmine staining of pollen used for pollen tube growth analysis. Pollen that is stained red and is round and turgid is considered viable. The scale bar indicates 0.5 mm. **b** Pollen tube staining of genotypes in the study to assess pollen tube growth in the pistil, two days after pollination viewed at 4X magnification. The scale bar indicates 0.1 mm. Pollen tubes stained with aniline blue dye fluoresce when excited with 358 nm light. Red dashed lines indicate where the pollen tube front ends. White arrows point toward the longest pollen tube

Tuber Phenotyping for Morphology and Yield

A variation in tuber appearances was observed in this study, as well as significant differences in tuber yield (Fig. 3). Most genotypes in this study have a round tuber shape with white-cream or tan skin color typical of cultivated potato. DMRH-S5-28–5 has long tubers and 1S1 has round tubers with red eyes (Fig. 3a) commonly found in *S. tuberosum* Grp Phureja germplasm (Lindqvist-Kreuze et al. 2015). DMRH-S5-28–5 produced the highest average tuber number per plant (29.4 tubers per pot, $\alpha < 0.05$); however, the individual tuber weight is among the lowest of the genotypes tested (11.2 g per tuber, $\alpha < 0.05$) (Fig. 3b). MSEE824-04 had the highest individual tuber weight with an average of 63.8 g per tuber ($\alpha < 0.05$) and MSEE737-05 did produce some tubers similar in size to MSEE824-04, but the average individual tuber size was second highest in the study (Fig. 3c). 1S1, DMRH-S5-28–5, M6 and UW-W4 had significantly lower average individual tuber weights compared to the other three lines ($\alpha < 0.05$). The total tuber yield per genotype, averaged from 5 clones, ranged from 51.8 to 565.0 g with 1S1 as the lowest yielding and MSEE737-05 the highest (Supplementary Table 1).

In vitro Regeneration Efficiency

All selected genotypes for this study were able to form calli from both sources of explants except for M6, which had a significantly lower callus formation rate from internode explants and no callus formation from leaf explants (Table 3, $\alpha < 0.05$). The regeneration rate of the genotypes used in this study ranged from 0–87% for internodes and 0–100% for leaves (Table 3). 1S1, DMRH-S5-28–5, MSEE720-04, MSEE737-05 and UW-W4 presented regeneration rates greater than 75% for internodes explants. For leaf explants, only MSEE720-04 and MSEE737-05 had regeneration rates greater than 75%. M6 was the only genotype that was not able to regenerate from either internode or leaf explants (Fig. 4, Table 3). Although UW-W4 did produce shoots for both internode and leaf explants, the stems of the shoots produced were thinner than the other genotypes (Fig. 4). For the genotypes that could produce shoots, the number of shoots per explant ranged from 0–10 with 1S1 having the highest average for internode explants with 6 shoots per regenerating explant and MSEE737-05 having the highest average for leaf explants with 10 shoots per regenerating explant.

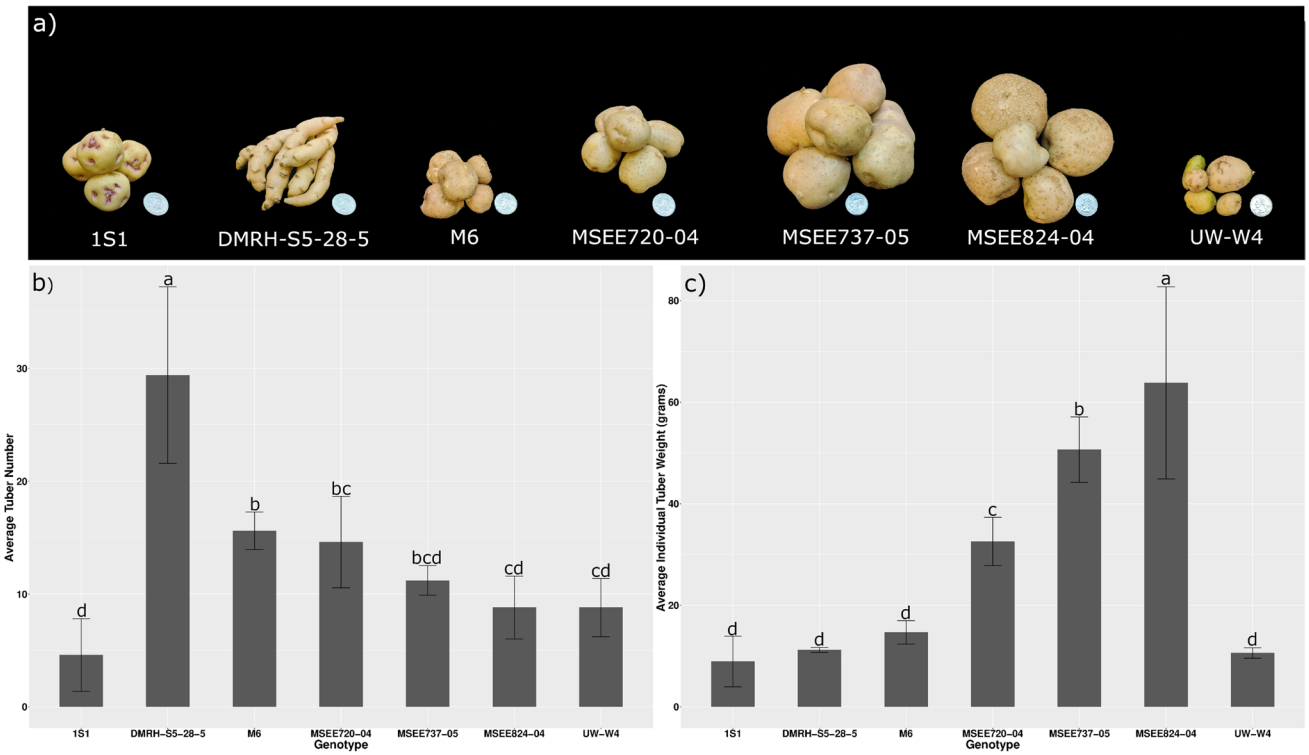

Fig. 3 Tuber related traits for the selected diploid potatoes. **a** Tuber appearance and morphology **b** Tuber number per pot ($N = 5$). **c** Average individual tuber weight is the average of the total tuber yield divided by the number of tubers ($N = 5$). Values with the same letters are not significantly different according to the least significant difference test ($\alpha < 0.05$). Quarters are used as scale to indicate the tuber size. Error bars represent standard deviation

Table 3 Regeneration efficiency of selected diploid germplasm

Genotype	Callus %[a]	Regeneration %[b]	Average shoots per regenerating explant
Internode Explants			
1S1	97.8% a*	84.4% ab	6
DMRH-S5-28–5	100% a	86.7% ab	4
M6	75.6% b	0% c	N/A
MSEE720-04	100% a	91.1% a	4
MSEE737-05	100% a	75.6% ab	5
MSEE824-04	100% a	68.9% b	4
UW-W4	100% a	82.2% ab	3
Leaf Explants			
1S1	93.3% a	17.8% d	2
DMRH-S5-28–5	100% a	53.3% c	3
M6	2.22% c	0% e	N/A
MSEE720-04	100% a	82.2% b	4
MSEE737-05	100% a	100% a	10
MSEE824-04	62% b	8.9% de	2
UW-W4	100% a	48.9% c	2

*The same letters are not significantly different according to the least significant difference test ($\alpha < 0.05$)

[a]number of explants producing calli divided by the total number of explants × 100

[b]number of explants producing shoots divided by the total number of explants × 100

N/A = no shoots could be counted for genotypes that could not regenerate

Transformation Efficiency of 1S1, MSEE737-05, and UW-W4

The lines 1S1, MSEE737-05 and UW-W4 were selected based on their SC, tuber-related traits and high regeneration ability for further assessment of transformation efficiency. All three genotypes were capable of being transformed under the experimental conditions in this study using either internode or leaf explants. Events were considered transformed if they displayed rooting in kanamycin selection media, were PCR positive for *nptII,* which is contained in the T-DNA borders of the pTRANS_220d binary vector, and negative for contamination by *Agrobacterium* used for transformation as determined by PCR for the *glyA* gene contained on the C58 chromosomal DNA of *Agrobacterium* strain GV3101 (Fig. 5).

1S1 had a high number of false positive events, resulting in only 1 true event collected from internode explants although 80 shoots had rooted in selection media. For MSEE737-05 only leaf explants produced transformed shoots and for UW-W4 only internode explants resulted in transformed shoots (Table 4). Overall, MSEE737-05 had the highest transformation efficiency of the genotypes tested when using leaf explants.

Discussion

Diploid breeding and genetics is a future vision for potato improvement with multiple research programs across the world contributing to the generation of valuable germplasm for breeding and genetic research. The ultimate goal is to develop potato diploid inbred lines with advanced tuber traits, in addition to being amenable to modern breeding and functional genomics research, including gene-editing. There is a need for model potato lines for the application of reverse genetic approaches to complement and expedite the goals of developing diploid inbred lines with advanced tuber traits. The current impediment to the use of genetic engineering or editing in diploid potato is that most of the germplasm available has low regeneration efficiency, is self-incompatible,

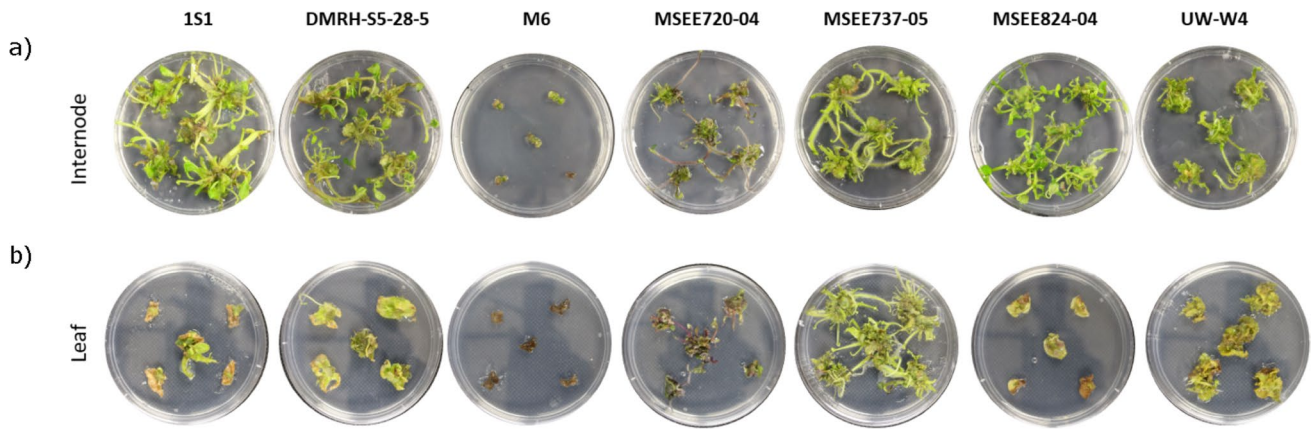

Fig. 4 Selected diploid germplasm on regeneration media **a** Internode and **b** leaf explants after 4 weeks on regeneration media. Regenerating shoots are observed from internode and leaf explants in all genotypes except for M6

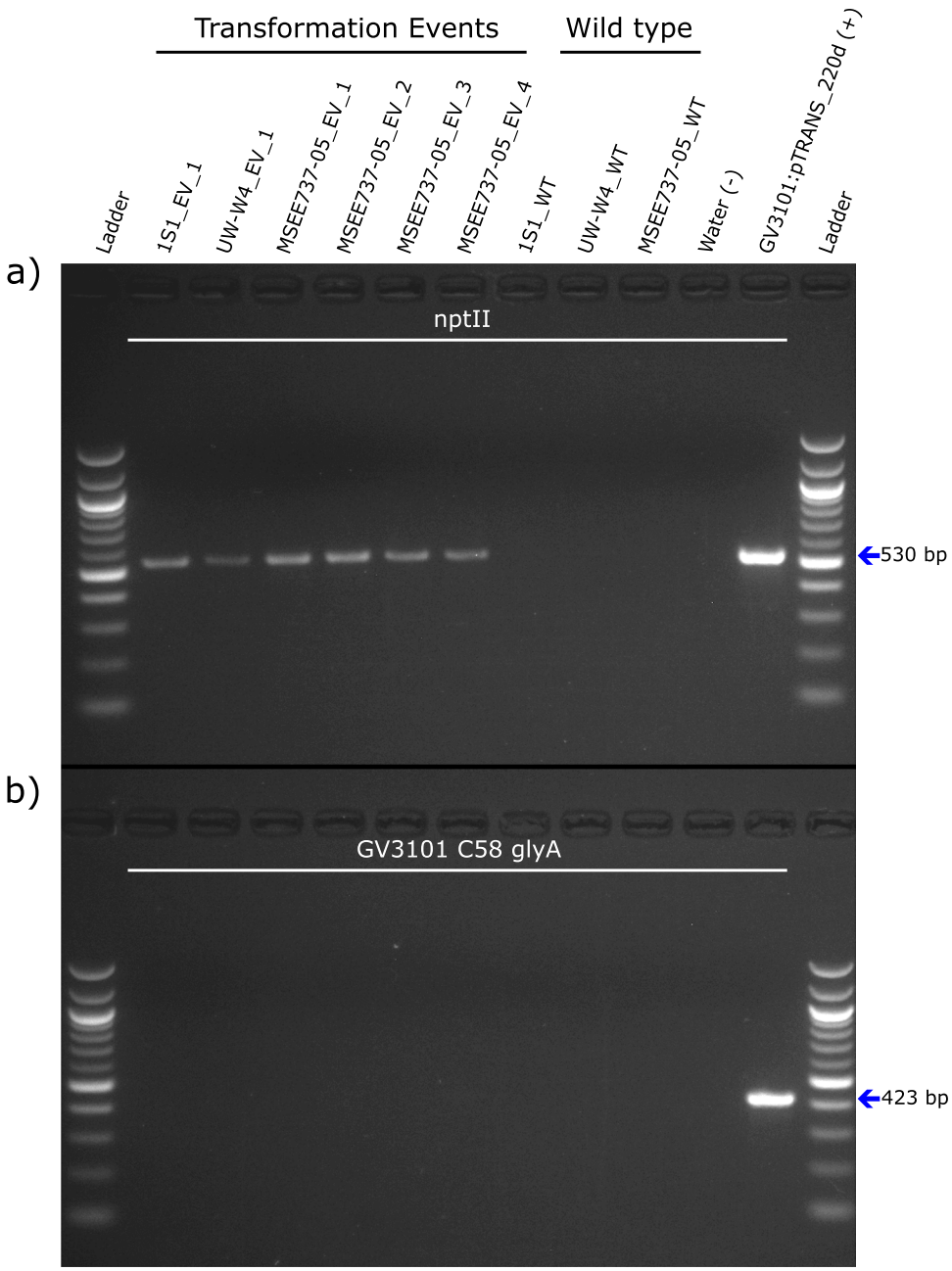

Fig. 5 **a** PCR screening of selected transformation events. One, one and four transformation events were validated for 1S1, UW-W4 and MSEE737-05, respectively using PCR amplification of *nptII* with the presence of an expected 530 bp product. Positive control is GV3101 containing the pTRANS_220d empty binary vector and negative wild type and water controls. **b** The presence of contaminant GV3101 which would cause false positive *nptII* bands was validated using GV3101 specific primers with an expected band size of 423 bp. Only the GV3101 positive control had a band, indicating GV3101 contamination was not present in the plant DNA samples

or has tuber qualities that do not reflect the economically important tuber traits found in cultivated potato, such as high yield, shallow eyes and desirable skin appearance.

Previously, the germplasm generated from Virginia Tech has been a resource for genetic engineering in diploid potatoes, due to its selection based on amenability to tissue culture techniques (Hardigan et al. 2016). DM x RH derived lines have been successfully used for transformation and gene knockouts (Ko et al. 2018; Enciso-Rodriguez et al. 2019). However, the DM x RH material has significant limitations associated with tuber shape and size. 1S1, on the other hand, is amenable to tissue culture, can produce viable fruit upon selfing and produces round tubers typical of Andean cultivated potatoes, although further considerations in optimizing a transformation system are necessary. Most self-pollinations in 1S1 did not result in fruit, because of pollen tube arrest in the style (Fig. 2.), suggesting that the GSI machinery could be controlling self-pollen rejection and not other mechanisms, supported also by the presence of homozygous recessive *sli*, previously characterized in 1S1 (Kaiser et al. 2021). Also, as previously observed in *S. tuberosum* Group Phureja material, even individuals that displayed self-fertility, did not always produce fruit upon selfing, and additional considerations such as environment

Table 4 Transformation efficiency of selected diploid potato germplasm

Genotype	Explant	Number of explants	Explants shooting (%)[a]	Rooting Shoots[b]	Number of PCR positive events	Transformation efficiency (%)[c]
1S1	Internode	199	69%	80	1	0.5%
	Leaf	150	15%	16	0	0%
MSEE737-05	Internode	100	0	N/A	N/A	0%
	Leaf	100	58%	4	4	4%
UW-W4	Internode	148	33%	1	1	0.7%
	Leaf	124	5%	0	N/A	0%

[a]number of explants forming at least one shoot after 4 weeks of culture on Step II media divided by the total number of explants × 100

[b]number of shoots collected that rooted in selection media

[c]number of explants producing a transformed shoot divided by the total number of explants × 100

N/A = no events could be collected for explants that did not shoot or for shoots that didn't root in selection

and plant age also play an important role in self-fertility-related traits (Kaiser et al. 2021; Enciso-Rodriguez et al. 2019; Peterson et al. 2016). The material chosen from University of Wisconsin was selected for this study based on previous reports of being reliably SC (De Jong and Rowe, 1971; Clot et al. 2020; Kaiser et al. 2021; Jansky et al. 2016; Leisner et al. 2018). However, M6 was found to be recalcitrant to regeneration media as well as limited in desirable tuber traits. Different strategies have been made to optimize conditions for genetic transformation of M6, such as hairy root-mediated transformations using *Agrobacterium rhizogenes* (Butler et al. 2020), but a robust method of recovering regenerated events has yet to be described for M6. The material from MSU has been selected for several generations specifically based on its SC and tuber shape and size (Alsahlany et al. 2021). Although they had not been specifically selected based on regeneration efficiency, several genotypes that are promising candidates for this application have been described in this study. Most notably, MSEE737-05 has high regeneration rates, strong SC, acceptable tuber appearance and is amenable to *Agrobacterium*-mediated genetic transformation.

1S1, MSEE737-05 and UW-W4 were all transformable under these experimental conditions, although further optimizations of the protocol could be made to increase the transformation efficiency. 1S1 displayed a clear resistance to kanamycin leading to the proliferation of escapes, under-estimating its true transformation potential. Alternative antibiotics for selection, hygromycin or G418, can be used instead to elicit a stronger lethality response in 1S1 for screening putative transformation events (Supplementary Fig. 1). As seen in Fig. 2, the regeneration of UW-W4 produces large calli and thin shoots, leading to its detriment in transformation where shoots produced on selection media were too weak to survive. To improve its transformation efficiency, the regeneration media can be altered to optimize the generation of UW-W4 vigorous events by modifying the ratio of auxins to cytokinins to promote stronger stems and smaller calli (Ohnuma et al., 2020). Several additional adaptations can be made in further studies to improve the *Agrobacterium*-mediated transformation efficiencies of these lines. Different strains of *Agrobacterium* can be tested to find the one that elicits the strongest response (Bakhsh 2020; Dönmez et al. 2019; Petti et al. 2009). The source of explants is also an important factor in transformations, here only leaf and internode explants were tested but younger tissue, such as that of cotyledons, may be more amenable to transformation than more developed tissue (Becerra et al. 2004).

Plant transformation and tissue culture regeneration are the most limiting steps for applying genetic engineering and genome editing to non-genetic model plants. Transformation efficiency and tissue culture regeneration is genotype dependent and ideal conditions have to be optimized to genetically transform a given genotype. While *Agrobacterium*-mediated transformation is currently the most common method of genetic transformation, a notable amount of work is currently being invested into developing alternative transformation methods for plants that are not as limited by tissue culture, such as meristem induction method, use of viral vectors and nanoparticle mediated transformations (Maher et al. 2019; Ellison et al. 2020; Nadakuduti and Enciso-Rodríguez 2021).

Three diploid potato genotypes have been identified in this study that are amenable to plant transformation, also presenting SC and desirable tuber-related traits that could be used for applications of new breeding techniques like genetic engineering and genome editing. This study also demonstrates foundational research in the development of a new model for genetic research in potato. Although further optimization is recommended to create a robust system for plant transformation, our preliminary findings suggest that

the selected lines could be used for future functional analysis or introduction of advanced traits into diploid potato.

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Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

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