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KNOX genes influence a gradient of fruit chloroplast development through regulation of *GOLDEN2-LIKE* expression in tomato

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SUMMARY

The chlorophyll content of unripe fleshy fruits is positively correlated with the nutrient content and flavor of ripe fruit. In tomato (*Solanum lycopersicum*) fruit, the *uniform ripening* (*u*) locus, which encodes a *GOLDEN 2-LIKE* transcription factor (*SlGLK2*), influences a gradient of chloroplast development that extends from the stem end of the fruit surrounding the calyx to the base of the fruit. With the exception of the *u* locus, the factors that influence the formation of this developmental gradient are unknown. In this study, characterization and positional cloning of the *uniform gray-green* (*ug*) locus of tomato reveals a thus far unknown role for the Class I *KNOTTED1-LIKE HOMEOBOX* (*KNOX*) gene, *TKN4*, in specifying the formation of this chloroplast gradient. The involvement of KNOX in fruit chloroplast development was confirmed through characterization of the *Curl* (*Cu*) mutant, a dominant gain-of-function mutation of *TKN2*, which displays ectopic fruit chloroplast development that resembles *SlGLK2* over-expression. *TKN2* and *TKN4* act upstream of *SlGLK2* and the related gene *ARABIDOPSIS PSEUDO RESPONSE REGULATOR 2-LIKE* (*SlAPRR2-LIKE*) to establish their latitudinal gradient of expression across developing fruit that leads to a gradient of chloroplast development. Class I *KNOX* genes typically influence plant morphology through maintenance of meristem activity, but this study identifies a role for *TKN2* and *TKN4* in specifically influencing chloroplast development in fruit but not leaves, suggesting that this fundamental process is differentially regulated in these two organs.

Keywords: *Solanum lycopersicum*, *KNOX* transcription factors, *GOLDEN 2-LIKE* transcription factors, chloroplast development, fruit development, gene expression gradients.

INTRODUCTION

In plants, many biochemical pathways, including those associated with photosynthesis and synthesis of primary and specialized metabolites, are located within the chloroplast. Leaves are typically the primary source of photosynthate, although it is estimated that photosynthesis by fleshy fruits contributes up to 20% of the total fruit carbohydrate (Blanke and Lenz, 1989; Hetherington *et al.*, 1998). Enhanced chloroplast development and chlorophyll content of unripe fruits increases their photosynthetic capacity, leading to greater accumulation of starch (Dinar and Stevens, 1981; Powell *et al.*, 2012; Sagar *et al.*, 2013). Furthermore, the starch content of unripe fruit is positively correlated with the sugar content in ripe fruit, which directly contributes to fruit quality by influencing nutritional value and taste (Davies and Hobson, 1981). Thus, chloroplast development and the carbohydrate status of unripe fruits affect the composition and quality of ripe fruit.

In tomato, several loci influence the development of fruit chloroplasts and the subsequent quality of ripe fruit. Mutations at the *high-pigment 1* and *2* (*hp-1* and *hp-2*) loci, which encode negative regulators of photomorphogenesis, possess an increased number of chloroplasts and plastid compartment size, leading to fruits with higher levels of chlorophyll and carotenoids (Mustilli *et al.*, 1999; Cookson *et al.*, 2003; Liu *et al.*, 2004; Kolotilin *et al.*, 2007; Rohrmann *et al.*, 2011). Alteration of phytohormone biosynthesis and responses, including those associated with auxin, ABA, cytokinins and brassinosteroids (BRs), can also have a positive impact on chloroplast development that influences the quality of ripe fruit (Martineau *et al.*, 1994; Galpaz *et al.*, 2008; Sagar *et al.*, 2013; Liu *et al.*, 2014). In contrast, mutation at the *uniform ripening* (*u*) locus has a negative impact on the development of fruit chloroplasts, due to loss of the green shoulder that surrounds the calyx end of

developing fruit, leading to ripe fruits with a reduced sugar content (Powell *et al.*, 2012). Positional cloning of *u* revealed a mutation in a tomato homolog of the *GOLDEN 2-LIKE* (*GLK*) transcription factor of maize, designated *SlGLK2* (Powell *et al.*, 2012). *GLK*s are required for chloroplast development (Fitter *et al.*, 2002; Waters *et al.*, 2008) and *SlGLK2* is preferentially expressed in the shoulder of the fruit, where in *U*/*U* genotypes it is involved in enhancing chloroplast development while ectopic expression of *SlGLK2* results in homogeneously dark-green fruit (Powell *et al.*, 2012). Similarly, over-expression of *ARABIDOPSIS PSEUDO RESPONSE REGULATOR 2-LIKE* (*SlAPRR2-LIKE*) in tomato, which is related to *SlGLK2*, causes enhanced development of fruit chloroplasts (Pan *et al.*, 2013). How these loci interact to jointly influence the development of fruit chloroplasts is not fully understood, although recent evidence suggests a role for both auxin and BRs in regulating *GLK* expression (Sagar *et al.*, 2013; Liu *et al.*, 2014). Furthermore, except for the involvement of *SlGLK2*, the factors that establish the latitudinal gradient of gene expression and chloroplast development in tomato fruit remain undefined.

Fruits of the *uniform gray-green* (*ug*) mutant of tomato are similar to *u* and lack the green shoulder surrounding the calyx (Bohn and Scott, 1945). In this study, positional cloning revealed that *UG* encodes *TKN4*, a Class I KNOTTED1-LIKE HOMEOBOX (KNOX) transcription factor. *KNOX* genes typically influence plant morphology, and particularly leaf shape, through maintenance of stem cell populations within apical meristems (Hay and Tsiantis, 2010). However, the present work identifies a previously undefined role for these homeobox proteins in the development of fleshy fruit, and in particular in the establishment of a gradient of chloroplast development through regulation of *SlGLK2* expression.

RESULTS

Positional cloning of *ug* defines a role for *KNOX* in fruit chloroplast development

The *ug* mutant lacks the green shoulder surrounding the calyx end of developing fruit (Figure 1a). Chlorophyll levels are reduced in the pericarp of *ug* fruit (Figure 1b–e). The development of individual chloroplasts in *ug* fruit is also impaired, with a reduction in size and in the number of thylakoids per granum (Figure 1f–k). The phenotypes of *ug* are similar to those of fruits with the *u* mutant allele (Powell *et al.*, 2012) (Figure 1a–d,g,j). Genetic mapping assigned the *ug* locus to the long arm of chromosome 1, with tight linkage to the marker C2_At1g56345. Fine mapping refined the *ug* locus to a 14.6-kb interval between the markers *Solyc01g100500* and *Solyc01g100520* that spans nucleotides SL2.40chr1:82268632–82283258 and contains the single gene *Solyc01g100510*, which encodes the KNOX transcription factor TKN4. Sequencing of *TKN4* from flower cDNA of *UG*/*UG* and *ug*/*ug* genotypes revealed a single T → A nucleotide change within exon 2 of *TKN4* corresponding to a phenylalanine to leucine substitution at residue 137 (Figure 2a). Phenylalanine-137 represents an invariant amino acid within the KNOX2 domain of diverse Class I KNOX proteins (Figure 2b). The KNOX2 domain mediates protein–protein interactions between homeodomain proteins (Bellaoui *et al.*, 2001; Smith *et al.*, 2002). Genotyping of the single nucleotide polymorphism (SNP) associated with the *ug* phenotype from LA4025 and Purple Prince, which lack the green shoulder and are purported to carry the *ug* mutation, implied that the F137L substitution co-segregates with the mutant phenotype, indicating that the SNP is conserved in *ug*/*ug* genotypes (Figure 2c and Figure S1 in Supporting Information). Attempts to genotype the *ug* mutant allele in the LA0021 accession, which lacks a green shoulder but is reported to be a *ug* mutant, failed (Figure S1). Similarly, amplification of *TKN4* from LA0021 flower cDNA was unsuccessful (Figure 2d). Subsequent amplification and sequencing of *TKN4* genomic fragments from LA0021 revealed the existence of a second mutant allele that contains a 2.4-kb internal deletion spanning nucleotides SL2.40chr1:82280365–82282785 of the tomato genome reference assembly (Figure 2e). Although mutations in Class I *KNOX* genes typically cause phenotypes associated with altered morphology, and particularly leaf shape (Parnis *et al.*, 1997; Venglat *et al.*, 2002; Hay and Tsiantis, 2010), the effect of *ug* is subtle and plants carrying the *ug* mutant allele are indistinguishable from the wild type (WT) except for the lack of a green shoulder in developing fruit. No impact was observed on leaf shape or leaf chlorophyll in *ug* (Figure S2a–c). *TKN4* is expressed in flower pistils and mature green fruits but expression is low in leaves, supporting the lack of an altered phenotype in leaves of the *ug* mutant (The Tomato Genome Consortium, 2012) (Figure S2d, e). As the *ug* and *u* fruit phenotypes are virtually identical (Figure 1a), germplasm lacking a green shoulder was also genotyped for the *u* mutant allele. While LA4025 is homozygous for both *u* and *ug* mutant alleles, LA3539, LA0021 and the Purple Prince cultivar possess the WT (*U*/*U*) allele (Figures 2c and S1). These data indicate that the lack of a green shoulder in these accessions is the result of mutations in *TKN4* and not mutations at the *u* locus.

KNOX over-expression alters fruit development and has a positive impact on chlorophyll accumulation

Additional support for the role of *KNOX* genes in regulating the development of fruit chloroplast is evident from characterization of the *Curl* (*Cu*) mutant. *Cu* represents a dominant gain-of-function mutation resulting from ectopic expression of *TKN2*, leading to altered plant morphology (Parnis *et al.*, 1997). Developing *Cu* fruit possess elevated chlorophyll levels that extend across the entire surface of the fruit (Figure 3a–e). In addition to the increased

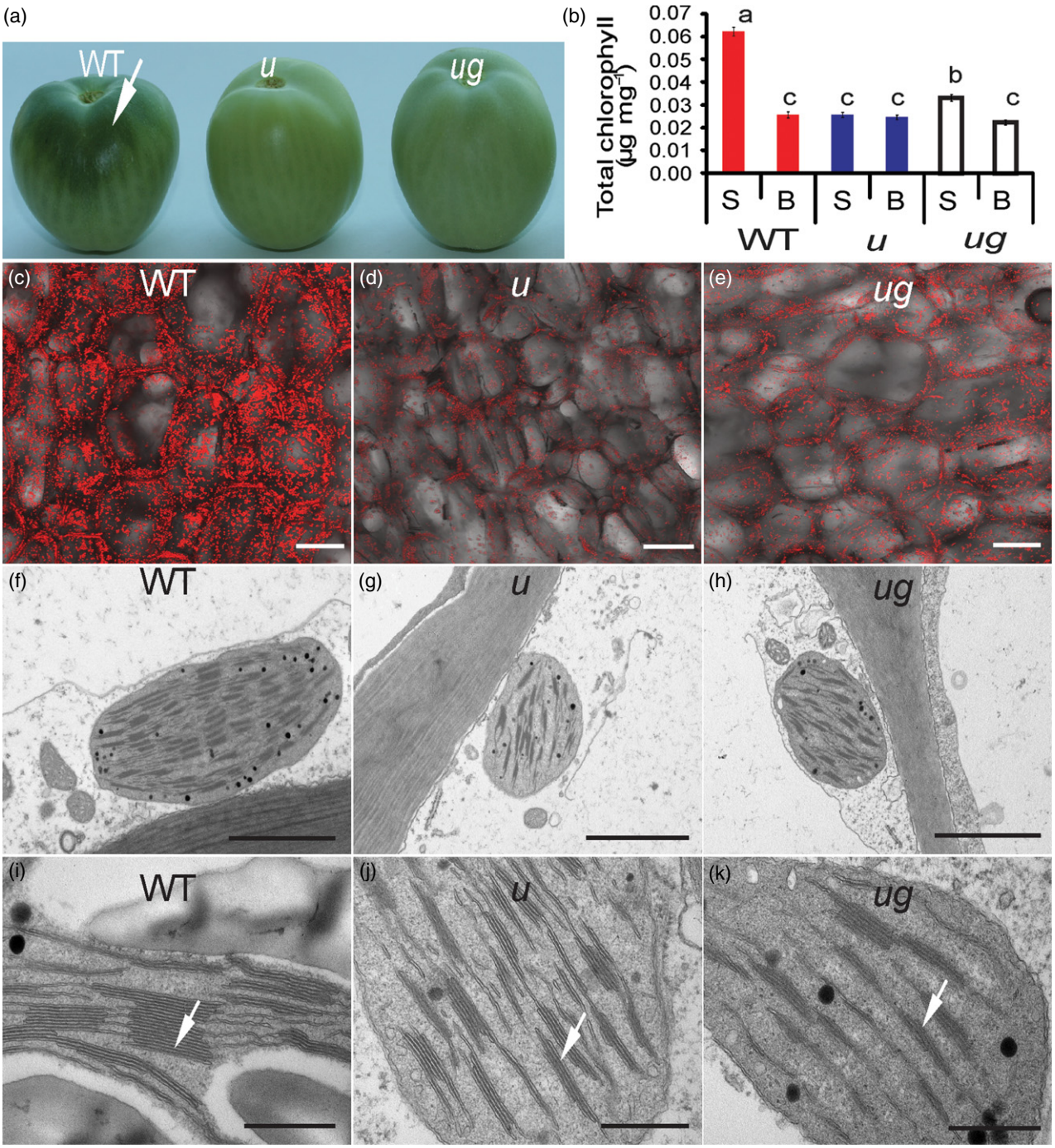

Figure 1. Fruit phenotype and chloroplast development in the *uniform gray-green* (*ug*) mutant.
(a) Phenotypes of the *uniform ripening* (*u*) and *ug* mutants compared with Ailsa Craig wild type (WT). The arrow highlights the presence of the green shoulder in WT fruit. (b) Chlorophyll content in the pericarp isolated from the shoulder (S) and base (B) of WT, *u* and *ug* fruit at 28 days post-anthesis (dpa). Data are presented as the mean ± SEM of $n = 5$ and means with different letters are significantly different (least squares means, $P < 0.05$). (c)–(e) Confocal laser scanning microscopy images of the shoulder pericarp of WT, *u* and *ug* fruit showing chlorophyll autofluorescence. Scale bar: 10 μm. (f)–(k) Transmission electron micrographs of 28-dpa fruit chloroplasts of WT, *u* and *ug*, Scale bars: (f)–(h), 2 μm; (i)–(k), 0.5 μm).

chloroplast number and higher chlorophyll content in *Cu* fruit, the size of the chloroplasts is larger than in WT and there is a general increase in the number of thylakoids per granum (Figure 3f–i). The phenotypes of *Cu* fruit are reminiscent of *SlGLK2* and *SlAPRR2-LIKE* over-expression lines (Powell *et al.*, 2012; Pan *et al.*, 2013). To assess the potential relationship between *Cu* and the *SlGLK*s and *SlAPRR2-LIKE*, the expression of these genes was examined in the

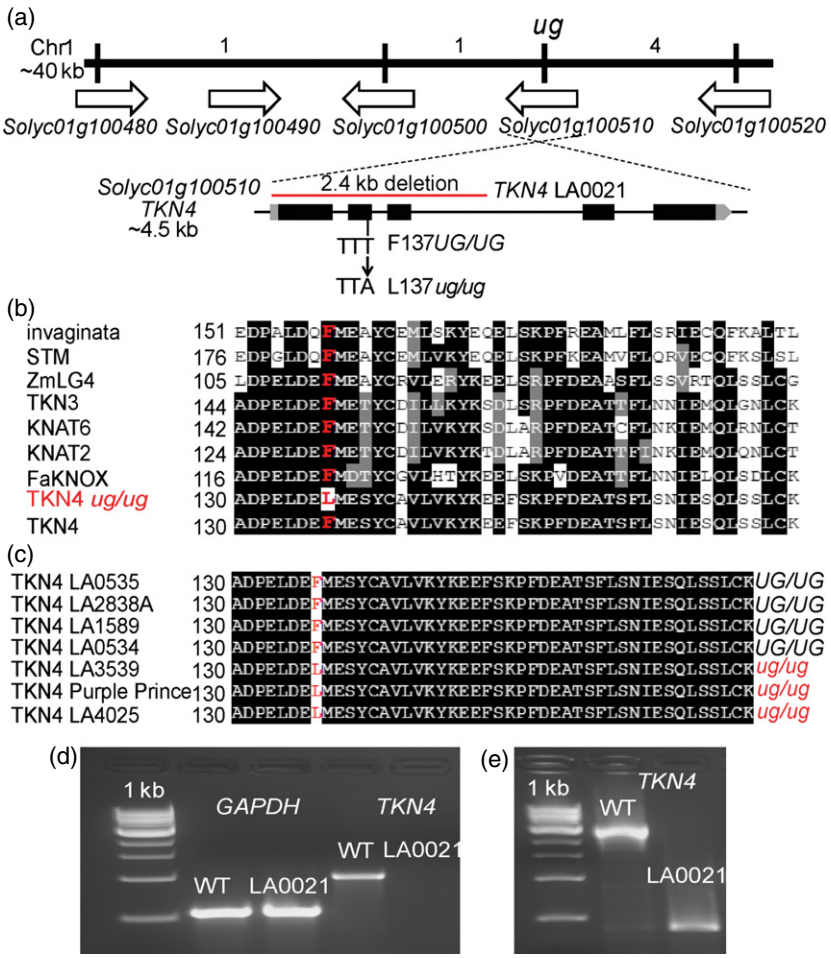

Figure 2. Genetic mapping of the *uniform gray-green* (*ug*) locus.
(a) Genetic and physical map of the *ug* locus identifying a single candidate gene, *Solyc01g100510*, which encodes *TKN4*. The numbers of recombinant individuals between genetic markers are provided. Sequencing of *TKN4* from wild type (WT) (*UG*/*UG*) and mutant (*ug*/*ug*) plants identified a single nucleotide polymorphism that converts phenylalanine 137 to leucine.
(b) Alignment of the KNOX2 domain of phylogenetically distinct KNOX proteins. The invariant phenylalanine residue within this domain and its substitution to leucine in *ug*/*ug* are shown in red.
(c) Correlation of L137 with the *ug*/*ug* phenotype in selected accessions of tomato.
(d) Reverse transcriptase-PCR amplification of *TKN4* from Ailsa Craig (WT) and LA0021 flower cDNA.
(e) *TKN4* amplification from WT and LA0021 genomic DNA highlighting 2.4-kb deletion in LA0021. This deletion spans the first three exons of *TKN4* and is highlighted by the red bar in (a).

pericarp of WT and *Cu* fruit. The expression of *TKN2*, *SlGLK*s and *SlAPRR2-LIKE* is elevated in *Cu* fruit (Figure 3j–m). However, *SlGLK1* expression, which is typically lower than *SlGLK2* expression in tomato fruit (Powell *et al.*, 2012), was not statistically different between WT and *Cu.* These data suggest that *SlGLK2* and *SlAPRR2-LIKE* act downstream of KNOX activity to regulate the development of fruit chloroplasts. In support of this hypothesis, fruit of a double mutant constructed from a cross between *Cu* and *u* partially suppressed the *Cu* mutant phenotype (Figure 3n,o). The *Cu* fruit are smaller in size (WT = 37.61 ± 2.53 g; *Cu* = 17.24 ± 1.69 g; $n = 8$) with a thinner pericarp and smaller cell size that results in an increased density of cells per unit area (Figure 3d,p,q). Congruent with the phenotype of the *ug* allele (Figure S2a–c) the *Cu* allele has no impact on leaf chlorophyll content (Figure S3a), and while the expression of the *SlGLK*s remains unchanged the expression of *SlAPRR2-LIKE* is slightly elevated in *Cu* leaves (Figure S3b–e).

KNOX loci establish gradients of *SlGLK2* expression that affect chlorophyll accumulation in fruit

The presence of a green shoulder in immature tomato fruit highlights the existence of a latitudinal gradient of chloroplast development that correlates with the increased expression of *SlGLK2* at the calyx end of the fruit relative to the stylar end (Powell *et al.*, 2012). To assess the role of *KNOX* genes in regulating the formation of this gene expression gradient and its associated differential pattern of chlorophyll accumulation, fruit pericarp was divided into whole, shoulder, middle and base, and the expression of *SlGLK2*, *SlAPRR2-LIKE*, *TKN2* and *TKN4* investigated (Figure 4a). In WT fruit, chlorophyll levels are higher in the fruit shoulder than in the middle and base of the fruit (Figure 4b), and the expression of each gene matches the gradient in chlorophyll accumulation with higher transcript levels observed in the shoulder compared with the middle and base, although this pattern is more pronounced for *SlGLK2*, *TKN2* and *TKN4* (Figures 4c–f and S4). The *Cu* and *ug* mutations disrupt both the chlorophyll gradient (Figure 4b) and the expression gradient that is present in WT fruit. For example, the expression level of *SlGLK2* is positively affected by *Cu* and negatively affected by *ug*, and the typical gradient of expression is disrupted (Figure 4c). In contrast, the *ug* locus has no impact on the expression of *SlAPRR2-LIKE*, and while the expression of this gene is elevated in *Cu* the expression gradient is not

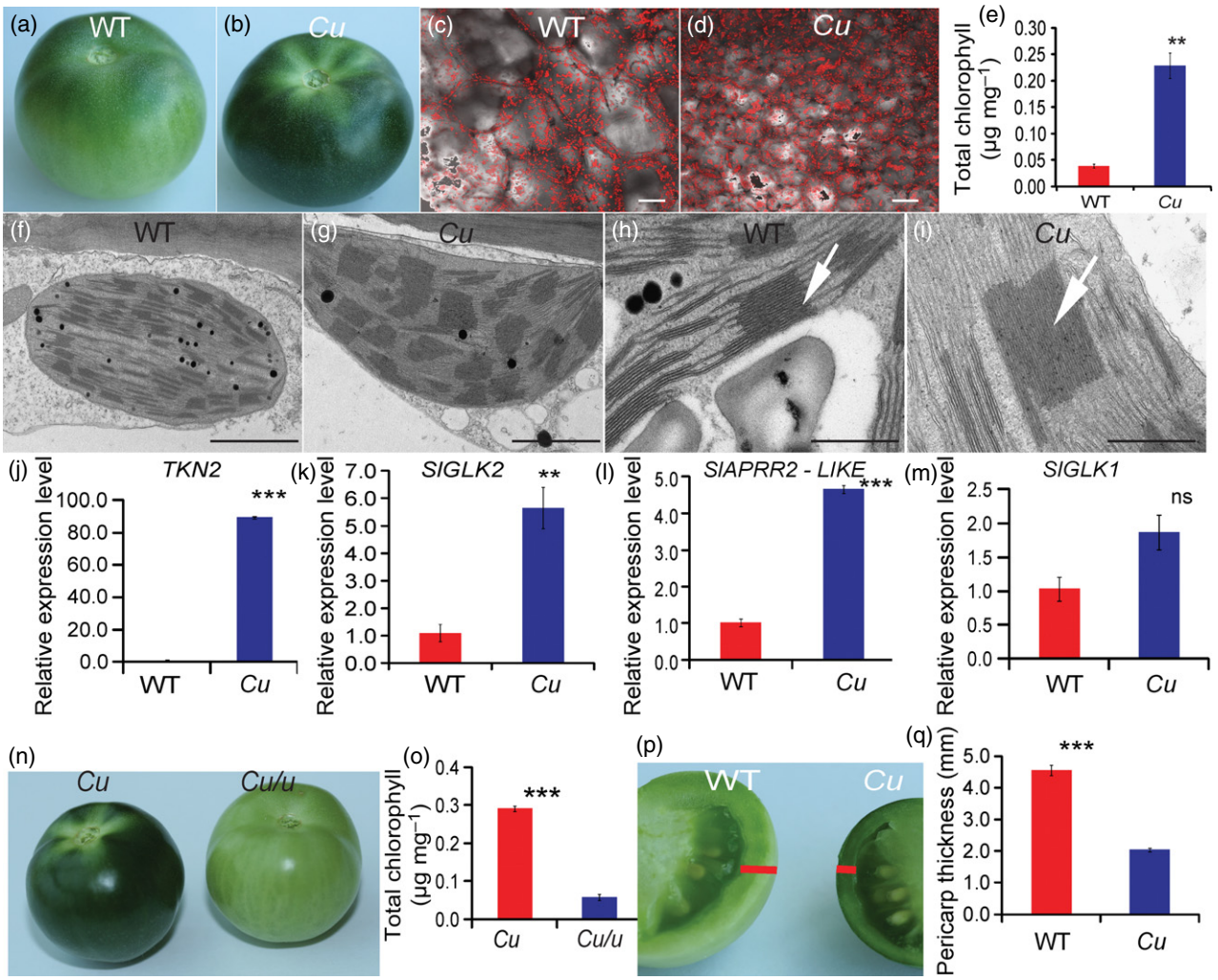

Figure 3. Enhanced chloroplast development in *Curl* (*Cu*) fruit.
(a), (b) Variation in fruit phenotype between Ailsa Craig wild type (WT) and *Cu* at 16 days post-anthesis (dpa). (c), (d) Confocal laser scanning microscopy images of WT and *Cu* fruit shoulder pericarp showing chlorophyll autofluorescence. Scale bar: 10 µm. (e) Chlorophyll content of WT and *Cu* fruit pericarp ($n = 5$). (f)–(i) Transmission electron micrograph of 28-dpa fruit chloroplasts of WT and *Cu*. Scale bars: (f), (g), 2 µm; (h) and (i), 0.5 µm. (j)–(m) Quantitative RT-PCR analysis of gene expression in WT and *Cu* fruit pericarp. (n) Fruit phenotype of *Cu* and *Cu/u*. (o) Chlorophyll content of *Cu* and *Cu/u* fruit pericarp ($n = 3$). (p), (q) Pericarp thickness of WT and *Cu* fruit ($n = 6$). Data are presented as the mean ± SEM. Asterisks denote significant differences (***$P < 0.001$; **$P < 0.01$) as determined by Student's *t*-tests. ns, not significant.

disrupted (Figure 4d). Together, these data not only support the role of *KNOX* genes in regulating both chloroplast development and gene expression gradients but also highlight that while *SlGLK2* and *SlAPRR2-LIKE* both have a positive impact on fruit chloroplast development (Powell *et al.*, 2012; Pan *et al.*, 2013) they are regulated by overlapping, yet distinct, mechanisms.

Several studies implicate phytohormones in mediating fruit chloroplast development in tomato, and in the case of auxin and BRs enhanced fruit chloroplast development is positively correlated with increased *SlGLK* expression (Martineau *et al.*, 1994; Galpaz *et al.*, 2008; Sagar *et al.*, 2013; Liu *et al.*, 2014). In addition, auxin gradients are involved in several developmental processes, and disruption of auxin signaling in the *arf6*/*arf8* double mutant results in ectopic *KNOX* expression and altered floral morphology (Benkova *et al.*, 2003; Petersson *et al.*, 2009; Tabata *et al.*, 2010). The potential role of auxin in regulating the *KNOX*-mediated chloroplast gradient in tomato fruit was investigated utilizing *SlARF4*-silenced lines, which possess increased starch content, enhanced chloroplast development and elevated *SlGLK1* expression (Sagar *et al.*, 2013). The *SlARF4*-silenced lines were developed in the Micro-Tom background (Kobayashi *et al.*, 2014), which is homozygous for the *u* mutation and lacks a functional *SlGLK2*. Unexpectedly, and unlike the *u* mutant allele in the Ailsa Craig background (Figure 1a and b), a decreasing gradient of chlorophyll content from the shoulder to the base of the

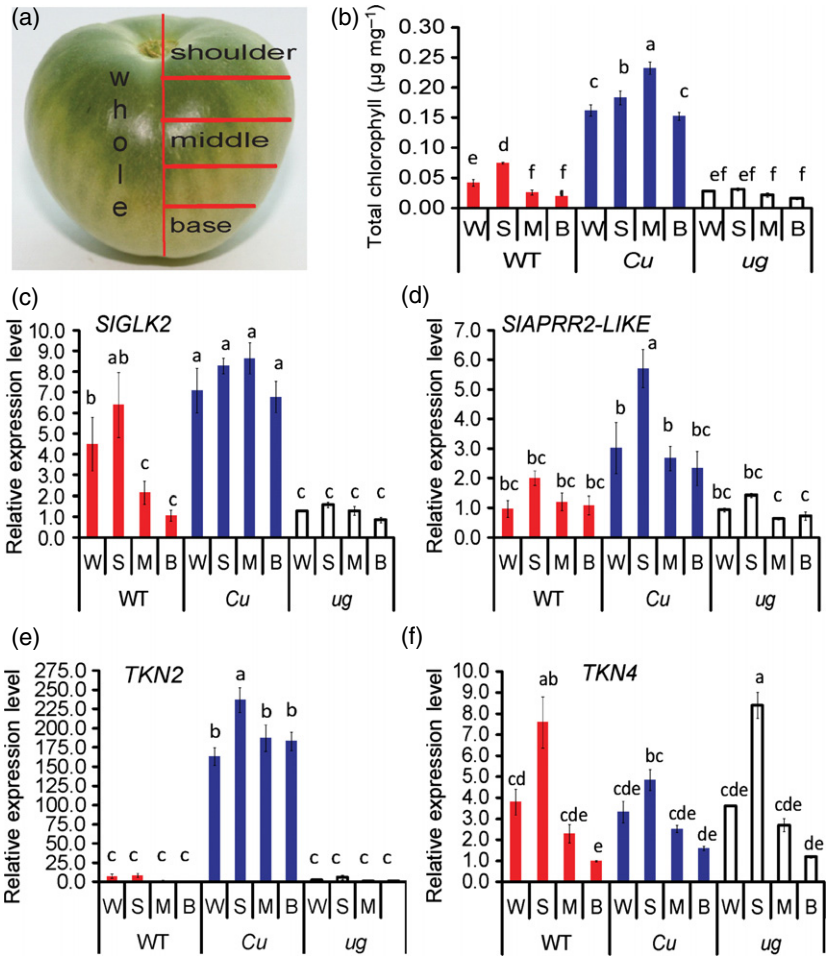

Figure 4. Mutations at *knox* loci disrupt the gradient of *SlGLK2* expression and affect fruit chloroplast development.
(a) Depiction of the sampling strategy. (b) Chlorophyll content in the pericarp isolated from the whole (W), shoulder (S), middle (M) and base (B) of Ailsa Craig wild-type (WT), *Curl* (*Cu*) and *uniform gray-green* (*ug*) mutant fruit at 28 days post-anthesis ($n = 5$). (c)–(f) Quantitative RT-PCR analysis of expression of *SlGLK2*, *SlAPRR2-LIKE*, *TKN2* and *TKN4* in the same tissue samples as described in (b) relative to the expression level in the base of WT fruits ($n = 3$). Data are presented as the mean ± SEM and means with different letters are significantly different (least squares means, $P < 0.05$).

fruit was observed in both Micro-Tom and a *SlARF4*-silenced line, with the latter displaying the expected elevated chlorophyll content (Figure 5a). The expression of *SlGLK2* and *SlAPRR2-LIKE* was elevated in the *SlARF4*-silenced line, particularly in the shoulder, and whereas the expression of *TKN2* and *TKN4* was also enriched in the fruit shoulder, the difference in expression levels between Micro-Tom and the *SlARF4*-silenced line are minor (Figure 5b–e). These data highlight the existence of a gradient of chlorophyll accumulation in Micro-Tom that occurs independently of *SlGLK2* and suggest that *SlARF4* negatively regulates the expression of *SlGLK2* and *SlAPRR2-LIKE*.

The fruit phenotype of *hp-1* is partially suppressed by *u* and *ug*

Fruits of the *hp-1* mutant have an increased number of chloroplasts, an increased plastid compartment size and a higher chlorophyll content (Cookson *et al.*, 2003). The contribution of *KNOX* and *SlGLK2* to the *hp-1* phenotype was assessed using a combination of single and double mutants in both WT and the *hp-1* background. Both *u* and *ug* partially suppress the *hp-1* phenotype, although suppression by *u* is greater than that achieved by *ug* and a slightly additive phenotype is apparent when *u* and *ug* are combined (Figure 6a,b). Congruent with the elevated levels of chlorophyll, expression of *SlGLK2*, *SlAPRR2-LIKE*, *TKN2* and *TKN4* is higher in *hp-1* fruit pericarp than WT (Figure 6c–f). Together, these data indicate that both *SlGLK2* and *TKN4* contribute to the *hp-1* mutant phenotype and are also suggestive of a role for *SlAPRR2-LIKE* in determining the elevated chlorophyll content of *hp-1* fruit, possibly via regulation by *TKN2*. The reduced suppression of *hp-1* by *ug* compared with *u* may be due to the residual expression of *SlGLK2* observed in the *ug* mutant (Figure 4c), which may indicate that the *ug* allele (accession LA3416), which carries the F137L substitution, is not a complete null or that additional unknown factors regulate *SlGLK2* expression. Furthermore, evidence for the involvement of *SlAPRR2-LIKE* or additional transcription factors in determining the elevated chlorophyll content of *hp-1* fruit is supported by the observation that while chlorophyll levels of the *hp1*/*u* mutant are reduced compared with those observed in

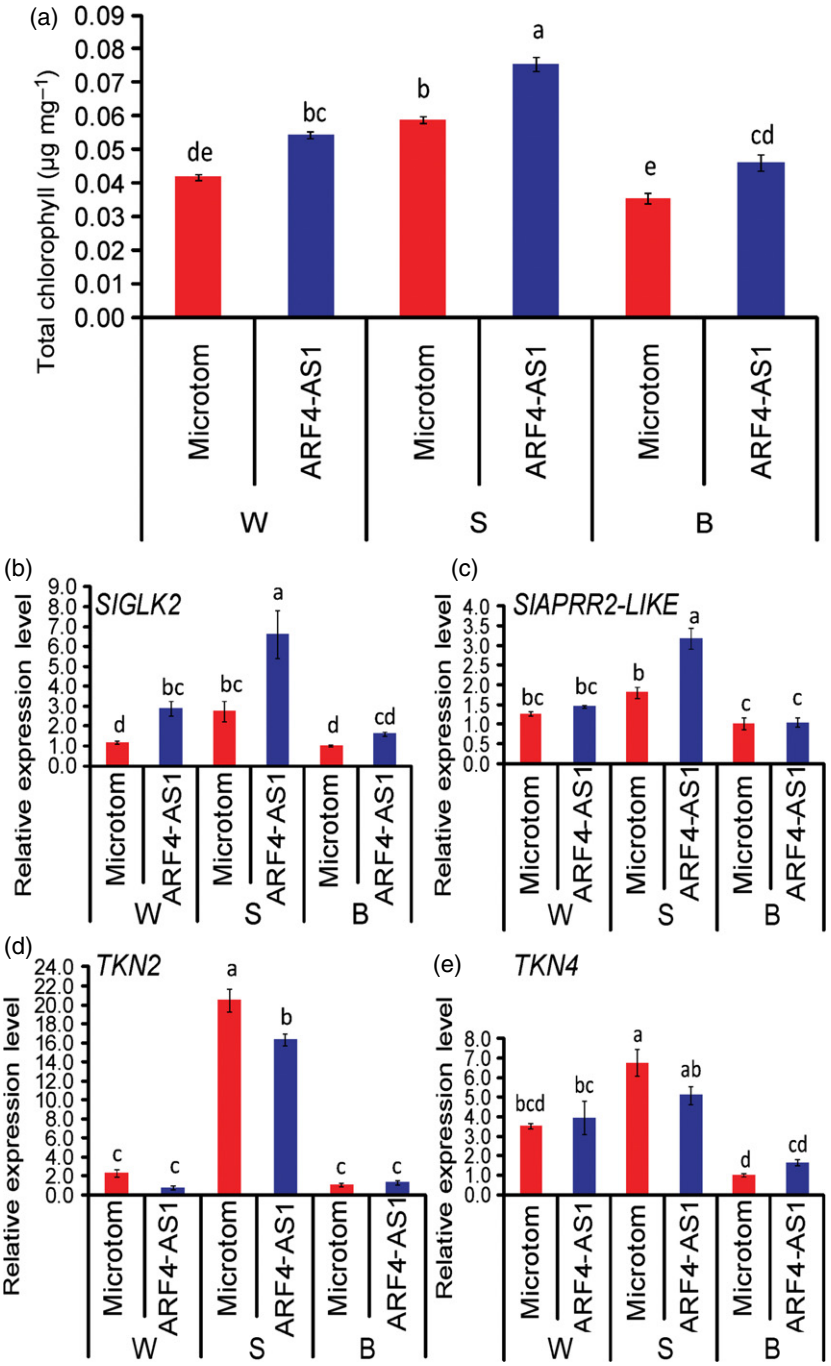

Figure 5. Silencing of *SlARF4* affects fruit chlorophyll and expression of *SlGLK2* and *SlAPRR2-LIKE*.

(a) Chlorophyll content in whole (W), shoulder (S) and base (B) pericarp of Micro-Tom and *SlARF4* antisense lines at 21 days post-anthesis ($n = 5$).

(b)–(e) Relative expression level of *SlGLK2*, *SlAPRR2-LIKE*, *TKN2* and *TKN4* in same samples described in (a). Data are presented relative to the expression level in base of Micro-Tom fruits ($n = 3$). Means ± SEM with different letters are significantly different (least squares means, $P < 0.05$).

WT fruit, they are not reduced to the levels observed in the *u* mutant (Figure 6b).

DISCUSSION

A role for *KNOX* genes in the development of fleshy fruit

KNOX genes influence multiple aspects of plant morphology and are typically expressed within apical meristems, where they maintain pluripotent cell populations required for organ initiation (Jackson *et al.*, 1994; Long *et al.*, 1996; Byrne *et al.*, 2002; Belles-Boix *et al.*, 2006; Hay and Tsiantis, 2010). Loss of KNOX activity can lead to inhibition of shoot formation, whereas ectopic *KNOX* expression results in pleiotropic phenotypes including increased lobe formation in simple leaves and increased complexity of compound leaves (Lincoln *et al.*, 1994; Hareven *et al.*, 1996; Long *et al.*, 1996; Hay and Tsiantis, 2006). In addition to roles in shoot apical meristem formation and leaf development, *KNOX* genes influence plant height, disrupt apical dominance and alter patterns of cell division and

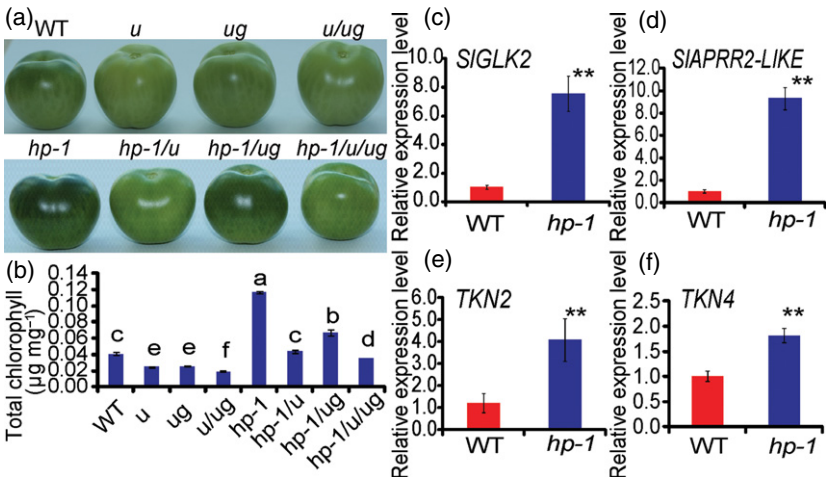

Figure 6. The *uniform ripening* (*u*) and *uniform gray-green* (*ug*) loci partially suppress *high-pigment 1* (*hp-1*).
(a) Variation in fruit phenotype between single, double and triple mutant combinations of *hp-1*, *u* and *ug* at 21 days post-anthesis (dpa).
(b) Chlorophyll content in the pericarp of genotypes in (a) ($n = 5$).
(c)–(f) Relative expression level of *SlGLK2*, *SlAPRR2-LIKE*, *TKN2* and *TKN4* in wild type (WT) and *hp-1* pericarp at 21 dpa. Data are presented as the mean ± SEM. Means with different letters are significantly different (least squares means, $P < 0.05$) and asterisks denote significant differences ($^{**}P < 0.01$) as determined by Student's *t*-test.

elongation (Douglas *et al.*, 2002; Venglat *et al.*, 2002). The Arabidopsis *KNOX* genes, *SHOOT MERISTEMLESS* (*STM*), *BREVIPEDICELLUS* (*BP*), *KNAT2* and *KNAT6*, also influence development of the inflorescence and carpels together with the architecture of abscission zones (Scofield *et al.*, 2007; Ragni *et al.*, 2008; Shi *et al.*, 2011). The role of individual *KNOX* genes in tomato remains obscure, but identification of the underlying basis of the *ug* mutant has revealed an atypical role for *TKN4* in influencing fruit chloroplast development and specifically the formation of the green shoulder (Figure 1). This role is supported through characterization of the fruit phenotypes of *Cu* that result from ectopic expression of *TKN2* (Figure 3). *TKN2* is closely related to the *STM* gene of Arabidopsis, which is required for both vegetative and reproductive meristem development (Long *et al.*, 1996; Scofield *et al.*, 2007; Hay and Tsiantis, 2010). As observed by *TKN2* over-expression in the *Cu* mutant, ectopic expression of *STM* also leads to aberrant meristem activity and altered plant morphology (Parnis *et al.*, 1997; Gallois *et al.*, 2002; Scofield *et al.*, 2013). Within the shoot apical meristem, both *TKN2* and *STM* function in part through modulation of hormone activity, and particularly by influencing the levels of cytokinins and gibberellins (Hay *et al.*, 2002; Jasinski *et al.*, 2005; Yanai *et al.*, 2005; Hay and Tsiantis, 2010; Shani *et al.*, 2010). At present the role of these hormones in contributing to the altered phenotypes observed in the fruit of the *Cu* mutant (Figure 3) remains to be defined. *TKN4* does not possess an apparent ortholog in Arabidopsis, although previous phylogenetic analysis suggested that it is closely related to *FaKNOX1* from strawberry and *NTH1* in tobacco (Chatterjee *et al.*, 2011). Manipulation of *FaKNOX1* expression in transgenic strawberry and Arabidopsis alters plant morphology, including leaf, flower and meristem architecture. However, altered expression of additional *KNOX* family members was also apparent in *FaKNOX1*-silenced lines, raising the possibility that the observed phenotypes could, in part, be due to altered expression of other strawberry *KNOX* genes (Chatterjee *et al.*, 2011). Similarly, over-expression of *NTH1* in tobacco altered plant morphology, manifested by crinkling of the leaves and altered phyllotaxy, phenotypes that are typical of *KNOX* over-expression (Tamaoki *et al.*, 1999). Interestingly, the *ug* mutant does not display any obvious phenotypes related to organ morphology (Figure S2), which may be the result of partial redundancy, a phenomenon often observed between *KNOX* genes (Byrne *et al.*, 2002; Belles-Boix *et al.*, 2006) and which is supported by the overlapping expression patterns of the four tomato Class I *KNOX* genes *TKN1–4* in different tissues and organs (The Tomato Genome Consortium, 2012). However, functional specificity also exists between the tomato *KNOX* genes, as although *TKN2* and *TKN4* display increased expression in the shoulder of the fruit (Figures 4 and S4), *TKN2* does not substitute for reduced TKN4 activity in the *ug* mutant. Furthermore, characterization of *Cu* revealed a broader role for *KNOX* genes in fleshy fruit development as *TKN2* over-expression not only promoted chloroplast development but also affected fruit size, pericarp thickness and pericarp cell density (Figure 3). Identification of a loss-of-function allele of *TKN2* or fruit-specific gene silencing of this gene will facilitate improved understanding of its role in fruit development, including chloroplast development.

Differential regulation of chloroplast development in tomato leaves and fruits

Both *u* and *ug* affect the gradient of chloroplast development in tomato fruit, although neither locus influences chloroplast development in leaves (Figures 1 and S2a–c). In the case of the *u* locus the lack of impact on leaf chloroplast development is likely to be due to redundancy and the overlapping expression of *SlGLK1* and *SlGLK2* in tomato leaf (Powell *et al.*, 2012), whereas the lack of impact of *ug* on leaf chlorophyll content may be due to the absence of *TKN4* expression in leaves (The Tomato Genome Consortium, 2012). However, over-expression of

TKN2 in the *Cu* mutant does not increase chlorophyll content in leaves but affects chloroplast development and the accumulation of chlorophyll in fruit (Figures 3 and S3). These data suggest differential regulation of chloroplast development in fruits and leaves of tomato, a hypothesis supported by data indicating that over-expression of *GLK*s, and *SlAPRR2-LIKE*, together with suppression of *SlARF4*, has a positive impact on chloroplast development in fruits but not leaves (Powell *et al.*, 2012; Pan *et al.*, 2013; Sagar *et al.*, 2013). The underlying basis for this differential regulation of chloroplast development in tomato is unknown but may be due to saturation of chloroplast development in tomato leaves, as these contain levels of chlorophyll that are approximately 20 times higher than in fruit (Figures 1b and S2c) and therefore possibly less amenable to further enrichment. In addition to the apparent differential regulation of chloroplast development in tomato leaves and fruits, parallel pathways regulate fruit chloroplast development. For example, the impact of *SlAPRR2-LIKE* over-expression on fruit chloroplast development is independent of *SlGLK2*, as experiments were performed in Micro-Tom which is homozygous for the *u* mutant allele (Pan *et al.*, 2013; Kobayashi *et al.*, 2014). Similarly, although *u* and *ug* suppress the *hp-1* mutant phenotype, they do not restore chlorophyll to levels detected in the single mutants in the absence of *hp-1* (Figure 6a,b). This discrepancy may be due in part to the elevated expression of *SlAPRR2-LIKE* in the *hp-1* background (Figure 6d). The availability of *SlAPRR2-LIKE* mutant alleles or silencing of *SlAPRR2-LIKE* in WT and mutant backgrounds will facilitate greater understanding of the role of this gene in chloroplast development, together with its relationship to *KNOX* genes and *SlGLK2*.

TKN4 influences the gradient of chloroplast development in tomato fruit through regulation of *SlGLK2*

Transcription factor gradients play important roles in the development of multicellular organisms but remain poorly understood in plants (Stathopoulos and Levine, 2002; Sozzani *et al.*, 2010; Chen *et al.*, 2012). In grasses, leaf differentiation proceeds basipetally and is accompanied by a gradient of differential gene expression, chloroplast development and photosynthetic competency (Leech *et al.*, 1973; Li *et al.*, 2010). Similarly, the present study has investigated a tissue-level macro-gradient of chloroplast formation that occurs during the development of tomato fruit that leads to the formation of a green shoulder. The physiological role of this chloroplast gradient is unknown, but its formation is increased under shade conditions and the shoulder possesses higher photosynthetic capacity than the remaining pericarp tissues, suggesting that this may be an adaptive trait that contributes to fruit photosynthesis (Smillie *et al.*, 1999). Manipulation of KNOX activity, either through mutation in *TKN4* in *ug* or over-expression of

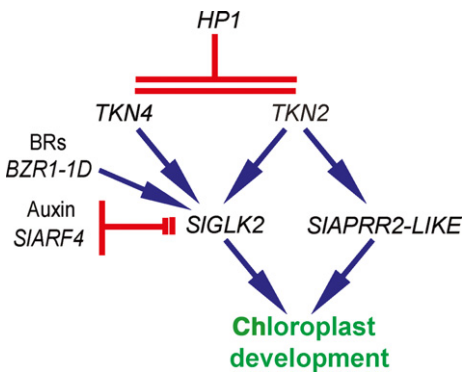

Figure 7. A proposed model for KNOX activity in developing tomato fruit. The *KNOX* genes *TKN2* and *TKN4* positively influence *SlGLK2* and *SlAPRR2-LIKE* expression to promote fruit chloroplast development. Auxin, via *SlARF4*, represses the expression of *SlGLK2* and *SlAPRR2-LIKE* whereas brassinosteroids (BRs), via expression of BZR1-1D, promotes *SlGLK2* expression (Liu *et al.*, 2014). Data indicate that *TKN2* influences the expression of both *SlGLK2* and *SlAPRR2-LIKE*, whereas *TKN4* only influences the expression of *SlGLK2*. *HP-1* acts upstream of these genes to repress their expression.

TKN2 in *Cu*, disrupts the gradient of chloroplast development in tomato fruit by altering the spatial expression of *SlGLK2* and *SlAPRR2-LIKE* (Figure 4). However, *TKN2* and *TKN4* possess overlapping and distinct activities, and while over-expression of *TKN2* influences the expression of both *SlGLK2* and *SlAPRR2-LIKE*, reduced *TKN4* activity only disrupts *SlGLK2* expression (Figures 4 and 7). These data promote a model (Figure 7) suggesting the existence of distinct regulatory modules that influence chloroplast development in tomato fruit, and it will be of interest to determine whether TKN2 and TKN4 bind directly to regulatory regions of *SlGLK2* and *SlAPRR2-LIKE*. Similarly, while suppression of *SlARF4* did not dramatically alter the expression of *TKN2* and *TKN4*, expression of both *SlGLK2* and *SlAPRR2-LIKE* was elevated (Figure 5). These data suggest multiple regulatory inputs that influence the expression of *SlGLK2* and *SlAPRR2-LIKE*, a hypothesis supported by the recent report indicating that activation of BR responses leads to elevated expression of *SlGLK2* and enhanced chloroplast development (Liu *et al.*, 2014). Although *TKN4* influences the latitudinal gradient of *SlGLK2* expression in tomato fruit, *TKN2* and *TKN4* also display a gradient-dependent expression pattern in developing fruit, suggesting that *KNOX* genes themselves do not establish the gene expression gradient that exists in this tissue and that as yet unidentified signaling pathways are required. Furthermore, KNOX proteins and their mRNAs are also trafficked through plasmodesmata (Lucas *et al.*, 1995), raising the possibility that the fruit chloroplast gradient may, at least in part, also be regulated through cell to cell movement of these proteins as well as their gradient-dependent transcript accumulation. The impact of gene expression gradients in plants is not fully

understood, but this study has identified a role for *KNOX* genes, whereby they regulate the gradient-dependent expression pattern of transcription factors required for the development of fruit chloroplasts.

EXPERIMENTAL PROCEDURES

Plant material, growth conditions and chlorophyll extraction

Seeds of tomato (*Solanum lycopersicum*) cultivars, mutants and mutant combinations at the *u*, *ug*, *Cu* and *hp-1* loci (Table S1), together with *Solanum pimpinellifolium* accession LA1589, were obtained from the Tomato Genetics Resource Center, UC Davis, CA, USA or commercial vendors. Plants were grown in peat-based compost supplemented with fertilizer in greenhouses at Michigan State University, East Lansing, MI, USA under conditions of 16-h day (25°C) and 8-h night (20°C). Micro-Tom and the *SlARF4*-silenced line (Sagar *et al.*, 2013) were grown in a growth room under similar conditions. Flowers were tagged at anthesis and fruits subsequently harvested at defined ages based on days post-anthesis. The chlorophyll content was sampled from fruit pericarp and the terminal leaflet of expanding leaves of 4-week-old plants as previously described (Nadakuduti *et al.*, 2012).

Genetic mapping of the *ug* locus and genotyping

An interspecific F_2 population segregating for the *ug* locus was generated from a cross between *S. lycopersicum* (*ug*/*ug*) (LA3539) and *S. pimpinellifolium* (*UG*/*UG*) (LA1589). Fruits from 60 F_2 individuals were phenotyped for the presence or absence of a green shoulder and plants were genotyped with genetic markers spanning the tomato genome. Subsequent fine mapping was performed on further 1600 F_2 individuals using chromosome 1 genetic markers (Table S2). Genomic DNA was extracted as previously described (Barry *et al.*, 2005). Details of genetic and physical maps together with associated molecular markers are available through the Sol Genomics Network. Genotyping tomato accessions (Table S1) for the *ug* mutant allele was achieved using a cleaved amplified polymorphic sequence marker (Table S3) that was resolved by digestion with *Mse*I. Genotyping for the *u* mutant allele was achieved through sequencing of PCR fragments amplified using primers (Table S3) that span the single nucleotide insertion site (Powell *et al.*, 2012).

Molecular cloning and sequence analysis

Full-length cDNA clones of *TKN4* were amplified and cloned from flower cDNA of WT and the *ug* mutant as previously described (Nadakuduti *et al.*, 2012). Five independent clones from each genotype were selected for sequencing. The entire *TKN4* gene was sequenced from LA0021 genomic DNA. Sequences from the region with an unexpected band size containing the deletion were cloned, and four independent clones were sequenced and assembled using SEQUENCHER® version 4.7. Amino acid alignments were generated using MUSCLE (Edgar, 2004). Primers are listed in Table S3 and additional sequences utilized in multiple sequence alignments are provided in Table S4.

Quantitative RT-PCR analysis

Expression analysis was performed according to Nadakuduti *et al.* (2012) with the exception that 10-μl PCR reactions were assembled using a Biomek 3000 liquid handler (http://www.beckmancoulter.com) and amplified using an Applied Biosystems ABI Prism 7900HT Real-Time PCR System (http://www.lifetechnologies.com) with the following sequence: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Primers are listed in Table S3.

Microscopy

Chloroplast imaging using confocal laser-scanning microscopy (CLSM) and transmission electron microscopy (TEM) was performed as previously described (Barry *et al.*, 2012) with minor modifications. The CLSM images were captured using an objective with a numerical aperture of 0.5 and maximum intensity projection images were generated from a confocal z-series acquired through an average thickness of 170 μm in 10-μm increments. Samples for TEM imaging were embedded in Spurr resin.

Statistical analyses

Statistical analyses were performed using SAS. The genotypic constituents were evaluated by Student's *t*-test and least squares means.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Genotyping of *TKN4* and *SlGLK2* in various tomato accessions.

Figure S2. Leaf phenotypes and relative expression of *TKN4*.

Figure S3. Chlorophyll and relative expression of genes in *Cu* leaf.

Figure S4. Relative expression gradient of *TKN2* within the fruit.

Table S1. Tomato accessions used in the study.

Table S2. The PCR based genetic markers flanking the *ug* locus.

Table S3. Oligonucleotide primers used in the study.

Table S4. *KNOX* genes used for amino acid alignments.

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