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ORIGINAL PAPER

CaGLK2 regulates natural variation of chlorophyll content and fruit color in pepper fruit

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Abstract

Key message **We provide multiple evidences that *CaGLK2* underlies a quantitative trait locus controlling natural variation in chlorophyll content and immature fruit color of pepper via modulating chloroplast compartment size.**

Abstract Pepper fruit quality is attributed to a variety of traits, affecting visual appearance, flavor, chemical composition and nutritional value. Among the quality traits, fruit color is of primary importance because the pigments that confer color are associated with nutrition, health and flavor. Although gene models have been proposed for qualitative aspects of fruit color, large natural variation in quantitative pigment content and fruit color exists in pepper. However, its genetic basis is largely unknown which hampers its utilization for plant improvement. We studied the role of *GLK*2, a *GOLDEN2-like* transcription factor that regulates chloroplast development in controlling natural variation for chlorophyll content and immature fruit color of pepper. The role of *GLK2* in regulating fruit development has been studied previously in tomato using ectopic expression and the *uniform ripening* mutant analyses. However, pepper provides a unique opportunity to further study the function of this gene because of the wide natural variation of fruit colors in this species. Segregation, sequencing and expression analyses indicated that pepper *GLK2* (*CaGLK2*) corresponds to the recently reported *pc10* QTL that controls chloroplast development and chlorophyll content in pepper. *CaGLK2* exerts its effect on chloroplast compartment size predominantly during immature fruit development. We show that the genetic background, sequence variation and expression pattern confer a complex and multi-level regulation of *CaGLK2* and fruit color in *Capsicum.* The positive effect on fruit quality predominantly at the green stage conferred by *CaGLK2* can be utilized to breed green pepper varieties with improved nutritional values and taste.

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Introduction

In plants, essential cellular processes associated with chloroplasts include photosynthesis, the production of carotenoids and tocopherols and differentiation of chloroplasts into chromoplasts during fruit ripening. Fruit biomass is mainly derived from translocation of photosynthate from leaves, but green pericarp tissue has chloroplasts capable of photosynthesis (Hetherington et al. 1998). Since the activity of both chloroplast and nuclear genes is required for chloroplast biogenesis and development, tight coordination between the cell compartments is needed (Kessler and Schnell 2009). Mutant analyses, mostly in Arabidopsis, have allowed the identification of a large number of organelle and nuclear-encoded genes that function in different aspects of chloroplast biogenesis (Pogson and Albrecht 2011).

In recent years, few transcription factors that regulate chloroplast development have been isolated. Among them,

GOLDEN2-like (*GLK*2) nuclear genes are members of the GARP super family of transcription factors (Fitter et al. 2002; Wang et al. 2013). In Arabidopsis, there are two *GLK* genes, *AtGLK1* and *AtGLK2,* that are functionally redundant. Both single-gene mutants are phenotypically indistinguishable from wild type and only the double mutant has a pale green leaf phenotype with reduced thylakoid membrane and grana stacking (Fitter et al. 2002; Wang et al. 2013). In Arabidopsis siliques, *Atglk2* and *Atglk1 Atglk2* mutants have similar pale green phenotypes (Fitter et al. 2002). Transcriptome analyses have indicated that *GLK2* coordinates the expression of a suite of photosynthetic genes involved in chlorophyll biosynthesis, light harvesting and electron transport (Waters et al. 2009).

Early work on *GLK* genes function focused on their role in regulating chloroplast development in leaves of maize, Arabidopsis and rice (Hall et al. 1998; Rossini et al. 2001; Fitter et al. 2002). Recent studies focused on the function of *GLK2* in the fleshy fruit of tomato (Powell et al. 2012; Nadakuduti et al. 2014; Nguyen et al. 2014). The monogenic mutation *uniform ripening* (*u*) has been widely used to breed uniform, light green tomato fruits that convert to evenly ripe fruits. Tomato *SlGLK2* is mutated at *u*, while the dominant *U* allele is associated with increased expression of *SlGLK2* in the pedicel attachment side of the fruit and with a dark green shoulders phenotype (Powell et al. 2012). Expressing either *AtGLK1* or *AtGLK2* in tomato *u* resulted in dark green fruit with increased chlorophyll content and chloroplast number, indicating functional conservation of *GLK* genes across taxa. Recently, a related but distinct transcription factor *ARABIDOPSIS PSEUDO RESPONSE REGULATOR2-LIKE* gene (*APRR2-like*) with similar phenotypic effects to *SlGLK2* in fruit was identified in tomato and pepper (Pan et al. 2013).

Pepper fruit quality is attributed to a variety of traits, affecting visual appearance, flavor, chemical composition and nutritional value (Guzman et al. 2011; Paran and Fallik 2011). Together, these traits determine consumer acceptance of the fruit. The pepper fruit contains a complex of beneficial phytochemical compounds, including carotenoids, flavonoids, ascorbic acid, phenolics, tocopherols, sugars, organic acids and volatiles. Variation in the contents of these compounds in *Capsicum* enables breeding for improved quality (Wahyuni et al. 2011). However, progress towards utilization of this variation for crop improvement is often hampered because its genetic and molecular basis is complex and little studied.

Among the quality traits, fruit color is of primary importance because the pigments that confer color are also associated with nutritional, health and flavor values. The major pigments in the pepper fruit include carotenoids, which determine primarily the mature fruit color, and anthocyanins and chlorophyll, which determine primarily the immature fruit color, but can also affect ripe fruit color (Borovsky and Paran 2008). While qualitative variation in pepper fruit color is well characterized and genes conferring various fruit colors were identified (Paran and van der Knaap 2007), large variation in quantitative pigment content exists (Hornero-Mendez et al. 2000; Levy et al. 1995; Mejia et al. 1988; Wall et al. 2001), but its genetic basis is unknown.

The most dramatic variation in pigment content of pepper fruit is associated with the green immature fruit stage. This was demonstrated in a cross of parents with 18-fold difference in their chlorophyll content (Brand et al. 2012). Quantitative trait locus (QTL) mapping for chlorophyll content allowed the identification of two major QTLs, *pc8.1* and *pc10.1*, that control this trait. *pc8.1* (termed *pc8* in the present study) was found to exert its effect via modulating chloroplast compartment size (Brand et al. 2012). In the present manuscript, we provide evidences that the gene governing *pc10.1* (termed *pc10* in the present study) is the pepper ortholog of *SlGLK2* (*CaGLK2*). By surveying the allelic diversity of pepper *GLK2* in a germplasm representing wide natural variation in chlorophyll content, we show that it has a role in determining chlorophyll content and immature fruit color in *Capsicum*. Furthermore, the effect of genetic background, sequence variation and expression pattern implicate a complex and multi-level regulation of this trait in *Capsicum.*

Materials and methods

Plant material

The parents used in the present study include *C. annuum* line 1154 with an immature dark green fruit (Brand et al. 2012), *C. chinense* PI 152225 (from hereafter PI 225) with a light green fruit and *C. chinense* PI 593611 obtained from the pepper USDA collection, Griffin, GA (from hereafter PI 611) with a typical medium green fruit (Fig. 1). Ripe fruits of the three parents have red color. Two sets of NILs for *pc10* were prepared by marker-assisted selection and backcrossing program using 1154 as a recurrent parent and PI 225 and PI 611 as donor parents. *pc10* NILs were derived from BC_4F_3 heterozygous individuals with the most significant marker at the QTL (Brand et al. 2012) and subsequent selfing to obtain homozygous plants for the recurrent and donor alleles. Additional lines used for sequencing and expression included (all *C. annuum* except when marked differently): 1902 (CGN21469), 1901 (CGN23289), CA4 (*C. chinense* PI 159234, USDA collection Griffin), Maor (The Volcani Center), 1150 (Cv. Planika, Enza Zaden), 13-2 (Grif 9094, USDA collection Griffin), 16-2 (PI 357539, USDA collection Griffin), 21-1

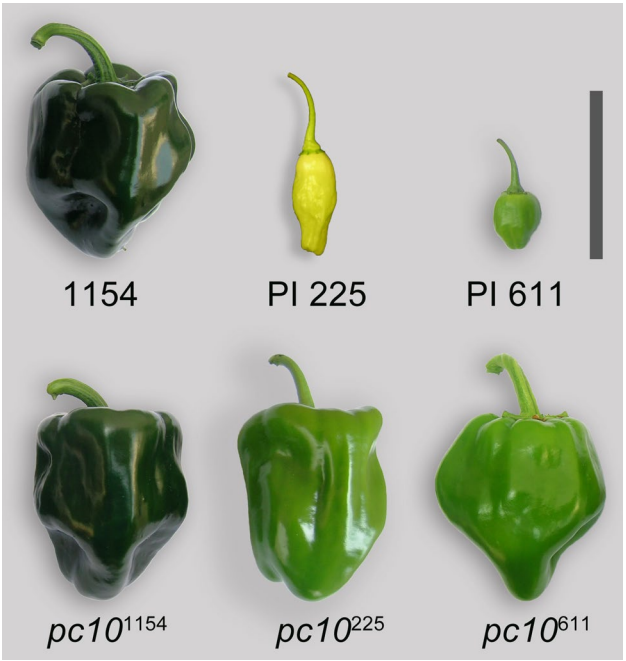


Fig. 1 Fruits of the recurrent (1154), donor parents (PI 225 and PI 611) and *pc10* NILs used in this study. *Bar* 5 cm

(PI 586677, USDA collection Griffin), 1134 (The Volcani Center), Cora-14 (referred to as Cora), Nayoi-3 (referred to as Nayoi) and 1202, (the last three are *C. annuum* var *glabriusculum* from Dr. Jose Luiz Luna, Aguascalientes, Mexico).

Plastid and pigment measurements

Number and area of chloroplasts per cell of each genotype were measured from green immature fruits at 10 days after anthesis using Z-stacks of confocal optical sections captured from pepper tissues. Area and number of chromoplasts per cell were measured by light microscope using pericarp slices of red mature fruits. Chlorophyll and total carotenoid content was measured from mature green fruits at four weeks after anthesis and from ripe red fruits, respectively. In addition, chlorophyll content was measured in fully developed mature leaves. These measurements were previously described in more detail (Brand et al. 2012).

Transmission electron microscopy (TEM)

Preparation of pericarp tissue from young immature fruit (10 days after anthesis) for TEM was previously described (Powell et al. 2012). Image analysis and grana thickness measurements were obtained using ImageJ processing and analysis program (National Institutes of Health, Bethesda, MD, USA; http://imagej.nih.gov/ij/).

Total soluble solids (Brix), sugar content and yield measurements

For yield and total soluble solids analyses, plants were grown in the open field in the Volcani Center Experimental Station in the summer of 2011. Yield was measured by weighing the total fruit yield from ten individual plants 3 months after planting. Brix was measured in ten plants for each parent (three fruits per plant) in the mature green and ripe red stages using a refractometer on a drop of fresh juice extracted by a garlic crusher. The content of sugars (glucose, fructose, sucrose and sum of sugars) was measured by high-performance liquid chromatography (HPLC) as previously described (Petreikov et al. 2009).

Sequencing, multiple alignments and mapping of *CaGLK2*

We used the *C. annuum* transcript MGMT_Contig23879 (GenBank Accession JW074123; Ashrafi et al. 2012) and pepper genomic DNA scaffolds 79152.1 and 122916.1 (kindly provided by D. Choi, Seoul National University) as templates for sequencing the open reading frame (ORF) of *CaGLK2* in different accessions using primers CaGLK2-F and CaGLK2-R; CaGLK2-F1 and CaGLK2-R3 (Table S1). The ORF sequences (deposited in GenBank as accessions numbers KJ420379-KJ420393) were translated and used for multiple alignments by the web-based version of Clustal W (http://www.ebi.ac.uk/services). For mapping *CaGLK2* in *C. annuum* × *C. chinense* crosses, we used the primers CaGLK2-F4 and CaGLK2-R2 based on the sequence of scaffold 122916.1 Pepper sequence v0.9 containing the genomic sequence of *CaGLK2* kindly provided by D. Choi, Seoul National University, Korea. Following PCR amplification, the product was digested with the restriction enzyme *Hph*1 that allows distinction of the two alleles.

Expression of *CaGLK2* and *CaGLK1*

For expression analyses in different tissues and fruit development stages, total RNA was extracted as previously described (Cohen et al. 2012) from roots of 4-week-old seedlings, fully expanded leaves, ovaries at anthesis, green fruit at ten dpa (days post-anthesis), mature green fruit (28 dpa), and ripe red fruit. Total RNA was used for first-strand cDNA synthesis by semi-quantitative RT-PCR using primers CaGLK2-F and CaGLK2-R and *CaUBIQUITIN* (DQ975458.1) as a reference gene (Cohen et al. 2012). For quantitative real-time PCR (qRT-PCR), plants were grown in the net-house under natural light conditions during the summer and RNA was extracted from green fruit at ten dpa using five biological replicates. qRT-PCR was performed using the primers CaGLK2qRT-F and CaGLK2qRT-R

5′- as previously described (Cohen et al. 2012). The relative expression of *CaGLK2* was normalized against pepper *CaUBIQUITIN*. All expression values are presented relative to that of the dark green parent 1154 which was set as 1.0. Similarly, primers for *CaGLK1* were designed from mRNA sequences, GenBank Accession JF807944 (Powell et al. 2012).

Results

Characterization of *pc10* NILs for pigment content and plastid compartment size

To study the effect of *pc10* on fruit pigment content and plastid compartment size, we constructed two pair of NILs using the same recurrent parent and two donor parents different in their pigment content. Compared to the recurrent parent 1154 that has a dark green immature fruit color and chlorophyll content of 83 ± 4 μg g FW^{-1}, the donor parents PI 225 and PI 611 have lighter green immature fruits with chlorophyll content of 4 ± 0.2 and 14 ± 1 μg g FW^{-1}, respectively (Fig. 1). NIL $pc10^{225}$ and NIL $pc10^{611}$ have significantly lower chlorophyll content than 1154, 38 ± 2 (55 % reduction) and 30 ± 3 (64 % reduction) μg g FW^{-1}, respectively (Figs. 1, 2a). Significant differences in chlorophyll content in the leaves were observed between 1154 and the two *pc10* NILs; however, the difference (9 % reduction) was much smaller compared to fruit chlorophyll content (Fig. 2b).

To test whether the difference in fruit chlorophyll content between *pc10* NILs is associated with differences in chloroplast compartment size, we compared chloroplast number and size. While no significant difference was observed in chloroplast number between 1154 and the two NILs (Fig. 3a), plastid area was significantly larger in 1154 compared to the two NILs (Fig. 3b). Furthermore, cell index (% plastid) calculated from the ratio of total chloroplast area to cell area was significantly larger in 1154 (27 ± 3) compared to the light green NILs (11 ± 2 and 10 ± 1.5) for $pc10^{225}$ and $pc10^{162}$, respectively. A closer look at the chloroplast structure using TEM showed significantly thicker grana in 1154 compared to $pc10^{611}$ and $pc10^{225}$ (Fig. 3c, d). To test whether *pc10* affects also pigment content and plastid compartment size at the ripe fruit stage, we measured total carotenoids content and number and size of chromoplasts in red ripe fruits of the NILs. A tendency of increased total carotenoids content and chromoplast compartment size was observed in 1154 compared to *pc10* NILs; however, these differences were not statistically significant (Fig. S1a, b, c). Together, these results indicate that *pc10* exerts its effect predominantly during immature fruit development and that the QTL affects chloroplast compartment size but not chloroplast number.

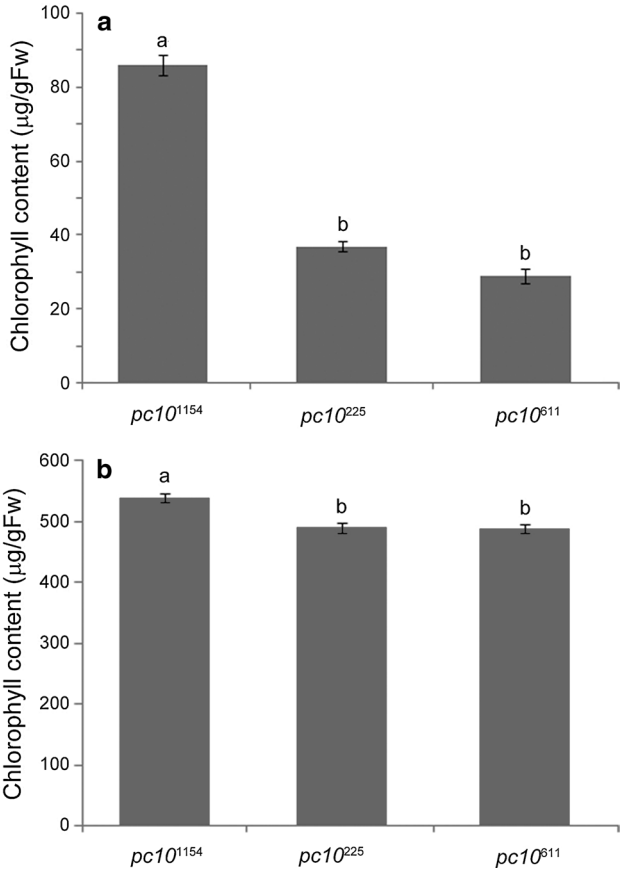


Fig. 2 Chlorophyll contents in mature green fruits (**a**) and leaves (**b**) in *pc10* NILs. Differences among means were determined by Tukey–Kramer range test at $P < 0.05$ and are indicated by different *lowercase letters*. Data for each group are means of five independent replicates ±SE

Characterization of *pc10* NILs for Brix, sugars and yield

To test whether increased chlorophyll content in leaves and fruit of 1154 compared to *pc10* NILs is associated with increased fruit photosynthate, we measured Brix and sugar content in green and red fruits. In the unripe green fruit, Brix was 28 % and 22 % higher in 1154 than in $pc10^{225}$ and $pc10^{611}$, respectively (Fig. 4a). In the ripe fruit, 1154 had significantly higher Brix content than $pc10^{225}$ but the difference was reduced to 6 % (Fig. 4a). Brix in ripe fruit was not significantly different between 1154 and $pc10^{611}$. Total sugar content as well as total fruit yield in red fruits did not differ significantly between 1154 and $pc10^{611}$ (Fig. 4b, c).

CaGLK2 is co-localized with *pc10*

Due to the phenotypic similarity of the dark green fruit color in 1154 and the dark green shoulders in the fruit of tomato *U* and the assignment of *SlGLK2* to the syntenic region of *pc10* in chromosome 10 of tomato, we hypothesized

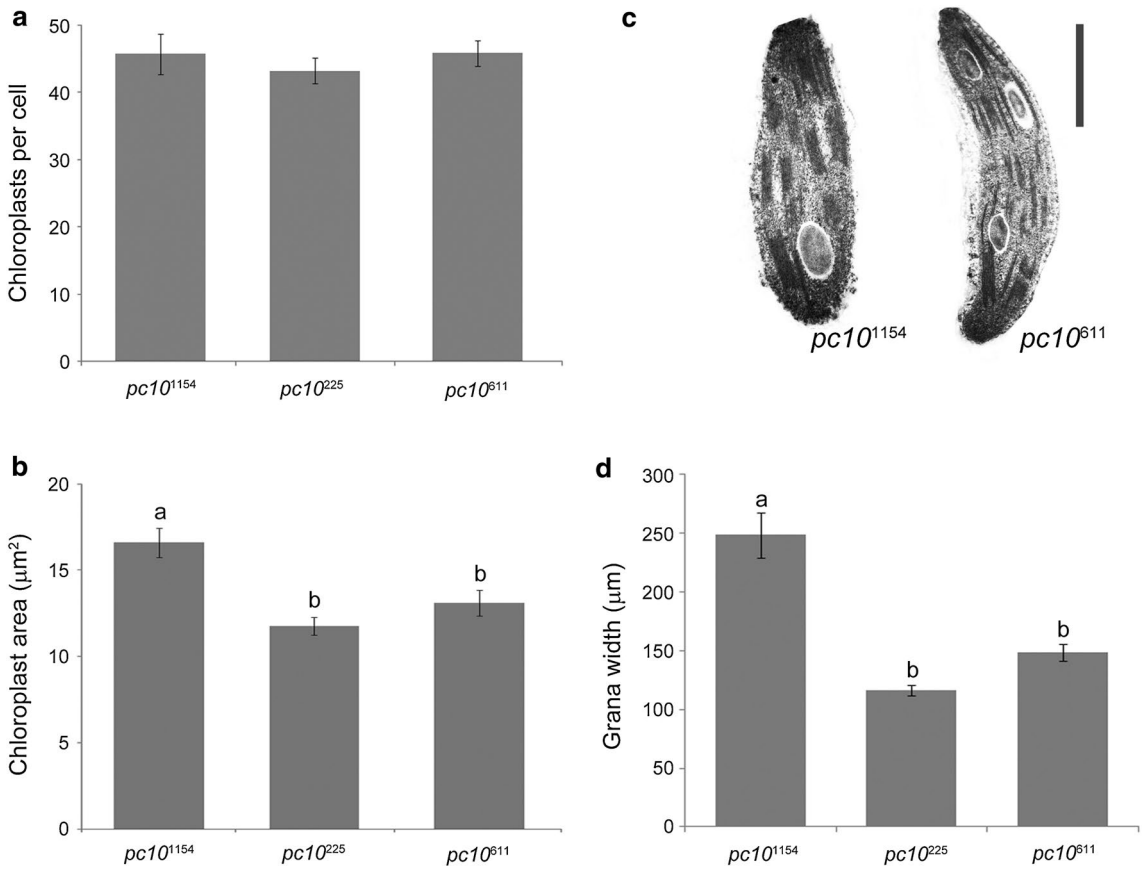


Fig. 3 Characterization of plastids in mature green fruits of *pc10* NILs. **a** Chloroplasts per cell. **b** Chloroplast area. **c** TEM image of chloroplasts. Bar = 1 μm. **d** Chloroplast grana width. Differences among means were determined by Tukey–Kramer range test at $P < 0.05$ and are indicated by different *lowercase letters*. Data for each group are means of ten independent replicates ±SE

that the pepper homolog of *SlGLK2* governs *pc10*. To test this hypothesis, we identified the *C. annuum* transcript MGMT_Contig23879 (https://pepper.ucdavis.edu/public/data.php) as the pepper ortholog of tomato *SlGLK2* and followed its segregation as a PCR marker with the fruit color phenotype in an F_2 population of $pc10^{611}$ NILs consisting of 237 plants. Pepper *GLK2* (*CaGLK2*) was significantly associated with chlorophyll content segregation in a dominant manner in the F_2 population ($P < 0.0{,}001$, $R^2 = 0.85$ tested by single marker analysis). Furthermore, when the fruit color phenotype was scored visually as a qualitative trait with three phenotypic classes (dark, light and intermediate), a Mendelian monogenic ratio was observed ($\chi^2 = 0.74$; $0.5 < P < 0.75$ for single-gene segregation) that completely co-segregated with *CaGLK2*. These genetic data support out hypothesis that *CaGLK2* governs *pc10*.

Null alleles at *CaGLK2* are associated with reduction of chlorophyll content in light green fruits

To determine whether sequence variation in *CaGLK2* is associated with fruit color variation, we sequenced the ORF of the three parents used as recurrent and donor lines for *pc10* NIL construction, the *pc10* NILs and additional lines exhibiting fruit color variation (Fig. 5). Sequence comparison of the ORF in all lines allowed the detection of three null alleles in the coding region of *CaGLK2* (Figs. 6, S2). Both PI 225 and PI 611 had stop codons in the third exon due to 6 bp insertion and 3 bp deletion, creating truncated proteins of 145 and 225 amino acids, respectively. A third null allele was observed in three *C. annuum* lines with light green/cream immature fruit color due to a single nucleotide change from A to G at position 489 of the ORF, creating a stop codon and truncated protein of 163 amino acids. Interestingly, several additional *C.annuum* light green lines including 1901, 21-1 and 13-2 and the wild *C. annuum* var *glabriusculum* light green lines Nayoi and 1202 did not carry any mutation in the ORF, indicating that the expression of *CaGLK2* may be down-regulated or other genes control the reduction of chlorophyll content in these lines. Furthermore, the dark green-fruited lines 1154 and Cora did not have sequence variation in the ORF compared to lines with typical medium green color such as Maor, indicating the possible up-regulation of *CaGLK2* in these lines.

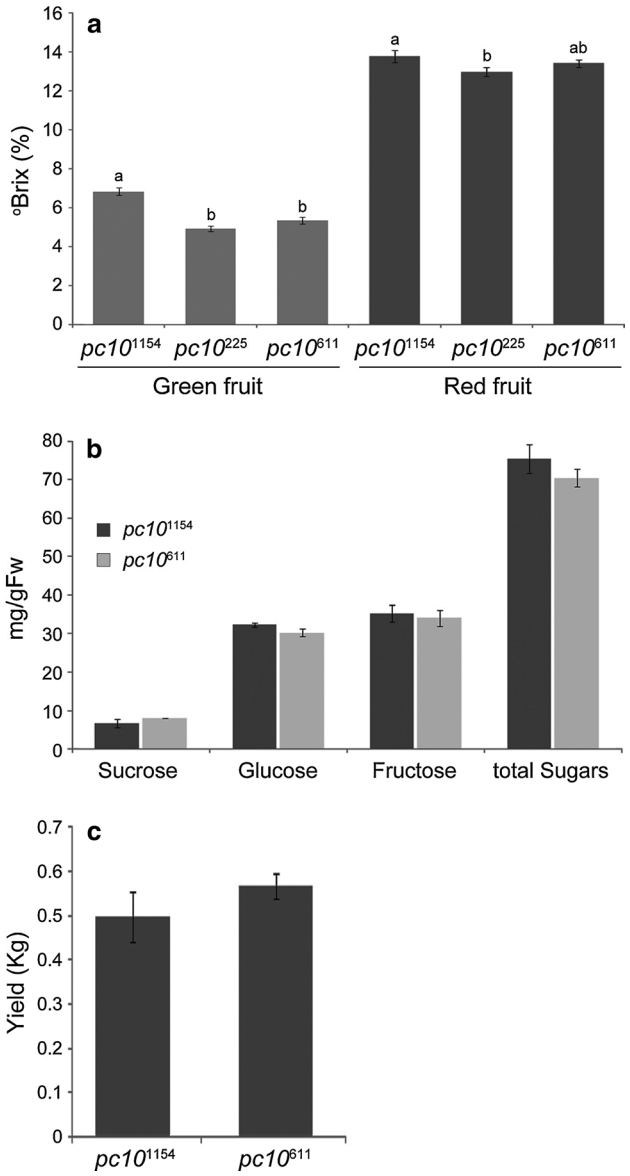


Fig. 4 Comparison of *pc10* NILs for total soluble solids, sugar content and fruit yield. **a** Total soluble solids (Brix) in mature green and ripe red fruits. **b** Sugar content in red ripe fruit. **c** Total fruit yield 3 months after planting. For total soluble solids and yield measurements, data for each group are means of ten independent replicates ±SE. For sugars measurements, data for each group are means of three independent replicates ±SE

Variation in chlorophyll content is associated with expression level of *CaGLK2*

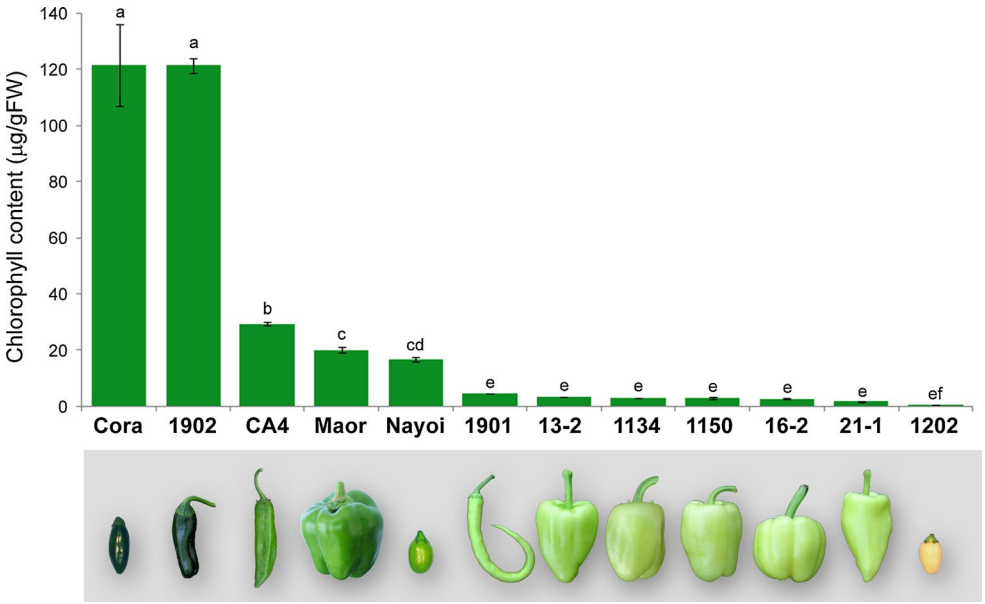
To determine whether up- or down-regulation of *CaGLK2* expression can account for variation in fruit color, we first determined the spatial and temporal pattern of the gene expression. During fruit development, *CaGLK2* is expressed at low level at anthesis and at ripe red stage (Fig. 7a), while it is predominantly expressed between 10 days post-anthesis (dpa) until mature green stage (30 dpa). *CaGLK2* is also expressed at low level in leaves and expression is not detected in the root. Furthermore, we show that as in tomato, *CaGLK2* is expressed in the fruit and the leaves and that *CaGLK1* expression is limited to leaves (Fig. S3). We then measured expression level of *CaGLK2* in lines for which no null alleles were detected (Fig. 7b). In general, the level of expression was positively correlated with chlorophyll content. The expression of *CaGLK2* was significantly higher in the dark green-fruited lines Cora, 1154 and 1902 compared to fruits with lower chlorophyll content. Low level of expression was detected in the light green-fruited line 13-2, while no expression was detected in the light green lines 1901, 1202 and Nayoi. An exception to this pattern was observed for light green-fruited line 21-1 for which the expression level of the gene was similar to that of Maor with medium green fruit color and no sequence variation was observed in the ORF of Maor, 21-1, 13-2 and 1901 (Fig. S2).

Discussion

Pepper is well known for its wide variation of fruit colors. At the immature fruit stage, various shades of green exit ranging from white-cream color with very low chlorophyll content to dark green fruit with very high chlorophyll content. This quantitative variation is indicative of the involvement of multiple genes that control the trait; however, the genes that control this variation are unknown in this species. Only recently, Brand et al. (2012) described two major QTLs, *pc8* and *pc10*, that control chlorophyll content in the immature pepper fruit, of which one of the QTLs *pc8* was shown to exert its effect by modulating chloroplast biogenesis. In the present study, we provide evidence that *CaGLK2*, a transcription factor regulating chloroplast biogenesis, controls the phenotype determined by the second QTL *pc10* and that this gene has an important role in controlling natural variation of pigment content in *Capsicum*.

The role of *CaGLK2* in controlling variation of chlorophyll content in pepper

Co-segregation of *CaGLK2* with fruit color phenotype and detection of multiple independent null allelic variants associated with fruit color variation provided a strong support to the correspondence of *CaGLK2* with *pc10*. Furthermore, expression levels of *CaGLK2* were generally correlated with chlorophyll levels in pepper lines, i.e., higher expression level in dark green fruits than in light green ones. However, exceptions to this pattern were observed indicating the likely involvement of additional genes in controlling this trait. For example, expression level in



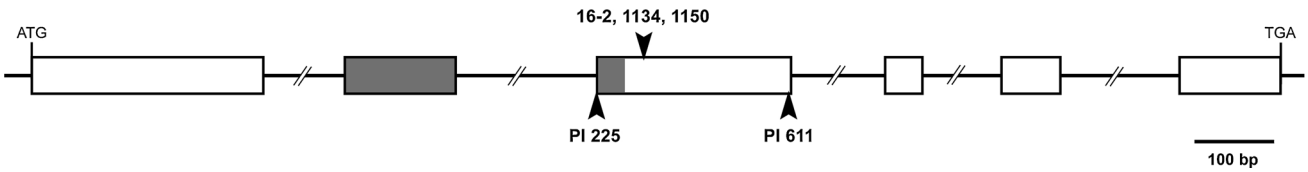


Fig. 5 Pictures and chlorophyll content of mature green fruits of pepper lines exhibiting variation in chlorophyll content. Differences among means were determined by Tukey–Kramer range test at $P < 0.05$ and are indicated by different lowercase letters. Data for each group are means of three independent replicates ±SE

Fig. 6 Gene structure of *CaGLK2* and locations of stop codon mutations in the coding region in different lines. Introns and exons are indicated by *solid lines* and *boxes*, respectively. The DNA binding site domain is indicated by *gray boxes*. The three stop codon mutations are located in the third exon and indicated by *arrowheads*

the light green line 21-1 was similar to that of Maor with medium green fruit and higher than other light green lines such as 13-2, 1901 and 1202 while all these lines had identical sequence of the ORF. Similarly, light green lines sharing the same haplotypes and expression level varied in color ranging from light green to cream color (for example 1901 vs 1202) suggesting a more complex regulation of pigmentation.

In addition to *CaGLK2*, we reported that the QTL *pc8* controls chlorophyll content in the immature pepper fruit (Brand et al. 2012). To date, we have not identified yet the gene governing *pc8*. However, a likely candidate is the recently identified *APRR2-like* that has similar function to *SlGLK2* in controlling chloroplast development and was mapped in tomato to the syntenic region of pepper *pc8* (Pan et al. 2013). Furthermore, a null mutation in the pepper ortholog of the gene was associated with reduced chlorophyll content and white-cream color of the immature fruit (Pan et al. 2013).

While our initial QTL analysis in the cross of 1154 × PI 225 revealed two QTLs, *pc8* and *pc10* (Brand et al. 2012), subsequent QTL analysis in the cross of 1154 × PI 611 revealed a phenotypic effect associated only with *pc10*. Furthermore, out of the two QTLs detected in the cross of 1154 × PI 225, *pc8* had larger phenotypic effect than *pc10*, implicating a strong effect of the genetic background on the expression of the QTLs. Collectively, our genetic, sequencing and expression data in various pepper lines demonstrate that *CaGLK2* has an important contribution to immature fruit color phenotype in pepper and that multiple alleles and influence of the genetic background play an important role in mediating the phenotypic effect of the gene.

Function of *CaGLK2* and its orthologs

CaGLK2 is expressed predominantly at the immature fruit stage as such affecting chlorophyll content mostly at this stage. Small but significant difference in chlorophyll content was observed in leaves of NILs differing for *CaGLK2* alleles, indicating contribution of this gene to chlorophyll accumulation in the leaves in accordance with its low level of expression in this tissue. We show that the expression patterns of *CaGLK1* in leaves and *CaGLK2* in fruit are similar to those of their homologs in tomato (Powell et al. 2012; Nguyen et al. 2014), although *CaGLK2* is more strongly expressed than its tomato ortholog (Kim

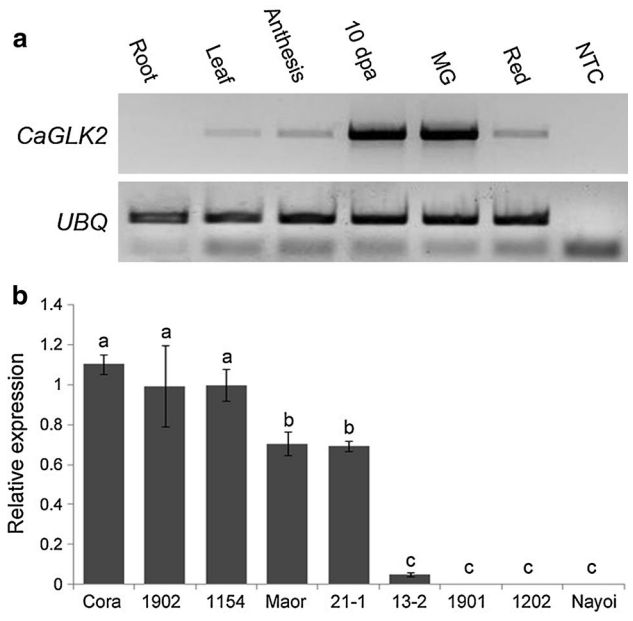


Fig. 7 Expression patterns of *CaGLK2*. **a** Semi-quantitative RT-PCR of *CaGLK2* transcripts in different tissues of 1154. *Dpa* days post-anthesis, *MG* mature green, *NTC* no template control. Expression of Ubiquitin (UBQ) was used as a control. **b** Quantitative RT-PCR expression analysis of *CaGLK2* transcripts in ten dpa green fruits of different lines. Expression levels were normalized to that of 1154. Differences among means were determined by Tukey–Kramer range test at $P < 0.05$ and are indicated by different *lowercase letters*. Data for each group are means of five independent replicates ±SE

et al. 2014). Constitutive expression of either *AtGLK1* or *AtGLK2* in tomato fruit produced similar dark green fruits and induced expression of similar sets of photosynthetic-related genes (Powell et al. 2012), supporting previous findings on functional redundancy of the two genes in Arabidopsis (Fitter et al. 2002).

CaGLK2 affects chloroplast compartment size by controlling chloroplast size and grana stacking but does not have an effect on chloroplast number. This indicates that *CaGLK2* controls chloroplast structure but does not affect chloroplast division or turnover. This result agrees with the suggestive function of *GLK* genes as promoting photosystem assembly in leaves of Arabidopsis (Fitter et al. 2002; Waters et al. 2009). In contrast to *CaGLK2*, NILs for *pc8* alleles had significant difference in both chloroplast area and number (Brand et al. 2012), indicating that the two pepper QTLs regulate a common process, fruit chloroplast biogenesis, but at different stages. This also indicates that the gene governing *pc8* operates upstream of or in parallel to *CaGLK2*. For example, *pc8* is likely required for chloroplast division or development prior to the *CaGLK2* regulated assembly of the light harvesting complex. Experiments testing the functional relationship of the two QTLs are currently in progress.

Pepper and tomato immature fruits have similar light green color due to null mutations at *GLK2*. Phenotypic differences between the two species are apparent in genotypes carrying the wild-type dominant alleles. In pepper, chlorophyll is accumulated evenly in the immature fruit regardless of the allelic state of *CaGLK2*. In contrast, in tomato *U*, chlorophyll is more abundant in the calyx (shoulder) part of the fruit than in the stylar part, while in *u* chlorophyll is accumulated evenly throughout the fruit but at a lower level than in *U*. The developmental gradient observed in chloroplast development in *U* is associated with increased expression of *SlGLK2* in the calyx than in the stylar part (Powell et al. 2012). Transcriptome analysis revealed that a large set of genes including photosynthesis and chloroplast biogenesis-related ones is differentially expressed along the latitudinal axis of the fruit (Nguyen et al. 2014). Furthermore, the involvement of the *KNOTTED1-LIKE HOMEOBOX* (*KNOX*) genes *TKN4* and *TKN2* that act upstream to *SlGLK2* in controlling the fruit chloroplast developmental gradient has been established, implicating a complex yet partially known regulatory mechanism of chloroplast development in the fruit (Nadakuduti et al. 2014).

Contribution of *CaGLK2* to pepper fruit quality

In accordance with the low level of *CaGLK2* expression in ripe fruit and the minor effect of the gene on chromoplast compartment size, total carotenoid content in the ripe fruit did not differ significantly between the *pc10* NILs. The increased fruit photosynthate in the immature stage of 1154 conferred by *CaGLK2* was implicated by elevating chlorophyll and sugar content in the immature fruit. However, like carotenoid content, the effect on Brix and sugar content existed in the ripe fruit but was considerably reduced. The elevation of Brix in the dark green parent was not associated with significant differences in yield. These results are in contrast to tomato in which overexpression of Arabidopsis and tomato *GLK* genes in the fruit increased significantly Brix, sugar and carotenoid contents in red fruits (Powell et al. 2012; Nguyen et al. 2014). However, these impressive effects in tomato ripe fruit were achieved by overexpressing the genes under the 35S promoter that drives expression of the genes at high level in all tissues and, therefore, cannot represent a naturally occurring situation such as described in pepper. Alternatively, the different outcomes of altering chloroplast development in unripe fruit on metabolite accumulation in the ripe fruit in pepper and tomato may represent variation in the metabolic pathways in the two species.

Concomitant increase of chlorophyll content and total soluble solids in pepper dark green fruits may not necessarily indicate that carbon fixation in the fruit is the major cause for this association. In tomato, the contribution of

fruit photosynthesis to carbon economy in the fruit is considered to be minor (up to 15–20 %) compared to leaf photosynthesis and sugar transport (reviewed by Cocaliadis et al. 2014). No data are available on the relative importance of fruit and leaf photosynthesis in pepper. Pepper *pc10* NILs show significant difference in chlorophyll content in the leaves (Fig. 2b), which may account for the elevated total soluble solids content.

Compared to tomato in which the dark green phenotype conferred by *GLK2* is restricted to the shoulders of the fruit in the *U* genotype, in pepper, the orthologous gene is expressed in the entire fruit and, therefore, provides the potential to improve fruit quality by exploiting natural variation for breeding. Because the major effect of *CaGLK2* is in the green fruit stage, exploitation of the benefits conferred by the activity of this gene such as elevation of sugar content (and thus expected improved taste), chlorophyll and carotenoids such as lutein and β-carotene that are accumulated in chloroplasts (Fig. 3 in Brand et al. 2012) will be maximized in cultivars carrying for example, the *STAY GREEN* gene (Borovsky and Paran 2008). This mutation inhibits chlorophyll degradation during ripening, resulting in conversion of the red color to brown, or to a green-ripe fruit at a yellow color background. Ripe-green cultivars are a relatively new product of several seed companies and have good potential to increase market share, especially if they will be marketed as improved quality peppers.

Author contributions IP and AVD conceived and designed research. AB, YB, TH, KAAR and AB conducted experiments and analyzed data. IP, AVD and TH wrote the manuscript. All authors read and approved the manuscript.

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