Mapping of the *CaPP2C35* Gene Involved in the Formation of Light-Green Immature Pepper (*Capsicum Annuum* L.) Fruits via GWAS and BSA

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Abstract

In pepper (*Capsicum annuum* L.), the common colors of immature fruits are yellowish white, milky yellow, green, purple, and purplish black. Some genes related to these colors have been cloned, but only those related to dark green, white, and purple immature fruits; few studies have investigated light-green immature fruits. Here, we performed a genetic study using light-green (17C827) and green (17C658) immature fruits. We found that the light-green color of immature fruits were controlled by a single locus-dominant genetic trait compared with the green color of immature fruits. We also performed a genome-wide association study and bulked segregant analysis of immature-fruit color and mapped the *LG* locus to a 35.07 kbp region on chromosome 10. Only one gene, *Capana10g001710*, was found in this region. A G-A substitution occurred at the 313th base of the *Capana10g001710* coding sequence in 17C827, resulting in the α -helix of its encoded PP2C35 protein to turn into a β -fold. The expression of *Capana10g001710* (termed *CaPP2C35*) in 17C827 was significantly higher than in 17C658. Silencing of *CaPP2C35* in 17C827 resulted in an increase in chlorophyll content in the exocarp and the appearance of green stripes on the surface of the fruit. These results indicate that *CaPP2C35* may be involved in the formation of light-green immature fruits by regulating the accumulation of chlorophyll content in the exocarp. Thus, this research lays the foundation for further studies and genetic improvement of immature-fruit color in pepper.

Key Message

Genome-wide association study, bulked segregant analysis, and genetic analysis delimited the *LG* locus controlling light-green immature pepper fruits into a 35.07 kbp region on chromosome 10. A strong candidate gene, *CaPP2C35*, was identified in this region.

Introduction

Fruit color is one of the most important traits of pepper (*Capsicum annuum* L.) appearance, as it is the main reference standard used by consumers for selecting and purchasing peppers (Jang et al. 2020). Thus, research on pepper fruit color has important practical significance for the development of economically efficient strategies of pepper cultivation.

Pepper fruit color can be divided into two categories depending on the developmental stage of the plant: immature and mature (Wahyuni et al. 2011). Most immature fruits are green because of the accumulation of chlorophyll in their chloroplasts (Paran et al. 2007). Nevertheless, many other pigments, such as lutein, β -carotene, violaxanthin, and anthocyanins, have been found in immature fruits, leading to fruit colors such as milky yellow, yellowish green, purple, and purplish black (Matsufuji et al. 2007; Lightbourn et al. 2008; Liu et al. 2020). As the fruit ripens, the chlorophyll gradually is degraded, and the chloroplasts are converted into chromoplasts in which carotenoids are synthesized and stored; this process results in orange, red, and yellow mature pepper fruits (Bouvier et al. 1994; Hugueney et al. 1996; Sun et al. 2018; Jeong et al. 2020; Lee et al. 2020).

Previous studies have shown that the genetics of white, yellowish-green, and deep green immature pepper fruit color is mainly controlled by the *SW1*, *SW2*, and *SW3* loci, respectively. *SW3* is dominant relative to *SW1* and *SW2*, while *SW2* is dominant relative to *SW1* (Lightbourn et al. 2008; Stommel et al. 2014). The dominance of these loci indicates that there are genetic differences among the different colors of immature pepper fruits. However, there have been few other studies on immature-fruit color in pepper plants. Brand et al. (2012) found two major quantitative trait loci (QTLs), *pc8.1* (also termed *pc1*, as subsequent mapping placed the QTL in chromosome 1 [Borovsky et al. 2019]) and *pc10.1*, that control the degree of dark green color saturation in immature fruits based on their chlorophyll content. In 2013, Pan et al. (2013) cloned the *APPR2-Like* gene in pepper, which positively regulates chlorophyll synthesis in pepper, and found that *APPR2-Like* and *pc8.1* were in the same region on the genetic map, indicating that *APPR2-Like* may be a candidate gene of *pc8.1*. However, Borovsky et al. (2019) used bulked segregant analysis (BSA) to find *CcLOL1*, a candidate gene of *pc1* (*pc8.1*), and observed that *CcLOL1* affected the formation of green-colored immature fruits by regulating the expression of genes related to photosynthesis and redox reactions. Brand et al. (2014) found that a possible candidate gene of *pc10.1* is *CaGLK2*. In addition, two genes (*MybA* and *Ca3GT*) were found to regulate anthocyanin accumulation in purple immature pepper fruits (Borovsky et al. 2004; Liu et al. 2020).

The molecular mechanism underlying the formation of green-colored immature pepper fruits is complex. The sequence analysis of the *CaGLK2* open reading frame (ORF) in light-green immature pepper fruits showed that six base insertions, one A-G substitution, and three base deletions occurred at three different positions of its third exon, indicating that there was diversity in the variation of *CaGLK2*. However, there was no variation in *CaGLK2* ORF in "1901", "21 – 1", "Nayoi", and "1202" pepper accessions, which were light-green immature fruits, indicating that *CaGLK2* may be down-regulated or that other genes are regulating the formation of immature-fruit color in these accessions (Brand et al. 2014). Borovsky et al. (2019) found that there was no difference in the ORF sequence of *CcLOL1* between *C. annuum* and *C. annuum* var *glabriusculum*, which have significant differences in chlorophyll content, indicating that *CcLOL1* has a limited ability to regulate immature-fruit color variation in pepper plants.

Although some studies have isolated genes related to the color of immature pepper fruits (Borovsky et al. 2004; Pan et al. 2013; Brand et al. 2014; Borovsky et al. 2019; Liu et al. 2020), only a few have reported specifically on the regulatory genes of light-green immature fruits. Among all pepper cultivars, there is a large variation in the hue of green-colored immature fruits, ranging from colors characterized by very low chlorophyll content, such as white or milky yellow, to dark green (Mejia et al. 1988; Levy et al. 1995; Hornero-Mendez et al. 2000; Wall et al. 2001). However, it is difficult to distinguish the different depths of green-colored fruits, especially of those that are yellowish green, milky yellow, light green, green, deep green, and dark green. This difficulty poses a challenge for conducting research on green-colored immature pepper fruits. Some studies have investigated pepper fruit color based on the pigment content (Brand et al. 2012; Borovsky et al. 2019). However, this method is tedious and easily affected by the sampling or determination technique.

Overall, the genetic and regulatory mechanisms of green-colored immature pepper fruits with different depths of saturation are complex, and there have been few studies on the genetic and mapping of the genes controlling light-green immature fruits. Considering this, we analyzed the heredity and formation of light-green immature pepper fruits in the present study with two *C. annuum* accessions, 17C827 (light-green immature fruits; red mature fruits) and 17C658 (green immature fruits; yellow mature fruits). Additionally, we utilized a genome-wide association study (GWAS) and BSA to perform gene mapping and functional analysis of the *LG* locus that controls light-green coloration in immature pepper fruits—the *LG* locus was identified based on visual and colorimetric analyses of the color phenotype. Finally, a molecular marker closely linked to the *LG* locus was developed. So this study provides a valuable reference for future studies on and genetic improvement of the color of immature pepper fruits.

Materials And Methods

Plant materials

In this study, we used *Capsicum annuum* accessions 17C827 (light-green immature fruits; red mature fruits) and 17C658 (green immature fruits; yellow mature fruits) to generate F₁ and F₂ populations (941 individuals) for mapping the *LG* locus which controls the light-green color in immature pepper fruits. In addition, 287 pepper accessions (Supplementary Table 1) were also used for the genome-wide association study (GWAS) (Wu et al. 2019). All the above-mentioned materials were provided by the pepper research laboratory in the College of Horticulture, China Agricultural University and were grown in the greenhouses at ShangZhuang experimental station of China Agricultural University (Beijing, China).

Determination of pigment content

Exocarp and endocarp (including mesocarp) of the immature pepper fruits in each accession were collected and stored at -80°C. 1.0 g sample was used for the pigment extraction. 25 mL of 95% ethanol was added into a 50 mL centrifuge tube containing the sample and then the tube was sealed with a sealing film and placed under dark conditions for 36 h after ultrasonic (100 W; 26°C) treatment for 1 h (Zhu et al. 2017; Vendruscolo et al. 2021). When the sample was whitened, the solution was mixed evenly and the supernatant was kept. Measurement was conducted using spectrophotometer under the 470 nm, 649 nm and 665 nm, respectively (Amorim-Carrilho et al. 2014). Finally, the contents of chlorophyll and carotenoid were calculated using the Aron formula method (Aron et al. 1969).

Chloroplast observation

The exocarp portion of pepper fruits were cut into a sample strip (1 mm × 2 mm × 5 mm) and then were immersed into 2.5% glutaraldehyde fixed solution and later were stored at 4°C overnight. The next day, ultra-thin sections were made according to the slice production process of Jeong et al. (2020). Finally, chloroplasts were observed and photographed by JEM-1400 Flash (JEOL, Tokyo, Japan) (120 kV) transmission electron microscope.

Color measurement of the immature pepper fruit

The color of immature fruits of the F_2 individuals as well as the 287 pepper accession was measured and recorded using colorimeter (CHROMAMETESR-400). Chroma (SD) and Shade (SG) were calculated with the formulas described by Yang et al. (2004). For each parameter, the value for each plant were collected from three fruits and for each fruit, the value was the average of three measurements.

Nucleic acid extraction

Genomic DNA of each plant was isolated from six fresh leaves using the cetyltrimethylammonium bromide (CTAB) method (Lee et al. 2017). DNA concentration and quality were determined by a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). DNA of which the concentration ≥ 20 ng/ μ L and the volume ≥ 30 μ L was used for sequencing (Wu et al. 2019). Furthermore, 30 individuals carrying light-green immature fruits and 30 individuals carrying green immature fruit were selected from 941 F_2 individuals. Equal amounts of DNA from each individual were mixed to generate the green pool and light-green pool for BSA analysis. For each pool, the final concentration was 40 ng/ μ L.

Total RNA was extracted from various tissues. For 17C827 and 17C658, RNA was extracted from stems, leaves, flowers, and three stages (9, 12 and 20 days after anthesis) of exocarp. For the virus-inoculated 17C827 used for VIGS analysis, RNA was extracted from light-green exocarp and green exocarp of immature fruit. For the control plants used for VIGS analysis, RNA was also extracted from exocarp of immature fruit. Total RNA was extracted using MG RNAzol kit (MGmed, Seoul, South Korea) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 2 μ g of RNA using the EasyScript Reverse Transcriptase kit (TransGen, Beijing, China) with oligo (dT) primers. The resulting cDNAs were used for further analyses.

GWAS and BSA analysis

The genome of 'Zunla-1' (<http://peppersequence.genomics.cn/page/species/index.jsp>, version 2.0) was selected as the reference genome for an electronic digestion prediction experiment. The genome size of 'Zunla-1' was ~3.36 Gb, and GC content was 34.97% (Cheng et al. 2014). We used the sequencing data of Wu et al. (2019) to perform the genome-wide association study of immature-fruit color (including yellowish white, milky yellow, yellowish green, light green, green, deep green, dark green, purple and purplish black) in the 287 pepper accessions. The detailed methods of GWAS were described in Wu et al. (2019). The BSA analysis was conducted according to Xu et al. (2016).

Genetic analysis, linkage map construction and QTL analysis

To determine the mode of genetic of fruit color, Chi-squared (χ^2) analysis was performed to test the phenotypic data for goodness-of-fit to Mendelian segregation ratios. A large number of SSR, InDel, CAPS and dCAPS markers within the candidate interval of *LG* were screened and seven polymorphic molecular markers were obtained (Supplementary Table 2). Using the phenotype and genotype data from the 941 F_2

individuals, the linkage analysis of *LG* was carried out by using JoinMap4 software, and a genetic linkage map was constructed with Mapmaker/EXP 3.0 command (LOD value ≥ 3.0) (Lander et al. 2009; Lander et al. 1987). In addition, Interval-mapping QTL analyses in the 315 F_2 individuals were performed for the *L**, *a**, *b**, *SD* and *SG* value of immature fruit with QTL IciMapping 4.0 software (Meng et al. 2015). Significance threshold level (LOD 3.0) for QTL detection was computed by permutation tests with 1,000 iterations at $P < 0.01$.

PCR was conducted using a 10- μ L system containing 1.0 μ L DNA template, 0.5 μ L forward primers, 0.5 μ L reverse primers, 5.0 μ L Taq polymerase mix (Beijing ComWin Biotech Co.,Ltd., Beijing, China), and 3 μ L ddH₂O. The thermal cycle was: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 35 s, and extension at 72°C for 30 s, before a final extension at 72°C for 5 min. HinfI was used to digest the PCR product of dCAP10-1 marker. The digested PCR product was separated by 7% polyacrylamide gel electrophoresis (Wu et al. 2019).

Gene expression and bioinformation analysis

Expression of *CaPP2C35*, *GSA*, *HEMA1*, *CHLD*, *CHLH*, *CAO*, *CHLG*, *CRD1*, *DVR*, *SGR1*, *PAO*, *NYC*, *RCCR*, *PPH*, and *SGR2* were measured by real-time PCR and primers were listed in Supplementary Table 2. The samples used for gene expression analysis were the exocarp of fruit and the reference gene was *UBI* (AY486137.1) gene. Gene relative expression data is calculated by $2^{-\Delta\Delta CT}$ method, with three biological and three technical repeats. Data is means of three biological replicates. RT-PCR, real-time PCR, and sequencing of PCR products were performed as described by Wang et al. (2019). DNAMAN (Lynnon Biosoft, USA) and MEGA7 software were used to analyze the sequence difference or evolution of related genes or proteins (Kumar et al. 2016). SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html) and I-TASSER (<https://zhanglab.ccmb.med.umich.edu/I-TASSER/>) software were used to analyze the primary structure, secondary structure and tertiary structure analysis of CaPP2C35 protein (Ballut et al. 2015; Roy et al. 2010; Yang et al. 2015).

Virus-induced gene silencing of *CaPP2C35*

To study the function of the *CaPP2C35*, virus-induced gene silencing (VIGS) was carried out in the 17C827 according to Cheng et al. (2018) with some modifications. A fragment of *CaPP2C35* CDS was selected as the target, and primers were designed using Primer 5 software (Supplementary Table 2). The constructs consisting of pTRV1, pTRV2, pTRV2-*PDS*, pTRV2-*CaPP2C35* were transformed into *Agrobacterium tumefaciens* GV3101, respectively. The transformed *Agrobacterium* were cultured for 12 h in Luria-Bertani (LB) medium (20 μ M acetosyringone, 50 mg L⁻¹ kanamycin, 25 mg L⁻¹ Rif and 10 mM MES) and then were harvested and suspended in the MS buffer (200 μ M acetosyringone, 10 mM MES, 10 mM MgCl₂ pH = 5.6) to a final OD₆₀₀ of approximately 1.0. A mixture of cultures containing 1:1 (v/v) of pTRV1 and pTRV2 was used as TRV control, and meanwhile pTRV1 and pTRV2-*PDS* were used as a reporter, and pTRV1 and pTRV2-*CaPP2C35* were used to silence the *CaPP2C35*. The effect of VIGS was determined according to Cheng et al. (2018).

Results

Low chlorophyll content in the exocarp of 17C827 affects light-green immature-fruit formation

We examined the cross-section of 17C658 (green immature fruits) and 17C827 (light-green immature fruits) immature fruits. As shown in Figure 1a, both 17C827 and 17C658 immature fruits formed a notable division of green coloration at the boundary between the exocarp and mesocarp; the green portion was located on the exocarp side, and the light-green portion was located on the mesocarp side. The degree of green in the 17C658 and 17C827 immature fruits is, therefore, mainly influenced by the exocarp.

Next, the pigment contents in the exocarp and endocarp (including the mesocarp) of the two pepper accessions were compared. We found that chlorophyll content (chlorophyll a [Figure 1b] and chlorophyll b [Figure 1c]) in the exocarp was significantly different between 17C827 and 17C658 fruits, and the content in 17C827 fruits was lower than in 17C658 fruits. However, there was no significant difference in chlorophyll content in the endocarp (including the mesocarp) between 17C827 and 17C658 fruits during the various periods of fruit development (Figure 1b, c). These results showed that low chlorophyll content in the 17C827 fruit exocarp was the main factor influencing the formation of its light-green immature fruits. Furthermore, chlorophyll content (chlorophyll a [Figure 1b] and chlorophyll b [Figure 1c]) in the exocarp and endocarp (including the mesocarp) of the two pepper accessions decreased from day 5 to day 9, increased from day 9 to day 12, and gradually decreased and eventually stabilized from day 12 to the M stage. The change in carotenoid content was similar to that in chlorophyll content (Supplementary Figure 1). The results showed that the degree of green in the immature fruits of 17C658 and 17C827 began to stabilize after day 12. Meanwhile, total chlorophyll content (0.048–0.382 mg/g·FW) in both 17C827 and 17C658 fruits was markedly higher than their carotenoid content (0.003–0.047 mg/g·FW) during the various periods of fruit development (Supplementary Figure 1), which indicates that the formation of green-colored immature pepper fruits is mainly affected by chlorophyll.

Abnormal chloroplast thylakoids in 17C827 exocarp affects light-green immature-fruit formation

To explore whether light-green immature-fruit formation of 17C827 plants was affected by chloroplasts, we first examined the number of chloroplasts in a single cell and the number of chloroplast grana in a single chloroplast. Regardless of the evaluation day—day 9 or day 20—there were no significant difference in the numbers of chloroplasts and chloroplast grana between 17C827 and 17C658 fruits (Supplementary Figure 2a). We then measured the perimeter and area of the chloroplast cross section in the exocarp and found that there were significant differences between 17C827 and 17C658 fruits (Supplementary Figure 2b, c). The chloroplast cross-section perimeter and area values of 17C827 fruits were higher than those of 17C658 fruits. Meanwhile, we calculated the increments of the chloroplast section perimeter and area from day 9 to day 20, and we found that the increments of these values in 17C827 were also higher than those in 17C658 (Supplementary Figure 2b, c). At day 9, dividing chloroplasts were found in both 17C827 and 17C658 fruits, indicating that both accessions were able to proliferate chloroplasts (Supplementary Figure 3a). At day 20, we also found that the chloroplast grana of

the mature chloroplasts in the light-green immature fruits of 17C827 were thinner and more scattered than those of 17C658, and there were fewer stroma lamella in 17C827 than in 17C658 (Supplementary Figure 3b). The above results indicate that the abnormal chloroplast thylakoids in the exocarp of 17C827 affected the formation of light-green immature fruits.

Light-green color of 17C827 immature fruits is controlled by a single dominant locus

In the present study, F_1 and F_2 were constructed from a cross between 17C827 (light-green immature fruits) and 17C658 (green immature fruits). All F_1 individuals showed a light-green immature-fruit color, which was the same as that of the female parent 17C827 (Figure 2). In contrast, the immature-fruit colors of the 941 F_2 individuals were segregated into light green (726 individuals) and green (215 individuals), with a ratio of 3:1 ($\chi^2 = 2.32 < 6.64$, $df = 1$, $p = 0.01$), which was similar to the separation ratio of red (701 individuals) and yellow (240 individuals) mature fruits. These results indicate that the formation of light-green immature-fruit color could be attributed to the genetics of a single dominant locus compared with the formation of green immature-fruit color. Finally, we found that F_2 could be segregated according to fruit color, light-green immature-fruit and red mature-fruit (534 individuals), light-green immature-fruit and yellow mature-fruit (174 individuals), green immature-fruit and red mature-fruit (167 individuals), and green immature-fruit and yellow mature-fruit (66 individuals) color, which followed the ratio of 9:3:3:1 ($\chi^2 = 1.45 < 11.35$, $df = 3$, $p = 0.01$). This result suggest that the genetic mechanisms of immature-fruit color (light green and green) and mature fruit color (red and yellow) are relatively independent from each other.

LG locus position revealed by GWAS and BSA

To efficiently map the *LG* locus, a GWAS on immature-fruit color, comprising yellowish white, milky yellow, yellowish green, light green, green, deep green, purple, and purplish black, in 287 pepper accessions was conducted (Supplementary Table 1). As shown in Figure 3a, there was a candidate region spanning from 157,194,038 bp to 190,675,776 bp on chromosome 10 that was significantly associated with immature-fruit color. Additionally, a GWAS of immature-fruit colors according to lightness (L^*), red and green coordinates (a^*), and yellow and blue coordinates (b^*) of 287 pepper accessions was conducted, and a candidate region was found on chromosome 10. The candidate region significantly associated with L^* spanned from 176,498,851 bp to 176,669,957 bp on chromosome 10, while that associated with a^* spanned from 55,687,612 bp to 62,184,580 bp, and that associated with b^* spanned from 175,987,065 bp to 186,812,911 bp. Therefore, we assumed that the *LG* locus controlling color in light-green immature fruits was likely to be located in the region spanning from 157,194,038 bp to 190,675,776 bp on chromosome 10. In addition, the BSA of the extreme mixed pool based on F_2 showed that there was a candidate region from 156,650,000 bp to 178,330,000 bp on chromosome 10 that was significantly associated with the light-green color of immature pepper fruits (Figure 3b). Finally, we selected the intersection of the GWAS candidate region and the BSA candidate region to determine that the *LG* locus was located in the region spanning from 157,494,038 bp to 178,330,000 bp (size: 20.8 Mbp) on chromosome 10. Seven primer pairs from the above region were used for linkage analysis of

the *LG* locus. The results showed that the *LG* locus was located between the InDel-106 and InDel-109 primers, with a physical distance of 35.07 kbp (from 174,787,932 bp to 174,823,007 bp) (Supplementary Figure 4a). The QTL analysis for the colorimetry indices (L^* , a^* , b^* , chroma [SD], and shade [SG]) of the immature-fruit surfaces of 315 F_2 individuals showed similar results (Supplementary Figure 4b).

***CaPP2C35* is the candidate gene of the *LG* locus**

Using the JBrowser tool of the Sol Genomics Network (<https://solgenomics.net/>), we found that there was only one gene, *Capana10g001710*, located between 174,787,932 bp and 174,823,007 bp on chromosome 10. Sequence analysis revealed a G-A substitution at the 313th base of the *Capana10g001710* coding sequence (CDS) in 17C827 (Supplementary Figure 5). Based on this base substitution, a derived cleaved amplified polymorphic sequence (dCAPS) molecular marker, dCAPS10-1, was designed. As shown in Figure 4a, b, dCAPS10-1 had a stable polymorphism between the two parents and was closely linked to *LG* (0.2 cM). Meanwhile, the QTL analysis of the colorimetry indices (L^* , a^* , b^* , SD, and SG) of the immature-fruit surfaces of 315 F_2 individuals showed that the QTLs of L^* , a^* , b^* , SD, and SG were all located near dCAPS10-1 (Figure 4c). Additionally, the percentage of variation explained (PVE%) of dCAPS10-1 for L^* , a^* , b^* , SD, and SG was 27.35%, 21.43%, 20.42%, 17.76%, and 18.14%, respectively. Furthermore, the expression of *Capana10g001710* in 17C827 with light-green pools was significantly higher than that of 17C658 with green pools (Figure 4d, e). Finally, we analyzed the structure of the protein encoded by *Capana10g001710* and found that a V-I substitution occurred at the 105th amino acid, which was also the last amino acid in the PP2C35 protein domain. This substitution caused the α -helix of the PP2C35 protein to turn into a β -fold in 17C827 (Supplementary Figure 6). Hence, *Capana10g001710* was named *CaPP2C35*. Evolutionary analysis showed that the PP2C35 proteins belonged to the C subgroup of the PP2C protein family (Supplementary Figure 7).

Silencing of *CaPP2C35* leads to green stripes on the surface of 17C827 immature fruits

To further explore the role of *CaPP2C35*, we silenced this gene in 17C827 (light-green immature fruits) via tobacco rattle virus (TRV)-mediated virus-induced gene silencing (VIGS). The immature fruits of 17C827 plants subjected to TRV2-*CaPP2C35* silencing continued to have green stripes on their surfaces that were unable to transition into the light-green color of the surface of the fruits (Figure 5). Additionally, chlorophyll contents in the green and light-green exocarps were determined for fruits treated with TRV2-*CaPP2C35*. The results showed that chlorophyll content in the green exocarp was 0.248 mg/g-FW and that in the light-green exocarp was 0.095 mg/g-FW; thus, there was a significant difference between them. In addition, the green and light-green parts of the exocarp of *CaPP2C35*-silenced fruits were sampled for *CaPP2C35* expression analysis. The exocarp of plants subjected to no treatment or TRV2 treatment was used as negative controls, and the exocarp of plants treated with TRV2-*PDS* was used as a positive control. The results revealed that the expression of *CaPP2C35* in the light-green exocarp of fruits treated with TRV2-*CaPP2C35* was not significantly different from that of the control treatments (Figure 6a). However, the expression of *CaPP2C35* in the green exocarp of fruits treated with TRV2-*CaPP2C35* was significantly lower than that in the light-green exocarp of the same fruits. In addition, the tissue-specific

expression pattern of *CaPP2C35* shows that it was specifically and highly expressed in the fruit tissues of 17C827 (Supplementary Figure 8). These results demonstrate that *CaPP2C35* plays a role in the formation of light-green immature pepper fruits.

***CaPP2C35* forms light-green immature fruits in 17C827 by affecting the accumulation of chlorophyll in the exocarp**

To investigate the effect of *CaPP2C35* down-regulation on the expression of genes involved in chlorophyll metabolism, we analyzed the expression of key chlorophyll metabolism-related genes in the green and light-green portions of the same fruits treated with *TRV2-CaPP2C35*. As shown in Figure 6b, the expression of the chlorophyll biosynthesis metabolism-related genes—*GSA*, *HEMA1*, *CHLD*, and *CAO*—in the green portion of the exocarp was significantly higher than that in the light-green portion. However, there was no significant difference in the expression of genes involved in chlorophyll degradation metabolism (Figure 6c). In addition, we analyzed the expression of chlorophyll metabolism-related genes between 17C827 and 17C658 (Supplementary Figure 9). The results showed that there was a significant difference in the expression of chlorophyll biosynthesis metabolism-related genes between 17C827 and 17C658 at day 9 or day 12, including the expression of *GSA*, *HEMA1*, *CHLD*, *CHLH*, *CAO*, *CHLG*, and *CRD1*, which were expressed at lower levels in 17C827 than in 17C658. Moreover, there was a significant difference in the expression of the chlorophyll degradation metabolism-related genes—*SGR1*, *PAO*, *RCCR*, and *SGR2*—between 17C827 and 17C658 at day 9 or day 12. In addition, the expression of some of them (*RCCR* and *SGR2*) was higher in 17C827 than in 17C658 at day 12. Finally, the co-expression analysis of *CaPP2C35* and the key chlorophyll metabolism-related genes showed that the expression levels of the genes (*HEMA1*, *CHLH*, *CAO*, *CRD1*, and *CHLD*) involved in the chlorophyll biosynthetic pathway were negatively associated with that of *CaPP2C35*. In contrast, the expression level of *SGR1*, involved in chlorophyll degradation, was negatively correlated to that of *CaPP2C35*, whereas *SGR2* was positively correlated to that of *CaPP2C35* (Figure 7 and Supplementary Figure 10). These results indicate that *CaPP2C35* may participate in the formation of light-green immature fruits in 17C827 by affecting the accumulation of chlorophyll in the exocarp.

Discussion

In pepper, the common immature-fruit colors are yellowish-white, milky yellow, green, purple, and purplish black. According to the level of color saturation, green-colored immature pepper fruits can be further subdivided into white, yellowish-green, light-green, green, deep-green, and dark-green fruits. Among them, the color of yellowish-green immature fruits is dominant over that of white immature fruits, while the color of deep green immature fruits is dominant over both yellowish-green and white immature fruits (Lightbourn et al. 2008). The color purple in immature fruits is dominant over green in immature fruits (Borovsky et al. 2004; Liu et al. 2020). Thus, it can be inferred that more saturated immature-fruit colors are likely to dominate those that are less saturated. However, in our study, the light-green immature-fruit color exhibited a single dominant locus compared with the green immature-fruit color, this result enriched the genetic theory of immature-fruit color in pepper. Therefore, there are genetic differences associated

with the color variations between green-colored immature pepper fruits, and these differences are relatively complex.

Considering the complexity of immature-fruit color genetics in green-colored immature fruits, Brand et al. (2012) studied the genetics between dark green and light-green immature fruits based on chlorophyll content and found two related major QTLs (*pc8.1* and *pc10.1*). In the present study, immature-fruit color was identified by using both visual observation and colorimetry. We verified that the QTL positions of L*, a*, b*, SD, and SG, which were located in the region spanning from 174,787,932 bp to 174,823,007 bp on chromosome 10, were consistent with the linkage analysis results of light-green immature fruits (LG) that were identified visually, which showed the reliability of the results. There was only one gene, *Capana10g001710* (also termed *CaPP2C35* because it encodes a PP2C35 protein), in this region, and its physical distance from the genes controlling dark green coloration of immature fruits (*CaGLK2*) and purple coloration (*Ca3GT*) was 166.45 Mbp and 18.56 Mbp, respectively (Brand et al. 2014; Liu et al. 2020). The expression of *CaPP2C35* in 17C827 with light-green pools was significantly higher than that of 17C658 with green pools, which confirmed that *CaPP2C35* was the candidate gene for the LG locus. We also found that there is a G-A substitution at the 313th base of the *Capana10g001710* coding sequence (CDS) in 17C827, which potentially changes the structure of the PP2C35 protein. This raised the possibility that the light-green phenotype results from the mutation or expression level or both, which will be studied in subsequent research.

We silenced *CaPP2C35* in the light-green immature fruits of pepper accession 17C827 to further analyze its function. After *CaPP2C35* was down-regulated, the key chlorophyll synthesis metabolism-related genes located in the green portion of the silenced fruits were up-regulated and promoted the synthesis of chlorophyll, deepening the green saturation on the fruit surface. In addition, *SGR2*, *PAO*, and *RCCR* were up-regulated in 17C827, though they were not in 17C658, whereas *CaPP2C35* and the chlorophyll synthesis metabolism-related genes (*HEMA1*, *GSA*, *CHLD*, *CHLH*, *CRD1*, *CAO*, and *CHLG*) were down-regulated in 17C827 (Supplementary Fig. 11). We speculate that the up-regulation of *CaPP2C35* in 17C827 promoted the degradation and inhibited the synthesis of chlorophyll, thus reducing the accumulation of chlorophyll in 17C827. These results revealed that *CaPP2C35* participates in the formation of light-green immature fruits in 17C827 by affecting the accumulation of chlorophyll in the exocarp, which is similar to the mechanisms of *APPR2-Like*, *CaGLK2*, and *CcLOL1* (Pan et al. 2013; Brand et al. 2014; Borovsky et al. 2019). *APPR2-like* indirectly regulated the accumulation of chlorophyll content in pepper fruit through the ABA signal pathway (Pan et al. 2013). *CaGLK2* affected the accumulation of chlorophyll by adjusting the size of the chloroplast chamber (volume) (Brand et al. 2014). *CcLOL1* affected the green color formation of immature fruits by regulating chloroplast size and chlorophyll content (Borovsky et al. 2019). In contrast, *CaPP2C35* may regulate chloroplast grana lamella stacking and stroma lamella formation, resulting in differences in the thylakoid area in the chloroplasts and affecting the metabolism and accumulation of chlorophyll, similar to the mechanisms underlying the formation of white immature cucumber fruits (Liu et al. 2016). Therefore, our findings shed light on the formation of light-green coloration in immature pepper fruits. The above results indicate that the

formation of immature pepper fruits with different green color saturation levels is mainly related to the development of chloroplasts or the content of chlorophyll.

In addition, we designed a marker, dCAPS10-1, and tested it using the F_2 population and 126 pepper accessions (Supplementary Fig. 12a and Supplementary Table 3). Interestingly, the phenotypic matching rates of dCAPS10-1 in F_2 and the 126 pepper accessions were 95.92% and 75.40%, respectively, which was a phenomenon related to the low phenotypic matching rates for a gene marker. Combined with the PVE% of dCAPS10-1 for L^* (27.35%), a^* (21.43%), b^* (20.42%), SD (17.76%), and SG (18.14%). A possible explanation is that *CaPP2C35* had a limited ability to regulate the formation of light-green immature pepper fruits, similar to the abilities of *CcLOL1* (Borovsky et al. 2019). Meanwhile, we found that there was no G-A substitution at the 828th base of the *CaPP2C35* sequence (313th base of the *CaPP2C35* CDS) in some light-green pepper accessions, such as 17C632 and 17C965, indicating that *CaPP2C35* may be up-regulated or that other genes may participate in the formation of light-green immature fruits in these accessions (Supplementary Fig. 12b). Similar results have been reported in studies on *CCS*, *CaGLK2*, and *CcLOL1* (Lefebvre et al. 1998; Brand et al. 2014; Borovsky et al. 2019). Therefore, dCAPS10-1 can be used to assist the selection of light-green immature pepper fruits at the seeding stage within a certain range.

In the current study, GWAS, BSA, and linkage analysis revealed that the *LG* locus controlling light-green immature-fruit coloration in pepper was located in the region between 174,787,932 bp and 174,823,007 bp on chromosome 10. Analysis of the *CaPP2C35* sequence and its expression confirmed that *CaPP2C35* was the candidate gene for *LG*. Silencing of *CaPP2C35* in 17C827 resulted in an increase in chlorophyll content in the exocarp and the appearance of green stripes on the surface of the fruits. These findings shed light on the formation of light-green coloration in immature pepper fruits. In subsequent research, we will further study the specific regulatory molecular mechanism of *CaPP2C35* to determine whether it indirectly regulates the development of chloroplast thylakoids and the accumulation of chlorophyll through the ABA signal pathway or related transcription factors. Such research will enrich our understanding of the mechanisms involved in immature-fruit color formation in pepper.

Declarations

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Author contributions

L.W. contributed to the experiments and writing of the manuscript. H.W. identified the dominance of light-green over green color and initiated the study. S.L. contributed to BSA and GWAS experiments. M.L.

contributed to candidate gene sequencing. J.L. assisted with the qRT-PCR. Y.W. assisted with the analysis of candidate gene mapping. L.S. contributed to TEM analysis. W.Y. revised the manuscript. H.S. supervised the entire process. All authors have read and approved the final manuscript.

Conflict of interest

The authors declare no competing interests.

Availability of data and material

The datasets and materials generated during or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

Figure 1

We examined the cross-section of 17C658 (green immature fruits) and 17C827 (light-green immature fruits) immature fruits

Figure 2

In the present study, F₁ and F₂ were constructed from a cross between 17C827 (light-green immature fruits) and 17C658 (green immature fruits). All F₁ individuals showed a light-green immature-fruit color, which was the same as that of the female parent 17C827

Figure 3

To efficiently map the LG locus, a GWAS on immature-fruit color, comprising yellowish white, milky yellow, yellowish green, light green, green, deep green, purple, and purplish black, in 287 pepper accessions was conducted

Figure 4

a derived cleaved amplified polymorphic sequence (dCAPS) molecular marker, dCAPS10-1, was designed

Figure 5

To further explore the role of CaPP2C35, we silenced this gene in 17C827 (light-green immature fruits) via tobacco rattle virus (TRV)-mediated virus-induced gene silencing (VIGS). The immature fruits of 17C827 plants subjected to TRV2-CaPP2C35 silencing continued to have green stripes on their surfaces that were unable to transition into the light-green color of the surface of the fruits

Figure 6

The results revealed that the expression of *CaPP2C35* in the light-green exocarp of fruits treated with TRV2-*CaPP2C35* was not significantly different from that of the control treatments

Figure 7

In contrast, the expression level of SGR1, involved in chlorophyll degradation, was negatively correlated to that of CaPP2C35, whereas SGR2 was positively correlated to that of CaPP2C3

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