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ORIGINAL ARTICLE

Genetic mapping and development of molecular markers for a candidate gene locus controlling rind color in watermelon

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Abstract

Key message ***ClCG08G017810 (ClCGMenG)*** **encoding a 2-phytyl-1,4-beta-naphthoquinone methyltransferase protein is associated with formation of dark green versus light green rind color in watermelon.**

Abstract Rind color is an important agronomic trait in watermelon [*Citrullus lanatus* (Thunb.) Matsum. and Nakai], but the underlying molecular mechanism for this trait is not fully known. In the present study, we identified a single locus on chromosome 8 accounting for watermelon rind color (dark green vs. light green). Genetic analysis of F_1, F_2, and BC_1 populations derived from two parental lines (9904 with dark green rind and Handel with light green rind) revealed that the watermelon rind color (dark green vs. light green) is controlled by a single locus, and dark green is dominant to light green rind. Initial mapping revealed a region of interest spanning 2.07 Mb on chromosome 8. Genetic mapping with CAPS and SNP markers narrowed down the candidate region to 31.4 kb. Gene annotation of the corresponding region in the reference genome revealed the *ClCG08G017810* gene sequence encoding the 2-phytyl-1,4-beta-naphthoquinone methyltransferase protein. The sequence alignment of the candidate gene with the two parental lines suggested a nonsynonymous SNP mutation in the coding region of *ClCG08G017810*, converting an arginine (R) to glycine (G). The SNP might be associated with rind color of 103 watermelon germplasm lines investigated in this study. The qRT-PCR analysis revealed higher expression of *ClCG08G017810* in dark green rind than in light green rind. Therefore, *ClCG08G017810* is a candidate gene associated with watermelon rind color. The present study facilitates marker-assisted selection useful for the development of cultivars with desirable rind color.

Introduction

Watermelon [*Citrullus lanatus* (Thunb.) Matsum. and Nakai ($2n = 2x = 22$)] is an important horticultural crop worldwide (Levi et al. 2001). The watermelon rind color is considered an important trait, influencing consumer preference, and breeders pay considerable attention this trait (Tang et al. 2018). Watermelon exhibits a wide range of rind colors: dark or light green, light green-gray, and yellow are the common colors (Guner and Wehner 2004; Gusmini and Wehner 2006; Kumar and Wehner 2011).

Various genes and QTL controlling rind color have been identified in crop plants. In cucumber, the *R2R3-MYB* gene locus is pleiotropic for orange mature fruit color (Li et al. 2013), while *Csa7G05143* and *Csa6G133820* gene loci are associated with cucumber light green rind (Lun et al. 2016; Zhou et al. 2015). The *Csa3G904140* gene locus is associated with immature rind color (Tang et al. 2018). The *Csa2G352940* gene sequence encodes the *MYB36* transcription factor associated with cucumber yellow green rind (Hao et al. 2018). In sweet cherry, the transcription factor *PavMYB10.1* was reported to determine fruit color (Jin et al. 2016). In tomato, the transcription factor *SlMYB12* has a key role in a flavonoid biosynthesis pathway and control of

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red phenotype (Adato et al. 2009; Ballester et al. 2010). Lin et al. (2014) demonstrated that a deletion of a cis-acting element impaired the transcription of *SlMYB12,* resulting in the absence of flavonoids and a colorless rind. The *CYC-B* gene is associated with the orange color in tomato, influencing the amount of β-carotene content in the fruit ripening stage (Hwang et al. 2016). Huh et al. (2001) identified a candidate gene, which encodes phytoene synthase imparting red and orange colors in pepper. A single locus on chromosome 5 is implicated in the control of rind color in wax gourd (Jiang et al. 2015). A major QTL associated with rind color was identified on chromosome 8 in pear (Yamamoto et al. 2014). In rice, the *Rc* and *Rd* genes are involved in proanthocyanidin synthesis, affecting the red and brown color (Furukawa et al. 2007). *MdMYB1a*, *MdMYB1*, *MdbHLH3-1*, *MdbHLH33-1*, *MdUFGT2-1*, and *MdUFGT4* are involved in anthocyanin synthesis, contributing to apple rind color (Meng et al. 2016).

Much attention has been given to regulatory mechanism underlying rind color in watermelon (Barham 1956; Kumar and Wehner 2011). Researchers have proposed diverse inheritance models of rind color and stripe patterns. It was reported that rind color was qualitatively inherited and that dark green was completely dominant to light green (Kumar and Wehner 2011; Porter 1937; Weetman 1937). Weetman (1937) and Poole (1944) proposed a model demonstrating that three alleles at a single locus were associated with inheritance of rind color and stripe patterns. Kumar and Wehner (2011) reported that two loci control the inheritance of solid dark green versus light green rind pattern in watermelon. Solid dark green rind is inherited in a duplicate dominant epistatic manner with light green rind (Kumar and Wehner 2011). A single recessive gene accounted for the inheritance of golden yellow rind color in the 'Royal Golden' (Barham 1956).

The development of high-throughput sequencing technology and the availability of reference genome provide new opportunities to elucidate the complex regulatory mechanism for watermelon (Davey et al. 2011; Guo et al. 2013; Levi et al. 2011). Based on the next-generation sequencing (NGS) technology and the draft genome assemblies for two diploid watermelon cultivars (97103 and Charleston Gray) (Wu et al. 2019), several genetic maps have been constructed in recent years (Cheng et al. 2016; Park et al. 2016; Ren et al. 2014, 2015; Shang et al. 2016; Zhang et al. 2018). Recently, a three-locus model for rind phenotype has been proposed in watermelon. The three independent loci, *S* (foreground stripe), *D* (depth of color), and *Dgo* (background rind color), followed the Mendelian segregation ratio (Yang et al. 2015). Moreover, the three rind-related loci were mapped on chromosome 6, chromosome 8, and chromosome 4 based on the genetic linkage maps, respectively (Park et al. 2016). The three physically separated loci are located on different chromosomes, suggesting that the three-locus model could be proved by molecular genetic analysis. Using BSA-seq and GWAS analysis, a locus for yellow rind color in watermelon has been anchored on chromosome 4 in a region of 59.8 kb (Dou et al. 2018). Until now, no stable markers and candidate genes have been documented for watermelon rind color, which are important for marker-assisted selection and for understanding the domestication of watermelon (Sebastian et al. 2010). Since the inheritance pattern is not fully understood, we constructed a high-density genetic map based on the whole-genome resequencing (WGR) of two parental lines and 126 recombinant inbred lines (RILs). In our previous study, one prominent locus accounting for watermelon rind color (dark green vs light green) was identified in a 2.07 Mb region at the end of chromosome 8 (Li et al. 2018).

In the present study, we investigated the inheritance of watermelon rind color in F_1, F_2, and BC_1 populations derived from '9904' (dark green) and 'Handel' (light green). We aimed to narrow down the candidate region to a small interval through genetic mapping based on CAPS and SNP markers. Finally, we identified the putative candidate gene for watermelon rind color (light green and dark green) through sequence alignment and qRT-PCR analysis. The present study facilitates marker-assisted selection and identification of putative gene loci associated with watermelon rind color.

Materials and methods

Plant materials and genetic mapping population

The preliminary mapping population consisted of 126 recombinant inbred lines (RILs, F_7), derived from a cross between the inbred lines '9904' (female parent) and 'Handel' (male parent) which have dark green and light green rind, respectively (Fig. 1) (Li et al. 2018). The F_2 population was used to perform genetic mapping. The backcross populations were produced by hybridizing an F_1 plant with each parent to create BC_1P_1 ($F_1 \times 9904$) and BC_1P_2 ($F_1 \times$ Handel) and used to validate genetic inheritance of rind color.

For genetic map construction and segregation analysis, the RIL population was grown together with the parental lines at two locations under three environments: Sanya Experimental Station in 2016 (Hainan, open field) and Xinxiang Experimental Station in 2017 (Henan, greenhouse and open field) (Li et al. 2018). The F_2 population was grown in 2017 (Hainan) and 2018 (Henan) during winter and spring seasons with 432 and 560 individuals, respectively. The 140 BC_1P_1 and 161 BC_1P_2 individuals were grown in 2018 autumn season (Table S1). The phenotype of rind color was determined by visual observation, and watermelons were categorized into dark green and light green groups based on their appearance at 35 days after pollination (DAP).

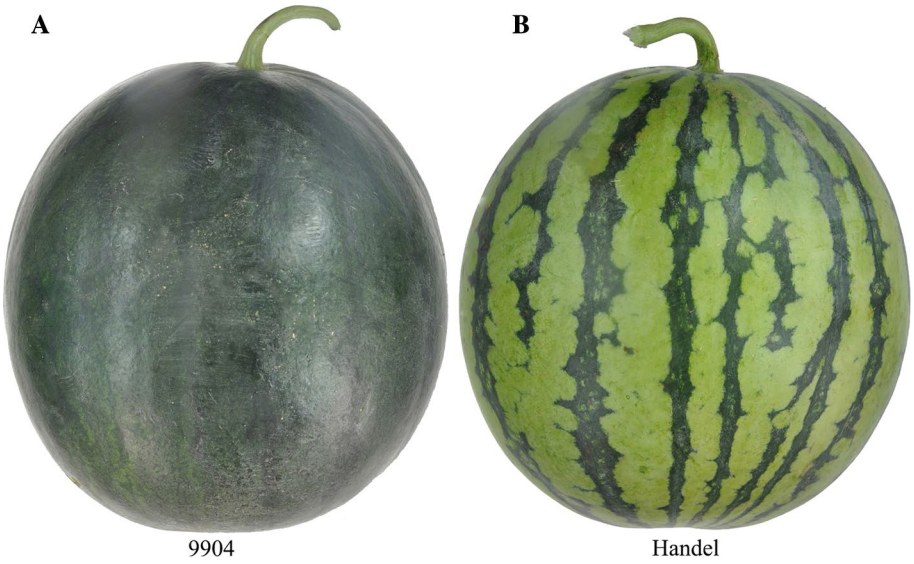

Fig. 1 Phenotype of rind color in parental lines. **a** Mature '9904' (P_1) fruit with dark green rind. **b** Mature 'Handel' (P_2) fruit with light green rind (color figure online)

Measurement of pigment content

The fruit rind was sampled at different developmental stages (5, 10, 18, 26, and 34 DAP), tissue samples of leaves and stems of the parental lines were collected, and the pigments were extracted with 80% acetone in the dark. The spectrophotometer (Persee, China) was used to measure the absorbance values of the chlorophyll a, chlorophyll b, and carotenoid at 663, 645, and 470 nm, respectively. The chlorophyll concentration was calculated according to Arnon's method (Arnon 1949), while carotenoid concentration was calculated according to Wellburn's method (Wellburn 1994). Three replicates were prepared for each sample.

Chloroplast phenotype analysis by transmission electron microscopy

Transmission electron microscopy was used to observe the number, morphology, and ultrastructure of the chloroplasts. Rind samples from watermelon fruit at 34 days after pollination were cut into thin pieces, immediately fixed with 2.5% glutaraldehyde and observed with a JEM-1400 (JEOL, Japan) transmission electron microscope. The number of chloroplasts was counted in every five cells for comparison between the two parental lines (Hao et al. 2018).

DNA extraction

Young leaves from two parental lines and the segregating population were collected and stored at − 80 °C until their DNA was extracted. The genomic DNA was extracted using the cetyltrimethyl ammonium bromide (CTAB) method (Murray and Thompson 1980). DNA was quantified with a NanoDrop-1000 spectrophotometer (NanoDrop, USA) and was evaluated by electrophoresis in a 1.0% agarose gel.

Molecular marker development and genetic mapping

Re-sequenced data were compared with the available watermelon reference genome (97103 and Charleston Gray) from the Cucurbit Genomics Database (https://cucurbitgenomics.org/) to identify reliable SNPs through a filter pipeline (Takagi et al. 2013). To narrow the candidate region and verify the accuracy of the preliminary mapping result from the genetic map, the corresponding cleaved amplified polymorphic sequence (CAPS) markers were developed based on SNPs (Table S2).

PCR amplification was performed in a 10-μl reaction with 1 μl DNA, 5 μl PCR master mix, 0.5 μl of 10 μM per primer, and 3 μl distilled water. The PCR protocol was performed under the following conditions: initial denaturation at 94 °C for 1 min and 30 s; followed by 30 cycles at 94 °C for 20 s, 57 °C for 20 s, 72 °C for 50 s; and a final extension at 72 °C for 5 min. Then, the corresponding restriction endonucleases were used to digest the amplified PCR products at 37 °C or 65 °C for 4–10 h following the manufacturer's instructions. The digested products were separated on 1.0% agarose gels and visualized with a Versa Doc (Bio-Rad). The markers with polymorphisms were used for fine mapping.

Sequence analysis and prediction of the candidate gene

The sequence and gene function were retrieved from the Cucurbit Genomics Database (https://cucurbitgenomics.org). A CAPS marker (Table S3) was developed according

to the candidate gene sequence and validated the linkage on 103 watermelon germplasms containing 30 watermelons with dark green and 73 with light green rind color. DNA and amino acid sequences were aligned using DNAMAN (version 9). All the germplasms were obtained from the Chinese National Watermelon and Melon Germplasm Resource Library (Zhengzhou, China).

RNA isolation and quantitative real-time PCR analysis of the candidate gene

The rind samples from different developmental stages (0, 5, 10, 18, 26, and 34 DAP) and other tissue samples, including roots, stems, leaves, and male flowers were collected from both parental lines. RNA was isolated using the plant total RNA purification kit (Tiangen, China) according to the manufacturer's instructions, and then, the first-strand cDNA was synthesized using a cDNA synthesis kit (Takara, Japan).

The gene-specific primers of the candidate genes and reference gene *Actin* (Kong et al. 2015) for quantitative real-time PCR (qRT-PCR) were designed based on the Cucurbit Genomic Database (https://cucurbitgenomics.org), using the software Primer Premier 5. The expression levels of the candidate genes were performed using a LightCycler480 RT-PCR system (Roche, Swiss) with a Real Master Mix (SYBR Green) kit (Toyobo, Japan) for qRT-PCR. Amplification was carried out in a 20-µl reaction mixture containing 1 µl cDNA, 1 µl forward and reverse primers (10 µM), 10 µl 2 × SYBR Green real-time PCR mixes, with nuclease-free water added to a total reaction of 20 µl. All reactions were performed in triplicate. The expression level was analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). And the primer sequences used in this study are listed in Supplemental Table S3.

Results

Inheritance and phenotypic characterization of rind color in watermelon

The parental inbred lines produced watermelons with a distinguishable rind color. Line 9904 produces watermelons with dark green rind color, while Handel produces watermelons with light green rind color (Fig. 1). All F_1 plants derived from the cross between these two parental lines are dark green without segregation, revealing dominant inheritance of the dark green rind color trait. In 2017, the F_2 population separated into 331 plants with dark green rind and 101 plants with light green rind, confirming a 3:1 Mendelian ratio ($\chi^2 = 0.60$, $P = 0.44$). The other F_2 population that was grown in 2018 contained 424 plants with dark green rind and 136 plants with light green rind, fitting the expected Mendelian ratio of 3:1 ($\chi^2 = 0.15$, $P = 0.70$). All 140 individuals of BC_1P_1 were dark green. For the BC_1P_2 population, there were 83 plants with dark green rind and 78 plants with light green rind color, showing a ratio of 1:1 ($\chi^2 = 0.16$, $P = 0.69$). The genetic analysis of rind color traits for dark green and light green is shown in Table S1. All the segregation results suggested that rind color (dark green and light green) was controlled by a single nuclear gene with complete dominance in the population.

Determination of pigment contents and observation of transmission electron microscopy

The chlorophyll and carotenoid contents of the rind samples were measured from two parental lines at different developmental stages (5, 10, 18, 26, and 34 DAP). The results showed that dark green rind contained significantly higher (~1.5-fold) chlorophyll content at 10 DAP (Fig. 2b); however, there is no significant difference at 5 DAP (Fig. 2a). Chlorophyll content in dark green rind was almost fourfold higher as compared to light green rind at 34 DAP (Fig. 2e). A negligible amount of carotenoids were detected in rind, and there was no distinct difference between the parental lines (Fig. 2). Similarly, the pigment content of the other tissues did not show a significant variation between the two parental lines which was consistent with the visual observations. However, chlorophyll content was nearly 11-fold higher in the leaves as compared to stem (Fig. 2f).

Transmission electron microscopy was used to analyze the number and ultrastructure of chloroplasts in the rind of two parental lines (Fig. 3). The result showed that more chloroplasts ($P < 0.05$) were observed in the dark green rind (4.4 ± 0.48/per cell) than in the light green rind (2.8 ± 0.75/per cell). The chloroplasts of the dark green rind had a distinctly enlarged size, and some chloroplasts showed an irregular shape (Fig. 3e) compared with that of the chloroplasts in the light green rind (Fig. 3f). In addition, the chloroplasts in the dark green rind stacked tightly and some were bound together, while the chloroplasts in the light green rind were loosely arranged. The number of grana was decreased in the light green rind (Fig. 3b) compared with that of dark green rind (Fig. 3a).

Genetic map construction

In our earlier study (Li et al. 2018), we constructed a high-density genetic map based on whole-genome resequencing of the RIL population and both parental lines. A total of 7.67 Gbp, 8.81 Gbp, and 177.08 Gbp of high-quality reads were obtained from the parental lines 9904 and Handel, and from 126 RIL families, respectively. The average

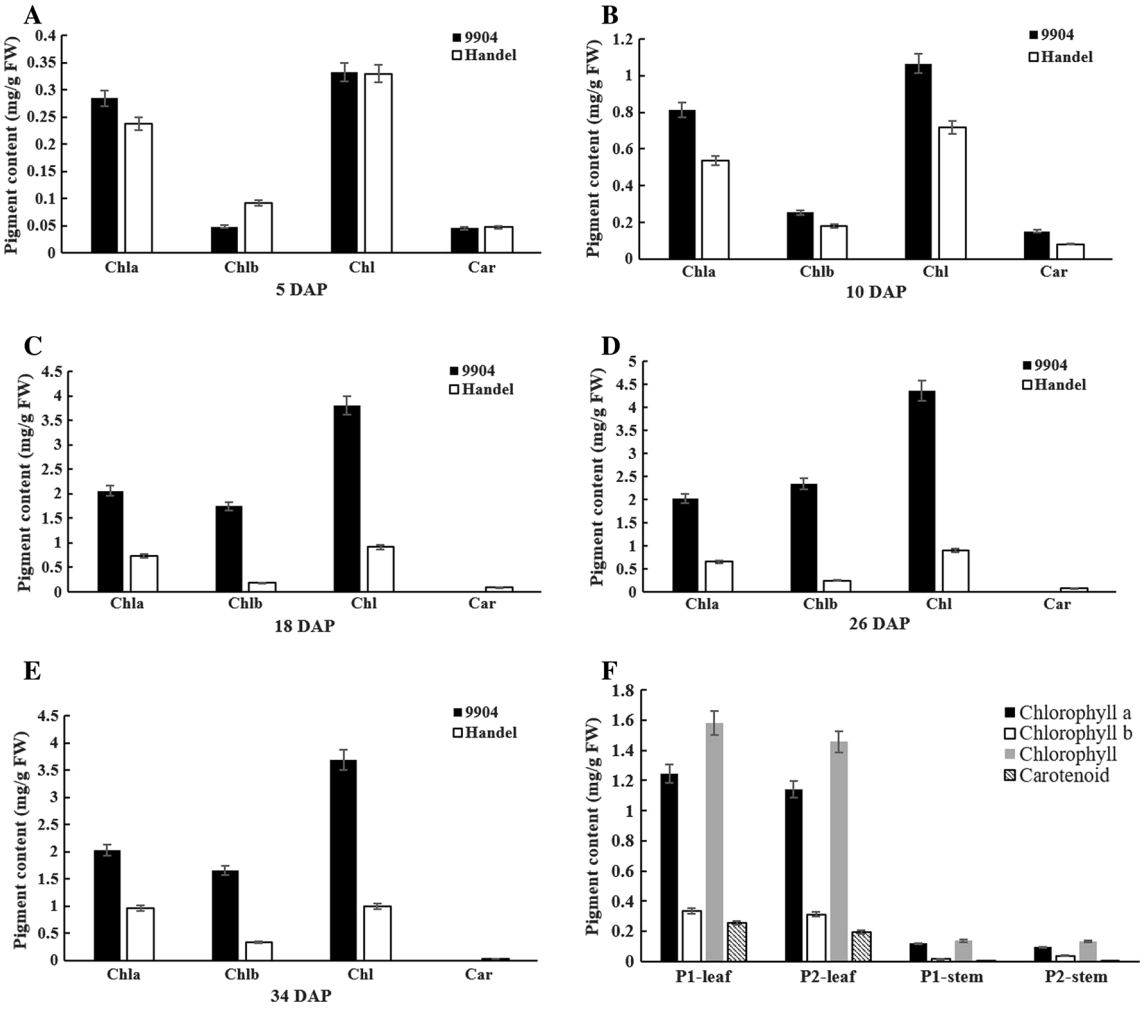

Fig. 2 Pigment content analysis of tissues in the two parental lines. **a, b, c, d, e** Pigment content of the rind at different stages (5, 10, 18, 26, 34 DAP). **f** Pigment content of different tissues. *Chl* chlorophyll, *Car* carotenoid

coverage depths of the markers were 19-fold for the male parent, 17-fold for the female parent, and threefold for the RIL population. A total of 178,762 SNPs with a sequencing depth greater than fourfold were detected by analyzing the parental lines. Additionally, 2132 recombination bin markers comprising 103,029 SNP markers were used to construct the genetic map containing 11 linkage groups (LGs). As compared to other recent studies in watermelon, we found that a greater number of SNP markers were mapped to this genetic map (Li et al. 2018). The final high-density genetic map had a total length of 1,508.94 cM, with an average distance of 0.74 cM between adjacent bin markers. Additionally, the haplotype, heat maps and collinearity of the genetic map with watermelon reference genome showed that the high-density genetic map was accurately assembled with good quality (Li et al. 2018). The LOD thresholds for determining significant loci were estimated from 1000 permutations, and a minimum LOD score of 2.5 was used to judge the presence of loci on the chromosome (Churchill and Doerge 1994).

A high-density genetic map identifies candidate genes located on chromosome 8

Based on that high-density genetic map and the phenotypes of the RIL population, we detected one prominent locus (qrc-c8-1) associated with rind color (Li et al. 2018). This locus was mapped to the end of chromosome 8 in a 2.07-Mb region ranging from 24.07 to 26.14 Mb with a high LOD score of 18.353 and explained 49.943% of the phenotypic variation (Fig. 4). Our mapping region results are consistent with the study of Park et al. (2016) which showed that the *D* locus (depth of rind color) is located on chromosome 8. These results indicate that a candidate gene accounting for watermelon rind color is located in this region.

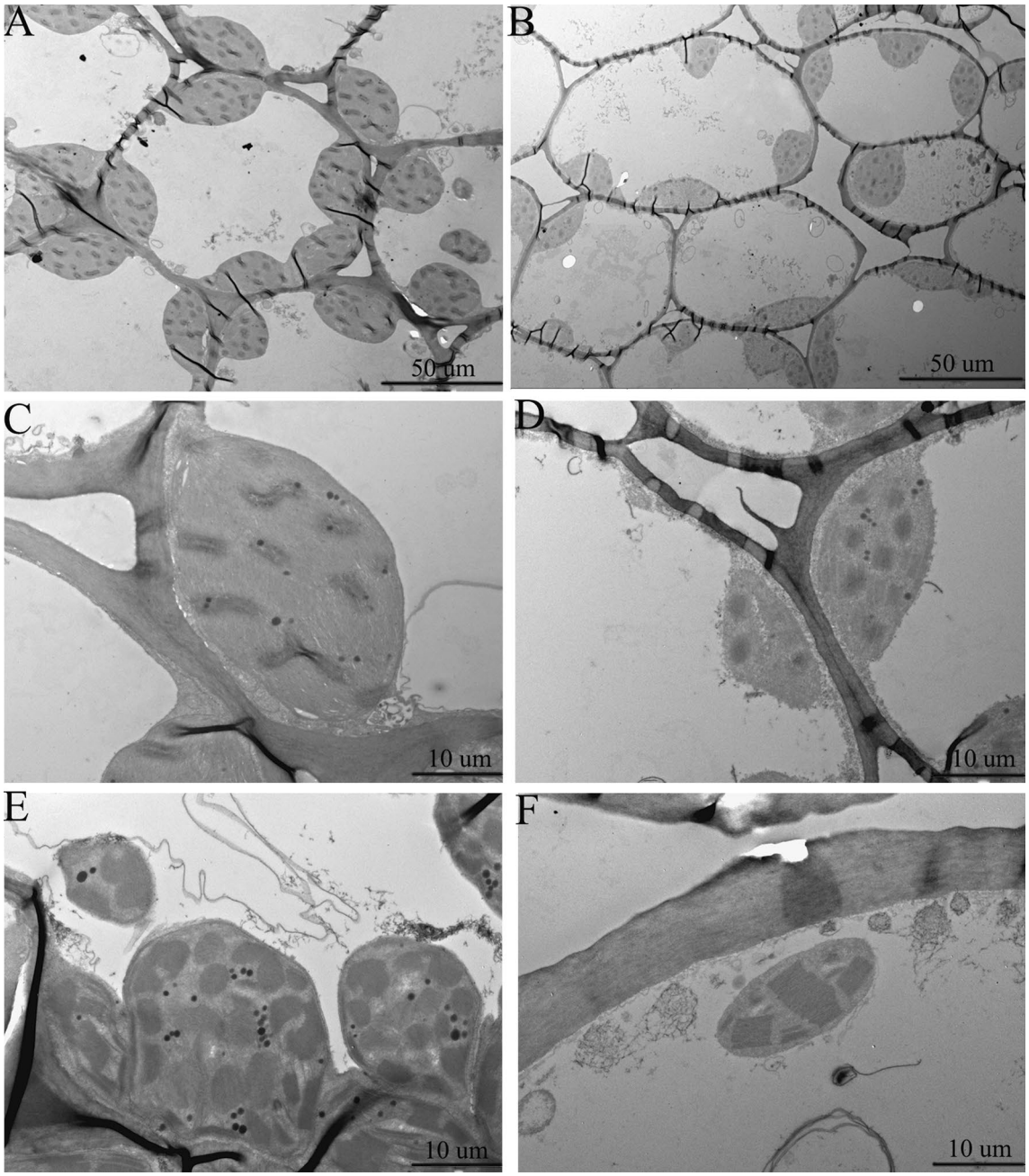

Fig. 3 Transmission electron micrographs of chloroplasts. **a** Epidermis chloroplast number and size of 9904. **b** Epidermis chloroplast number and size of Handel. **c** Epidermis chloroplast ultrastructure of 9904. **d** Epidermis chloroplast ultrastructure of Handel. **e** Epidermis chloroplast with irregular shape of 9904. **F** Epidermis chloroplast with normal shape of Handel

Genetic mapping of a candidate gene to a 31.4-kb region

To genetically map the candidate gene for rind color, two F_2 populations consisting of 432 and 560 individuals were developed during 2017 and 2018, respectively. In order to screen all F_2 segregating populations for polymorphic analysis, 431 CAPS makers based on the '97103' watermelon reference genome were developed for the candidate region on chromosome 8 (Table S2). Finally, the candidate gene was located next to the CAPS marker Chr8_26139204, with 14 recombinant individuals. We blasted the interval to 'Charleston Gray' watermelon reference genome (https://cucurbitgenomics.org/) and compared the two reference genomes. We found that the assembly of chromosome 8 in the 97103 watermelon reference genome is not intact and

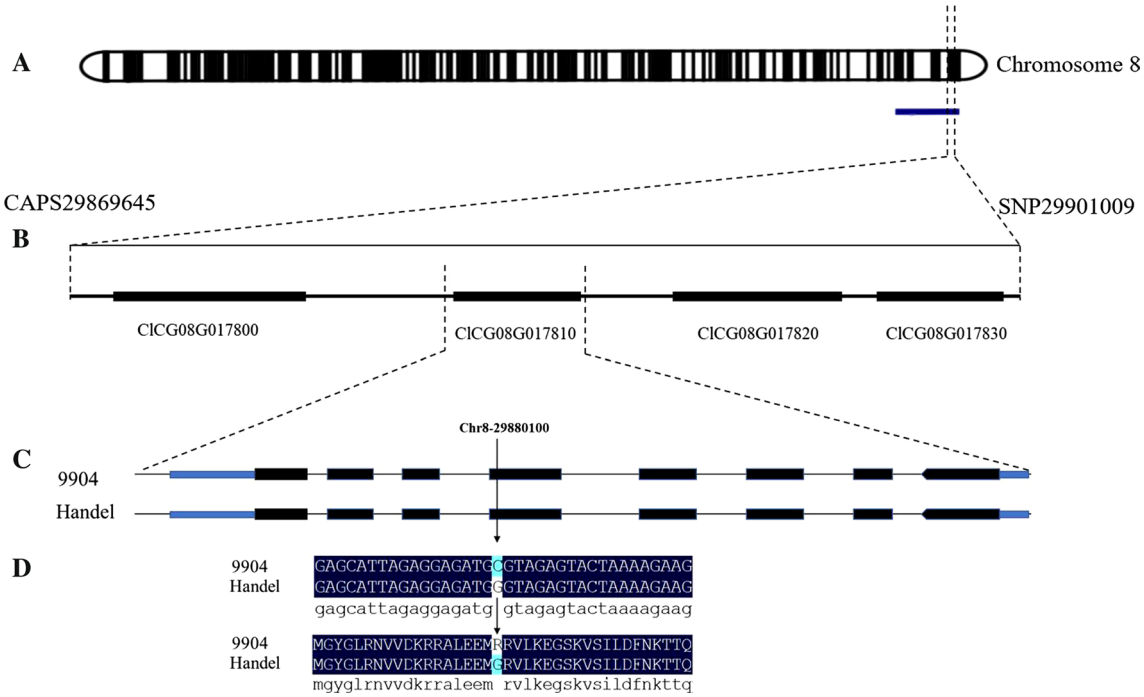

Fig. 4 Genetic mapping of the rind color gene and candidate gene analysis in watermelon. **a** The preliminary genetic map. The candidate gene was located at the end of chromosome 8. **b** Fine mapping of the candidate gene. The candidate gene was localized to a 31.4-kb region between the flanking markers CAPS29869645 and SNP29901009. **c** Structure of the *ClCG08G017810* gene. A nonsynonymous SNP mutant is located on the fifth exon region at 29,880,100 bp on chromosome 8. **d** The nonsynonymous SNP mutation (C→G) results in the conversion of an arginine (R) to glycine (G) at residue 171

contains a nearly 262-kb deletion on chromosome 8, comprising 34 additional genes at this chromosomal region in 'Charleston Gray'. Based on 'Charleston Gray' reference genome and the deletion fragment, 281 new CAPS and 7 SNP markers were developed to screen the F_2 populations (Table S2). Finally, the candidate gene was delimited to a 31.4-kb interval between CAPS29869645 and SNP29901009 on chromosome 8 with 5 recombinant individuals according to the 'Charleston Gray' watermelon reference genome (Levi et al. 2011; Wu et al. 2019; Table 1, Fig. 4).

Sequence and expression analysis of the candidate genes

The candidate genes were mapped to a region of 31.4-kb interval based on the genetic mapping. Four putative genes (*ClCG08G017800*, *ClCG08G017810*, *ClCG08G017820*, and *ClCG08G017830*) were found in the narrowed region. The 'Charleston Gray' watermelon genome database was used to predict the function of the genes (Table 2). Among the four genes, *ClCG08G017810* is highly homologous to *AT1G23360* (*MENG*) in *Arabidopsis thaliana* encoding a 2-phytyl-1,4-beta-naphthoquinone methyltransferase protein (https://www.arabidopsis.org/). This 2-phytyl-1,4-beta-naphthoquinone methyltransferase is involved in the biosynthesis of phylloquinone (vitamin K1), which is required for the conversion of 2-phytyl-1,4-beta-naphthoquinol to phylloquinol. It has been proven that phylloquinone is involved in the biosynthesis of chlorophyll and photosystem I (van Oostende et al. 2011). Thus, *ClCG08G017810* is likely the candidate gene associated with rind color.

To analyze the sequences of the four candidate genes, we developed primers to clone the four genes and the entire coding sequences (CDS) from both parental lines (Table S3). The sequence alignment of the four genes between dark green and light green parental lines showed that four SNP mutations existed in *ClCG08G017800*. Among those SNP mutations, 3 SNPs were found in the intron region, while one synonymous SNP mutation was in the exon region leading to no amino acid change. Similarly, the sequence alignment of the *ClCG08G017810* gene between the two parental lines revealed three SNP mutations in the intron region and one nonsynonymous SNP mutation (C→G) in the fifth exon region at 29,880,100 bp on chromosome 8, which resulted in the conversion of an arginine (R) to glycine (G) at residue 171 (Fig. 4). However, no mutation exists between the two parents for *ClCG08G017820* sequence, while a SNP was detected in the intron region

Table 1 Phenotypes and genotypes of recombinant individuals showing the recombinant breaking points

NO.	Phe	CAPS295098	CAPS298574	CAPS298609	CAPS298696	SNP298742	SNP298979	SNP299010	CAPS299047	CAPS299163	CAPS299426
		91	89	50	45	76	29	09	20	02	32
P1	D	A	A	A	A	A	A	A	A	A	A
P2	L	a	a	a	a	a	a	a	a	a	a
F1	D	H	H	H	H	H	H	H	H	H	H
2017-82	L	a	a	a	a	a	a	a	a	H	H
2018-66	D	H	H	H	H	H	H	H	H	a	a
2018-212	D	H	H	H	H	H	H	H	H	a	a
2018-15	L	a	a	a	a	a	a	H	H	H	H
2018-383	L	a	a	a	a	a	a	H	H	H	H
2018-297	D	a	a	a	a	H	H	H	H	H	H
2017-157	D	a	a	a	a	H	H	H	H	H	H
2018-476	L	H	H	H	H	a	a	a	a	a	a
2017-271	L	H	H	H	a	a	a	a	a	a	a
2018-322	D	a	a	a	H	H	H	H	H	H	H

Phe means the recombinant individuals' phenotypes. D indicates dark green rind color. L indicates the light green rind color. The alleles are abbreviated according to their origin: A: 9904 (dark green); a: Handel (light green); H: heterozygous.

Table 2 Cucurbit Genomic Database annotation of the candidate genes

Gene ID	Position	Cucurbit Genomics annotation
ClCG08G017800	Chr08: 29872343 … 29878958	Kinase family protein
ClCG08G017810	Chr08: 29879201 … 29881087	Ubiquinone/menaquinone biosynthesis methyltransferase UbiE like protein (2-phytyl-1,4-beta-naphthoquinone methyltransferase, chloroplastic)
ClCG08G017820	Chr08: 29881627 … 29884196	Homeobox transcription factor KN4
ClCG08G017830	Chr08: 29894263 … 29897127	Knotted-1 homeobox protein

and a synonymous SNP mutation was detected in the exon region for *ClCG08G017830*. Thus, we propose that *ClCG08G017810* is the candidate gene for dark green and light green rind color in watermelon.

Furthermore, expression analysis of *ClCG08G017810* was performed using qRT-PCR for rind samples at different developmental stages and other tissues including root, stem, leave, and male flower samples in both parental lines. As shown in Fig. 5, the expression level of *ClC08G017810* in the dark green rind was relatively higher than in the light green rind. *ClCG08G017810* had the highest expression level during the color accumulation stage (nearly 10 DAP). The expression level in dark green rind was almost twofold higher than in light green at this stage. The expression level of *ClCG08G017810* was relatively higher in leaves and stems than in root and male flowers. But *ClCG08G017810* did not show any significant differences in expression between the same tissues of the two parental lines, except in the rind. In addition, we sequenced the 5'-upstream sequence from CDS region for about 2300 bp to analyze the promoter region of *ClCG08G017810* from the parental lines based on the 'Charleston Gray' reference genome (Ye et al. 2017). A 16-bp deletion from the position of − 207 bp was detected in Handel compared with 9904 (Fig. S2). The results indicated that the different expression levels of *ClC08G1017810* in the rind could account for dark green and light green rind in watermelon. Therefore, *ClCG08G017810* is likely the candidate gene associated with dark green versus light green rind color in watermelon.

Identification of candidate gene in watermelon germplasm using a CAPS marker

A CAPS marker was developed based on the *SNP29880100* of *ClCG08G017810* (Table S3). To verify this marker, 180 individuals were selected from F_2 population to check the genotype. The result confirmed that the phenotype was consisted with the genotype (Table S4). This marker named RD-1D was used to screen 103 homozygous watermelon accessions to check the consistency of genotype with the phenotype. The 103 accessions contained 30 accessions with dark green rind and 73 accessions with light green rind. As predicted, the genotypes were completely consistent with the phenotype (Fig. S1, Table S4). Above all, these results further proved that *ClCG08G017810* could be the candidate

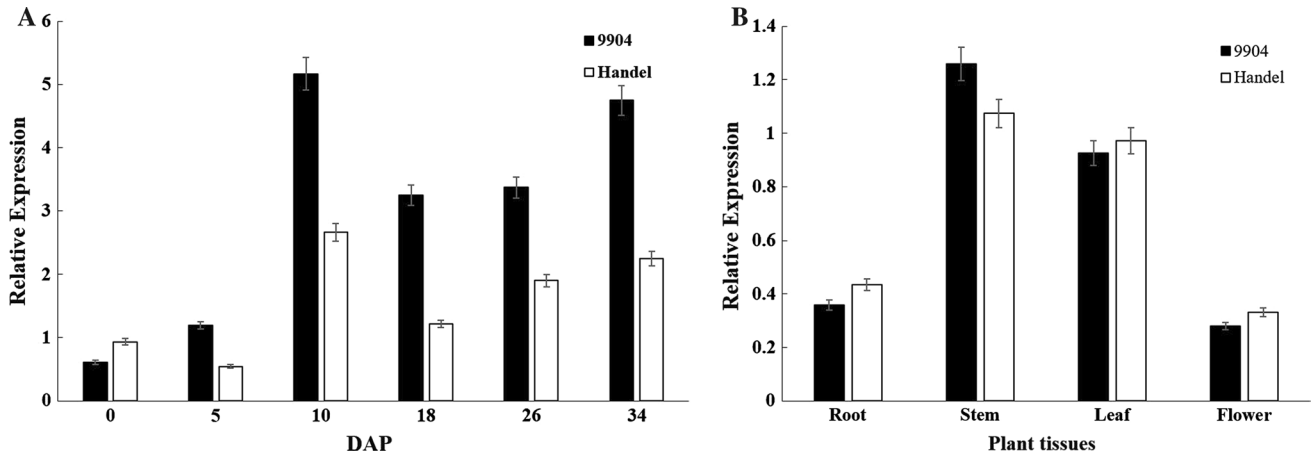

Fig. 5 Quantitative real-time PCR analysis of *ClCG08G017810*. **a** Relative expression level of *ClCG08G017810* in different development stages (0, 5, 10, 18, 26, and 34 days after pollination). **b** Relative expression level of *ClCG08G017810* in different tissues

gene conferring dark green versus light green rind color in watermelon.

Phylogenic analysis

To better understand the relationship between the protein coded by *ClCG08G017810* and its homologs, we used BLAST search in the NCBI database and MEGA 7 software to perform phylogenic analysis through bootstrap method with 1000 replications (Kumar et al. 2016). The result of the neighbor-joining tree indicated that *ClCG08G017810* from watermelon has a close phylogenetic relationship to the Cucurbitaceae family, including *Cucumis sativus*, *Cucumis melon*, *Cucurbita maxima*, and *Cucurbita moschata* (Fig. 6), which revealed that this gene was evolutionarily conserved within the Cucurbitaceae family. The sequence alignment analysis via UniProt (https://www.uniprot.org/) and SMART (https://smart.embl-heidelberg.de/) revealed that the *ClCG08G017810*, which shared 67.9% sequence identity with *MENG* in *Arabidopsis thaliana*, contained a conserved methyltransferase chemotaxis domain, and the amino acid mutation (R → G) is located in this domain (Fig. 6).

Discussion

Whole-genome resequencing (WGR) has become an important method for developing molecular markers, studying polymorphisms within the genome, constructing genetic linkage maps and mapping QTL of important traits (Huang et al. 2012; Jiao et al. 2012). The genome resequencing approach could effectively reduce the time and efforts required to genotype mapping populations (Lim et al. 2014). To increase marker saturation and develop marker resources for watermelon, we used WGR to construct a high-density genetic map using 126 RIL (F7) families derived from a cross between the inbred lines 9904 and Handel (Li et al. 2018). We identified a prominent locus for rind color in a 2.07-Mb region on chromosome 8 and a candidate gene sequence *ClCG08G017810,* named *ClCGMenG*, associated with watermelon fruit rind color (dark green vs. light green). The *ClCGMenG* is an ortholog of *MENG* in *Arabidopsis thaliana*, encoding the 2-phytyl-1,4-beta-naphthoquinone methyltransferase which takes part in conversion of 2-phytyl-1,4-beta-naphthoquinol to phylloquinol, known as vitamin K1, involved in the biosynthesis of the chlorophyll and photosystem I (van Oostende et al. 2011).

As an important horticultural crop all over the world, watermelon rind colors are mainly dark green or light green, light green-gray or yellow (Guner and Wehner 2004; Gusmini and Wehner 2006; Kumar and Wehner 2011). The inheritance of the watermelon rind color is a qualitative trait, but the genetic pattern and developmental mechanisms are complex (Dou et al. 2018; Kumar and Wehner 2011; Park et al. 2016). Although several studies described the inheritance model of watermelon rind color, to date, no study reported a candidate gene associated with this trait. Weetman (1937) suggested that the rind traits (stripe patterns and rind color) were controlled by three alleles at a single locus. Kumar and Wehner (2011) proposed that the solid dark green versus the light green in watermelon is controlled by duplicate dominant epistatic inheritance. The homozygous recessive genes, *g-1* and *g-2*, were identified to control light green rind color when both loci are homozygous recessive, while the presence of one dominant allele resulted in dark green rind pattern. A recessive gene *m* is responsible for the greenish white mottling of the rind color, resulting in gray types (Guner and Wehner 2004; Weetman 1937). Several researchers have

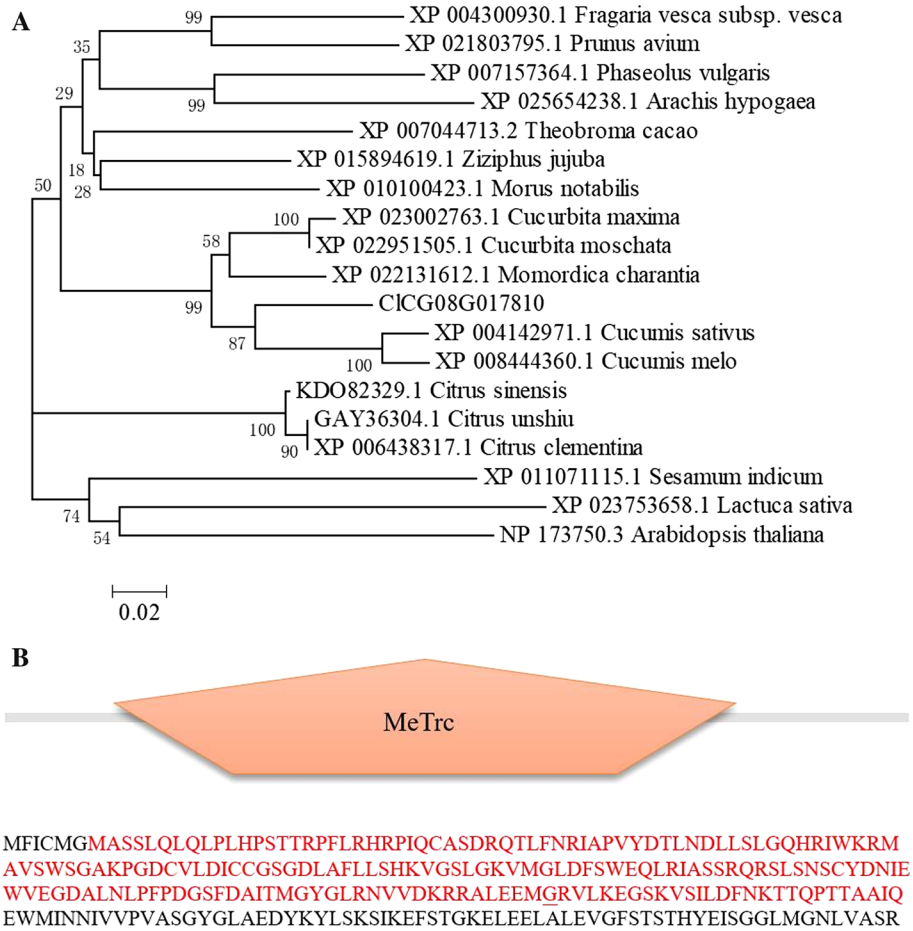

Fig. 6 The analysis of the phylogenetic and conserved domains of the candidate gene. **a** The phylogenetic tree of *ClCG08G017810* and its homologous proteins. The tree was constructed using MEGA 7 with 1000 bootstrap replications. **b** The conserved domains were analyzed by SMART software. The polygon and red amino acid sequence indicated a methyltransferase chemotaxis domain. The underline indicated the amino acid mutation

argued that the yellow rind color is controlled by a recessive gene (Barham 1956), while recent studies reported contrasting results that the yellow rind color is controlled by a single dominant gene based on a sequence-based genotyping method (Dou et al. 2018; Yang et al. 2015). Yang et al. (2015) reported that depth of rind color (*D*) is controlled by a single dominant gene and the *D* locus is located near the 26,061-kb region of the reference genome sequence on chromosome 8, concurring with our results. In our two-year study, we determined that watermelon rind color is controlled by a single dominant locus at the end of chromosome 8 and dark green was dominant to light green.

In the present study, the candidate gene for watermelon rind color was delimited to a 31.4-kb region at the end of chromosome 8 using CAPS and SNP markers (Fig. 4). The sequencing alignment between the parental lines showed one nonsynonymous SNP nutation in the CDS region of *ClCG08G017810*. Based on the SNP mutation, a CAPS marker was developed to screen 103 watermelon accessions, and the results revealed that the genotypes perfectly matched with the phenotypes (Fig. S1, Table S4). *ClCG08G017810* had a higher expression level in dark green rind than in light green rind. Furthermore, to understand the difference in expression between two parental lines, we analyzed the promoter region of *ClCG08G017810*. Compared with 9904, a 16-bp deletion region was detected in Handel (Fig. S2). The mutation in the promotor region could alter gene expression (Ye et al. 2017). Results indicated that *ClCG08G017810* was the candidate gene for watermelon rind color.

In general, the contents and the formation of pigments determined the color of leaves and fruit rind (Liu et al. 2007). Chlorophyll content is the primary factor for the green rind in Cucurbitaceae (Lancaster et al. 1997; Xiao-lei et al. 2003). Mutant plants with altered color in the fruit, leaf or other parts of the plant have been studied in different plants, which serve as important resources to study chlorophyll metabolism and chloroplast development (Liu et al. 2007; Mei et al. 2017). The *CSP41b* is a gene required for normal leaf color and chloroplast in rice (Mei et al. 2017). The mutants with a pale green peel had fewer and abnormally enlarged chloroplasts and accumulated lower amount of chlorophyll than the plants with a dark green peel in tomato (Forth and Pyke 2006). Fruit color can be influenced by the GLK transcription factor, which regulates chlorophyll levels in pepper (Brand et al. 2014). In the present study, we

found that chlorophyll is the major pigment for watermelon rind color, and the dark green rind contains a much higher level of chlorophyll than the light green rind (Fig. 2).

ClCG08G017810 identified as *ClCGMenG* is an ortholog of the *MENG* gene in *Arabidopsis thaliana* which is responsible for the rind color in watermelon. The *MENG* gene is involved in the biosynthesis of phylloquinone (vitamin K1) in *Arabidopsis thaliana*, encoding a 2-phytyl-1,4-beta-naphthoquinone methyltransferase that catalyzes the final step in phylloquinone biosynthesis, the conversion of 2-phytyl-1,4-beta-naphthoquinol to phylloquinol (Lohmann et al. 2006). Phylloquinone (vitamin K1) serves as an electron carrier in photosystem I (PSI) and as a cardinal redox cofactor, synthesized in some cyanobacteria and in the chloroplasts of plants (van Oostende et al. 2011). The significance and synthesis of phylloquinone have been studied using loss-of-function mutants. For instance, the *AtmenA* mutant in *Arabidopsis* showed the complete absence of phylloquinone resulting in reduced amount of chlorophyll and PSI, leading to an abundant reduction in photosynthesis efficiency (Shimada et al. 2005). The *AtmenA* mutant plants are incapable of growing in soil, showing the role of phylloquinone in PSI (Shimada et al. 2005). The phylloquinone loss-of-function mutants in *Synechocystis* cause a minor influence on photosynthesis or growth under low-light conditions, while the growth of *menA* and *menB* mutants is severely restrained under high-light conditions (Johnson et al. 2000). Furthermore, it was indicated that the 3-methyl group of phylloquinone plays an important role in retaining high efficiency of photosynthetic electron transport (Lohmann et al. 2006). Phylloquinone biosynthesis is associated with the metabolism of chlorophyll, salicylate, and tocopherols through crucial intermediates (J Basset et al. 2017; van Oostende et al. 2011). The albino leaves that result from a mutation in the DHNA phytyl transferase, which catalyzes the formation of 2-phytyl-1,4-naphthoquinone (demethylphylloquinone), showed smaller chloroplasts and fewer thylakoids than wild-type plants (Shimada et al. 2005). In the present study, we observed a larger size and a greater number of chloroplasts in the dark green rind. The phenotypes here are in concurrence with these in *Arabidopsis* (Shimada et al. 2005), supporting that *ClCGMenG* underlies the chloroplast variation. The chloroplasts in dark green rind stacked tightly and some were bound together, while the chloroplasts in light green rind were loosely arranged. In addition, some chloroplasts showed irregular shapes in the dark green rind. The number of grana was decreased in the light green rind compared with the dark green rind (Fig. 3). Therefore, we hypothesized that the differential expression of *ClCGMenG* might regulate the dark green and light green rind color in watermelon by influencing the biosynthesis of phylloquinone, resulting in different amounts of chlorophyll, number and morphology of chloroplasts.

Our present study indicated that pigment differences only existed between the rinds of the two parental lines (Fig. 2). Furthermore, the expression levels of *ClCGMenG* and the pigment content in the leaves and stems of the two parental lines did not show significant differences (Figs. 2, 5). In addition, the expression levels of *ClCGMenG* in green tissues (leaves and stems) were higher than in other tissues (root and male flowers). The plant color formation is a complex process and associated with different kinds of pigment biosynthesis pathways (Liu et al. 2007). Mutations in the functional genes can affect the biosynthesis and accumulation of pigment, leading to the variations of plant color (Mei et al. 2017). Previous studies proved that there was desynchronized color diversification between fruits and leaves color. Fruit and leaf colors are controlled by separate molecular mechanisms (Gao et al. 2016; Hao et al. 2018; Liu et al. 2016). For example, the different expression of *CsMYB36* explained the formation of yellow green rind in cucumber (Hao et al. 2018), while the difference in pigment and gene expression only occurred in the fruit rind. The gene expression level did not show significant difference in leaves, concurring with our result. The possible reason for these phenomena is that candidate gene exhibited a low expression level in the other tissues as compared to fruit rind and did not show significant difference. Therefore, it might slightly affect the color formation of the other tissues (Hao et al. 2018).

In summary, we identified a candidate gene (*ClCGMenG*) that accounts for the dark green versus light green rind color. The *ClCGMenG* is associated with chlorophyll and chloroplast development, which in turn controls watermelon rind color. The SNP and CAPS markers developed in this study should be useful in breeding programs for marker-assisted selection, and the data in this study should be useful for elucidating the mechanisms controlling rind color in watermelon and other cucurbit crops.

Author contribution statement WL conceived the research and designed the experiments. XL and NH developed the plants population. SZ, JD, and LG analyzed data. HG and AA checked the manuscript. WJ investigated the phenotype. BL performed most of the experiment and wrote the manuscript. All authors reviewed and approved this submission.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standard The experiments in this study comply with the current laws of China.

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