Fine-mapping and identification of a candidate gene controlling seed coat color in melon (Cucumis melo L. var. chinensis Pangalo)

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Abstract

Key message MELO3C019554 encoding a homeobox protein (PHD transcription factor) is a candidate gene that involved in the formation of seed coat color in melon.

Abstract Seed coat color is related to flavonoid content which is closely related to seed dormancy. According to the genetic analysis of a six-generation population derived from two parents (IC2508 with a yellow seed coat and IC2518 with a brown seed coat), we discovered that the yellow seed coat trait in melon is controlled by a single dominant gene, named CmBS-1. Bulked segregant analysis sequencing (BSA-Seq) revealed that the gene is located at 11,860,000–15,890,000 bp (4.03 Mb) on Chr 6. The F2 population was genotyped using insertion–deletions (InDels), from which cleaved amplified polymorphic sequence (dCAPS) markers were derived to construct a genetic map. The gene was then fine-mapped to a 233.98 kb region containing 12 genes. Based on gene sequence analysis with two parents, we found that the MELO3C019554 gene encoding a homeobox protein (PHD transcription factor) had a nonsynonymous single nucleotide polymorphism (SNP) mutation in the coding sequence (CDS), and the SNP mutation resulted in the conversion of an amino acid (A→T) at residue 534. In addition, MELO3C019554 exhibited lower relative expression levels in the yellow seed coat than in the brown seed coat. Furthermore, we found that MELO3C019554 is related to 12 flavonoid metabolites. Thus, we predicted that MELO3C019554 is a candidate gene controlling seed coat color in melon. The study lays a foundation for further cloning projects and functional analysis of this gene, as well as marker-assisted selection breeding.

Introduction

Seed coat color is an essential horticultural trait in melon. An earlier study revealed that seed coat color is related to antioxidant quantities and activities, as well as the biochemical characteristics of the seed (Malencic et al. 2012). Melons possess diverse seed coat colors. While yellow and brown seed coats are common in melon, white, yellowish-white, dark brown, reddish-brown, and other seed coat colors are also found. Flavonoids related to seed coat color formation, as well as the associated molecular mechanisms thereof, have been identified in Arabidopsis and the Brassicaceae family (Debeaujon et al. 2000; Li et al. 2010; Padmaja et al. 2014). Proanthocyanidin (PA), one type of flavonoid, was confirmed to be associated with seed coat color formation in earlier research (Dixon et al. 2005; Lepiniec et al. 2006). PAs are end products in the flavonoid biosynthesis pathway and have protective effects against pathogens (Winkel-Shirley 2001; Dixon et al. 2005).

There are far more studies on seed coat color in Arabidopsis, the Brassicaceae family, soybean, and other species than in melon. Research has reported that different genes control the formation of seed coat color in different species. In Arabidopsis, several genes related to the flavonoid biosynthesis pathway were found to be associated with seed coat color, including chalcone synthase, chalcone isomerase, dihydroflavonol reductase (DFR), and other genes (Yu 2013). In soybean, GmF3’H, which encodes flavonoid 3’ hydroxylase, is involved in the flavonoid pathway and results in pigmentation of the seed coat (Zabala and Vodkin 2003). In addition, seed coat color is also regulated by some
transcription factors. BrTT8 encodes a basic/helix-loop-helix (bHLH) transcription factor related to the accumulation of PAs, and a large insertion in BrTT8 is responsible for the yellow seed coat in *Brassica rapa* (Li et al. 2012). Ren et al. (2017) and Wang et al. (2016) found that TTG1 was the most likely candidate gene regulating seed coat color in *Brassica rapa*. In *Arabidopsis*, genes encoding PAP1, GL3, FUSCA3, ANL2, KAN4, and other transcription factors were identified that control seed coat color formation (Qu et al. 2013; Padmaja et al. 2014). Other genes related to seed coat color formation have been discovered. In *Brassica juncea*, Huang et al. (2016) confirmed that Bra036828 is likely linked to yellow seed coat formation. The gene encoding polyphenol oxidase (PPO) involved in black pigment synthesis was found to be a candidate gene of seed coat color in sesame (Wei et al. 2016). Cla019481, which encodes PPO, is the candidate gene for the formation of a black seed coat in watermelon (Li et al. 2019).

Melon (*Cucumis melo* L.) is an essential vegetable crop in the Cucurbitaceae family. Following the release of the melon genome (Garcia-Mas et al. 2012; Ruggieri et al. 2018), the associated sequence information has been employed in marker development, fine-mapping, and gene cloning. Some markers tightly linked to peel spot (Lv et al. 2018), chilling tolerance (Hou et al. 2018), gummy stem blight (GSB) resistance (Hassan et al. 2018), male sterility mutant (Sheng et al. 2017), sweet and sour (Zhang et al. 2016), and other agronomic traits have been developed in melon. Genes controlling cucumber mosaic virus resistance (Giner et al. 2017), GSB resistance gene (Hassan et al. 2018), fruit shape (Gur et al. 2017), fruit firmness (Nimmakayala et al. 2016), and other important traits have been mapped and cloned in melon.

In this study, we used six-generation populations derived from IC2508 (yellow seed coat) and IC2518 (brown seed coat) to investigate the inheritance of melon seed coat color. We used bulked segregant analysis sequencing (BSA-Seq) and constructed a genetic map with InDels and dCAPS markers from the F2 population to map the candidate gene. Based on gene function annotation analysis, sequence alignment, and quantitative real-time (qRT)-PCR, we predicted a candidate gene controlling melon seed coat color. The study contributes to the cloning of the *CmBS-1* gene and furthers our understanding of melon seed coat color formation.

**Materials and methods**

**Plant materials**

The inbred line IC2508 (*Cucumis melo* ssp. *agrestis* var. *chinensis*, with yellow seed coat) was the female parent, and the inbred line IC2518 (*Cucumis melo* ssp. *agrestis* var. *chinensis*, with brown seed coat) was the male parent (Fig. 1). The seed coats of IC2508 and IC2518 at 30 days after pollination were used to measure the flavonoid content and were subjected to flavonoid metabolomic analysis. The *F1* generation generated by crossing IC2508 and IC2518 was self-pollinated to produce the *F2* population. The *F1* (as the female) was backcrossed with IC2508 and IC2518 to obtain BC1P1 and BC1P2. To validate the genetic inheritance and map the gene, in the 2018 spring season, we planted 50 *P1*, 50 *P2*, 50 *F1*, 258 *F2*, 98 BC1P1, and 95 BC1P2 individuals. In the 2019 spring season, we screened the recombinant plants from a larger *F2* population (2262 individuals) to fine-map the target gene. All of materials were grown under greenhouse conditions in the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing. Plants were grown under natural light conditions. The greenhouse was maintained at daily temperatures between 17 and 33 °C; the relative humidity of day/night was about 55/85%.

**Phenotypic analysis**

Self-pollinated seeds of the six-generation family were collected in the mature period, and the seed coat color trait was.

![Fig. 1 Phenotype of seed coat color in the parents. a Mature IC2508 (P1) seed with a yellow seed coat. b Mature IC2518 (P2) seed with a brown seed coat. Bar, 2 cm](image)
analyzed for genetic inheritance. The coat color of mature melon seeds was examined visually. In addition, a colorimeter (Konica Minolta CM-3700D, Osaka, Japan) was used to measurement of F₀ seed coat color. The color parameters include *a*, a greenness to redness parameter, *b*, a blueness to yellowness parameter, *L*, a darkness to lightness parameter. *E*-value = \((a^2 + b^2 + L^2)^{1/2}\). SAS version 9.4 (SAS Institute, Cary, NC, USA) was used to conduct Chi-square tests on the F₁ (252 individuals) and BC₁P₂ (94 individuals) populations.

**Flavonoid content measurement**

We collected the seed coats of IC2508 and IC2518 at 30 days after pollination. Powdered freeze-dried samples (0.1 g) were weighted and extracted overnight at 4 °C with 1.0 mL 70% aqueous methanol. 1 mL extract aliquot (1 mg mL⁻¹) was mixed with 4 mL sterile ddH₂O and subsequently with 0.3 mL of 5% NaNO₂. The mixture was placed for 6 min, mixed with 0.3 mL of 10% AlCl₃, and stand for 6 min. Four milliliters of 4% NaOH was added to the mixture, and sterile ddH₂O was added to the mixture up to 10 mL. After mixed thoroughly, stand for 15 min. Absorbance of the mixture was determined at 510 nm against the water blank. We used rutin as the standard compound for the quantification of total flavonoids, and the results were expressed as rutin equivalents, with three biological and technical replicates measured per sample, respectively (Saravanan and Parimelazhagan 2014).

**Extraction and analysis of flavonoid metabolites**

Metabolites were extracted from the seed melon coats of IC2508 and IC2518 at 30 days after pollination, with three replicates per sample. Freeze-dried samples were used in our experiment for metabolome analysis. 0.1 g powder was extracted overnight at 4 °C with 1 mL 70% aqueous methanol. An ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI–MS/MS) system (UPLC, SHIMADZU CBM 30A, www.shimadzu.com.cn; MS, Applied Biosystems 6500 Q TRAP, www.appliedbiosystems.com.cn/) was used to analyze the extracts. We used a combination of the variable importance in the projection (VIP) score extracted from the orthogonal partial least squares-discriminant analysis (OPLS-DA) and absolute Log2FC (fold change) to determine the significant differential metabolites, with a screening criteria of VIP ≥ 1 and Log2FC ≥ 1 (Thévenot et al. 2015; Chong et al. 2018). We used SnpEff software (Cingolani et al. 2012) to annotate the identified metabolites using the Kyoto Encyclopedia of Genes and Genomes (KEGG) compound database (http://www.kegg.jp/kegg/compound/) and then mapped the annotated metabolites to the KEGG pathway database (http://www.kegg.jp/kegg/pathway.html) (Kanehisa and Goto 2000). Additionally, we fed the pathway into MSEA (metabolite sets enrichment analysis), with significance determined by the p-values of the hypergeometric test (Gong et al. 2013; Chong et al. 2018).

**DNA extraction**

A modified cetyltrimethyl-ammonium bromide (CTAB) method (Murray and Thompson 1980) that with the following modifications: 2% CTAB buffer (added 2% β-mercaptoethanol), washing with 70% ethanol twice, and dissolving the DNA pellet in 50 μL sterilized ddH₂O was applied to extract total genomic DNA from the young leaves of parents, F₁ individuals, F₂ individuals, and two bulks. The two bulks were constructed with 30 yellow seed coat F₂ individuals and 30 brown seed coat F₂ individuals. The DNA concentration was quantified using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and was diluted to 30–50 ng μl⁻¹. Electrophoresis in 1.0% agarose gel was used to evaluate the DNA.

**BSA-Seq and resequencing analysis**

The DNAs of IC2508, IC2518, the yellow bulk, and the brown bulk were extracted for resequencing on an Illumina HiSeq 2000 platform. The melon genome version 3.5.1 was used as the reference genome (https://melonomics.net/files/Genome/Melon_genome_v3.5.1/). Clean reads were obtained by removing the raw reads that contain adaptor sequence, reads with more than 10% N (unknown base type), and low-quality reads (bases of Q < 10 that were more than half of the entire read length). Clean reads were aligned to the melon reference genome using Burrows Wheeler Aligner (BWA, 0.7.12-r1039) with default parameters (Li and Durbin 2009; Table S1). The single nucleotide polymorphisms (SNPs) and insertion–deletions (InDels) were detected by the genome analysis toolkit (GATK, McKenna et al. 2010). This process included eliminating the influence of PCR duplication with the Mark Duplicates tool (http://sourceforge.net/projects/picard), followed by local realignment (to correct the mistakes in base alignment), base recalibration, variant calling (SNPs and InDels), and strictly SNP/InDel filtration (SNP cluster: if the distance between a SNP and InDel < 5 bp; if two SNPs were in 5 bp; InDel cluster: if the distance between 2 InDels < 10 bp). We compared the SNPs and InDels between samples with a Venn diagram and used SnpEff software (Cingolani et al. 2012) to annotate the localization of SNPs and InDels. Euclidean distance (ED) (median + 3SD as the threshold), ΔSNP index (99% quantile as the threshold), and ΔInDel index (95% quantile as the threshold) values were calculated to obtain a preliminary candidate interval associated with the seed coat color trait.
and the other analysis used default parameters. The Kosambi’s mapping function with QTL Icimapping 4.1 software (Meng et al. 2015) to morphic primers linked to the
CmBS-1 locus were analyzed marked as ‘u’. The segregation data associated with poly-
meric primers were then used to identify the geno-
types of the brown seed coat color individuals from the F₂ population.

The 10 μL PCR reaction mixture contained 1 μL DNA, 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), 3 μL ddH2O, and 5 μL PCR mix and was per-
formed under the following conditions: an initial 94 °C for 5 min; followed by 35 cycles at 94 °C for 15 s, 54 °C for 15 s, and 72 °C for 30 s; with a final extension at 72 °C for 6 min; followed by 4 °C. The dCAPS marker repeated the above steps, and the corresponding restriction endonucleases were added to the PCR-amplified products to digest the PCR products following the manufacturer’s instructions. Ampli-
cons were separated by 8% polyacrylamide gel electropho-
resis at 150 V for 2–2.5 h and then sliver-stained.

**Construction of genetic map**

The individuals of 252 F₂ planted in 2018 and 2262 F₂ planted in 2019 were used to construct the fine map of the CmBS-1 locus. For each polymorphic primer, individuals with the IC2508 allele were marked as ‘a,’ individuals with the IC2518 allele were marked as ‘b,’ individuals with the F₁ allele were marked as ‘h,’ and the unknown bands were marked as ‘u’. The segregation data associated with poly-
morphic primers linked to the CmBS-1 locus were analyzed with QTL Icimapping 4.1 software (Meng et al. 2015) to construct the genetic map. The Kosambi’s mapping function was used to count genetic distances (cM) (Kosambi 1944), and the other analysis used default parameters.

**Functional annotation and sequence analysis of the candidate gene**

The functional annotation and sequence analysis of the genes in the mapping region were based on the melon (DHL92) genome (https://melonomics.net/files/Genome/Melon_genome_v3.5.1/). Amino acid sequences were predicted using the ExPAsy-Translate tool (https://web.expasy.org/translate/). The gene and amino acid sequences were aligned using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and ClustalW in MEGA 7 software.

**Extraction of RNA and qRT-PCR**

Five cultivated agrestis (B₁–B₅) are all Chinese landraces from the group C. melo ssp. agrestis var. chinensis with brown seed coat color; they were ‘Hongmei’ (B₁), ‘Huang-
mibao’ (B₂), ‘Hongzihuangmigua’ (B₃), ‘Hongzikui’ (B₄) and ‘Hongzixiangwang’ (B₅). Five inbred lines (Y₁–Y₅) are all from the group C. melo ssp. agrestis var. chinensis with yel-
low seed coat color. They were planted in the autumn sea-
on of 2019 to measure the relative expression of the target gene using qRT-PCR. The mature seeds of the 10 melon accessions are shown in Fig. S2. The specific primers of the candidate gene and Actin for qRT-PCR were designed using Primer 5.0 software based on the melon genomic database (Table S2). Total RNA was extracted from the melon seed coat tissue using an RNAprep Pure Plant Kit (TianGen, China). We used a PrimeScript RT Reagent Kit (TaKaRa, Japan) to prepare cDNA. The SYBR Primer Ex Taq Kit (TaKaRa, Japan) was used to conduct the qRT-PCR. The amount of DNA was monitored using the StepOne Plus Real-Time Fluorescent Quantitative PCR system (ABI, Foster City, CA, USA). Three biological replicates and five technical replicates of each sample were used for qRT-PCR. The 2−(ΔΔCt) method was used to evaluate the quantification.

**Phylogenetic analysis**

The homologous protein sequence obtained by a BLAST search with default settings in the NCBI database (https://www.ncbi.nlm.nih.gov/). The ClustalW in MEGA 7 software was used to align the multiple sequence. We con-
structed the neighbor-joining phylogenetic tree in MEGA 7 software using the bootstrap method with 1000 replications (Kumar et al. 2016), and other parameters used defaults.

**Results**

**Determination of flavonoid content**

The flavonoid contents of the mature seed coat samples of the two parents were measured. The result showed that the flavonoid content of the yellow seed coat was 3.21 mg g⁻¹, while that of the brown seed coat was 8.09 mg g⁻¹ (Fig. 2). The flavonoid content of the brown seed coat was 1.5-fold higher than that of the yellow seed coat. The result revealed that melon seed color was related to flavonoid content in seed coat.
Genetic analysis of melon seed coat color

The seed coat color of all 50 individuals in the F₁ population was yellow, indicating that the yellow seed coat trait is dominant over the brown seed coat trait. In the F₂ population (252 individuals), 186 individuals exhibited a yellow seed coat and 66 individuals produced a brown seed coat. The Chi-square test suggested a 3:1 segregation ratio (Table 1). The analysis of E-value showed that the seed color of F₂ population conformed to bimodal distribution (Fig. S1). In the BC₁P₂ (F₁ × P₂) population (94 individuals), 56 individuals showed a yellow seed coat and 38 individuals showed a brown seed coat. The Chi-square test suggested that the segregation ratio was 1:1. The seed coat color of all 97 BC₁P₁ (F₁ × P₁) individuals was yellow (Table 1). The results confirmed that the yellow seed coat trait was controlled by a single dominant gene, which was named CmBS-1.

Preliminary mapping of CmBS-1 by BSA-Seq

We used BSA-Seq to rapidly identify the genomic region of CmBS-1. A total of 60.06 Gb clean reads were generated by re-sequencing, with an average sequencing depth of 33.37 × . The clean reads were aligned to the melon reference genome, and the coverage of the reference genome was 98.02%. A total of 123,456 SNPs and 49,004 small InDels were identified between the two parents, while 30,950 SNPs and 13,894 small InDels were identified between the two DNA bulks. The details of the SNPs and InDels between the two bulks as well as the parents are shown in Tables S3 and S4. We used the ED method (the threshold was 0.29) and ΔSNP index (the confidence value was 99%) to analyze the SNPs and found that CmBS-1 was mapped to a 4.03 Mb region from 11,860,000 to 15,890,000 bp on Chr 6 (Fig. 3). Similarly, we used the ED method (the threshold was 0.30) and ΔInDel index (the confidence value was 95%) to analyze the small InDels, and the result suggested that CmBS-1 was mapped to an interval of 11,770,000–16,270,000 bp (4.50 Mb) on Chr 6 (Fig. 4). The two regions overlapped. Therefore, we confirmed that CmBS-1 was located at the merged region from 11,860,000 to 15,890,000 bp on Chr 6 at a physical distance of 4.03 Mb.

Fine-mapping of CmBS-1 using InDel and dCAPS markers

In the preliminary mapping region, based on the re-sequencing data, we designed 1498 InDel primers and randomly chose 240 InDel primers to screen in the two parents and the two bulks to obtain polymorphic primers. Fifty-two of the 240 primers were polymorphic, and the polymorphism rate was 21.7%. We then chose eight co-dominant polymorphic markers that had clear bands and demonstrated stable amplification to map CmBS-1 in a smaller F₂ population (252 individuals) with 12 recombinant individuals (Tables S5 and S6). The result suggested that CmBS-1 gene was mapped in a 970.81 kb interval between M255 and L274 (11,109,147–12,079,958 bp).

In the interval of M255 and L274, we designed 35 InDel markers and two dCAPS markers based on the re-sequencing data, and eight of them were polymorphic markers. We then used the eight co-dominant polymorphic markers to fine-map the CmBS-1 in a larger F₂ population (2262 individuals) with 10 recombinant individuals (Tables S5 and S6).

Table 1 Genetic analysis of seed coat color trait in six-generation family

| Generation | Total no. of individuals | No. of yellow individuals | No. of brown individuals | Expected ratio | χ² (α=0.05) | p-value (α=0.05) |
|------------|--------------------------|--------------------------|--------------------------|---------------|-------------|--------------|
| P₁         | 50                       | 50                       | 0                        | –             | –           | –            |
| P₂         | 50                       | 0                        | 50                       | –             | –           | –            |
| F₁         | 50                       | 50                       | 0                        | –             | –           | –            |
| F₂         | 252                      | 186                      | 66                       | 3:1           | 0.1905      | 0.6625       |
| BC₁P₁      | 97                       | 97                       | 38                       | 1:1           | 3.4468      | 0.0634       |
| BC₁P₂      | 94                       | 56                       | 38                       | 1:1           | –           | –            |

Fig. 2 The flavonoid content of the two parental lines. Error bars represent the standard error of three independent biological replicates. Letter above the bars represent significant differences compared with each other using Student’s t-test (p < 0.05)
The result indicated that SNP2 and ZH39 were the closest markers to CmBS-1. The physical distance between the two markers was 233.98 kb (11,763,814–11,997,798 bp). We used QTL Icimapping 4.1 software to construct the genetic map with 16 markers (Fig. 5a).

**Prediction and sequence analysis of the candidate gene**

Based on the melon genome database, 12 genes were identified in the 233.98 kb region. The position and functional annotation of these genes are shown in Table 2. We analyzed the sequences of the 12 genes in the candidate region based on the parental re-sequencing data. The gene sequence alignment of the 12 genes between the two parental lines in the yellow seed coat and brown seed coat parental lines revealed that one nonsynonymous SNP and two InDel mutations were present in MELO3C019554. We found that both of the two InDel mutations were in the intron region at 11,965,686 bp (TA → T) and 11,966,210 bp (G → GA) on Chr 6, respectively (Table S7). The one nonsynonymous SNP was in the fifth exon region at 11,967,142 bp (C → T) on Chr 6, resulting in the conversion of an alanine (A) to threonine (T) at residue 534 (Fig. 5b–d; Table S7). However, we found that there were no mutations in exon of the other 11 genes between the two parental lines. The promoter sequence analysis of the 12 genes between the two parental lines showed that there were no mutations in any of the 12 genes. According to the melon genome database, MELO3C019554 encodes a homeobox protein that is a PHD transcription factor. Therefore, we predicted that MELO3C019554 was the candidate gene for the yellow seed coat and brown seed coat in melon.

**Expression analysis for verifying the candidate gene**

To verify the candidate gene, we performed expression analysis of MELO3C019554 using qRT-PCR in the two parents, F₁, and 10 melon germplasms with different seed

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Fig. 3 Mapping of CmBS-1 based on the application of the ED/ΔSNP index to SNP. a ED method of SNPs between IC2508, IC2518, the yellow bulk, and the brown bulk. The black line is the fitted ED value, and the red dotted line is the significance association threshold (0.29). b ΔSNP index analysis of SNPs between IC2508, IC2518, yellow bulk, and brown bulk. The black line is the fitted ΔSNP index, and the red, blue, and green lines represent the thresholds of 99%, 95%, and 90%, respectively.
coat colors. As indicated in Fig. 6a, the relative expression level of MELO3C019554 in the seed coat of IC2508 and F₁ was significantly lower than that in the seed coat of IC2518 at 5, 10, 15, and 20 d after pollination, while the expression level did not differ significantly between P₁(F₁) and P₂ on the other days. In IC2508, IC2518, and F₁, the relative expression level of MELO3C019554 was highest at the color accumulation stage (10 d after pollination). Thus, we analyzed the relative expression of MELO3C019554 using qRT-PCR in five yellow seed coat melon germplasms and five brown seed coat melon germplasms at 10 d after pollination. The result showed that MELO3C019554 exhibited significantly higher relative expression in the brown seed coat melon germplasms than in the yellow seed coat melon germplasms (Fig. 6b). Within the same seed coat color, the differing expression level may be due to the different shades of the coat (Fig. S2). MELO3C019554 exhibited different expression levels in the seed coat, suggesting that it may be a candidate gene in regulating melon seed coat color.

ΔInDel index analysis of InDels between IC2508, IC2518, yellow bulk, and brown bulk. The black line is the fitted ΔInDel index, and the red, blue, and green lines represent thresholds of 99%, 95%, and 90%, respectively.

The candidate gene is related to flavonoid metabolites

We analyzed flavonoid metabolites of the two parental lines (IC2508 and IC2518). A total of 19 significantly differentially abundant metabolites were detected between mature seed coat samples of IC2508 and IC2518, including anthocyanins, flavonols, flavonoids, chalcones, and flavonoid carbonosides (14 metabolites upregulated and 5 metabolites downregulated in IC2518; Table S8). KEGG enrichment analysis showed that the differentially expressed metabolites were mainly involved in biosynthesis of secondary metabolites (ko01110), metabolic pathways (ko01100), flavone and flavonol biosynthesis (ko00944), anthocyanin biosynthesis (ko00942), and flavonoid biosynthesis (ko00941) (Table S8). The results of flavonoid metabolites profiling fell in line with the results of total flavonoid content, which suggested that the flavonoid content was responsible for the difference in the seed coat color.
Fig. 5 Genetic mapping of the seed color gene and analysis of the candidate gene in melon. a The genetic map constructed using the F₂ population. b The 12 genes in the candidate region. c The structure of MELO3C019554. d The SNP mutation (G→A) resulted in the conversion of the amino acid (A→T) at residue 534

Table 2 Annotation of genes in the candidate region

| Gene ID      | Position                  | Functional annotation                                      |
|--------------|---------------------------|------------------------------------------------------------|
| MELO3C019545 | 11,771,441-11,774,714 (− strand) | Reticulon-like protein                                     |
| MELO3C019546 | 11,775,819-11,778,327 (− strand) | PRKR-interacting 1                                        |
| MELO3C019547 | 11,783,292-11,783,498 (− strand) | MGDG synthase type A family protein                        |
| MELO3C019548 | 11,851,782-11,853,751 (− strand) | Alcohol dehydrogenase, putative                           |
| MELO3C019549 | 11,868,402-11,868,584 (− strand) | Galactose oxidase/kelch repeat superfamily protein         |
| MELO3C019550 | 11,892,173-11,895,412 (+ strand) | Methylthioribose kinase 1                                  |
| MELO3C019551 | 11,897,392-11,897,999 (− strand) | FAD-binding Berberine family protein                       |
| MELO3C019552 | 11,900,852-11,902,620 (+ strand) | Coffea canephora DH200=94 genomic scaffold, scaffold_1     |
| MELO3C019553 | 11,924,765-11,929,982 (+ strand) | Alpha-L-fucosidase 2                                       |
| MELO3C019554 | 11,955,708-11,969,635 (− strand) | Homeobox protein                                           |
| MELO3C019555 | 11,956,237-11,956,524 (+ strand) | Zinc finger protein 1                                      |
| MELO3C019556 | 11,972,549-11,973,087 (− strand) | DNA polymerase epsilon catalytic subunit A                 |
protein was highly conserved in the Cucurbitaceae family. The sequence alignment analysis suggested that the region with the amino acid mutation (A → T) at residue 534 was conserved in the homologs (Fig. 7b).

Discussion

We measured the flavonoid content of the two parental lines. The result showed that the flavonoid content of IC2518 (brown seed coat) was significantly higher than that of IC2508 (yellow seed coat), which revealed that melon seed coat color is related to flavonoid content. In Arabidopsis and Brassica juncea, it has been verified that flavonoid content causes a difference in seed coat color (Dixon et al. 2005; Lepiniec et al. 2006; Huang et al. 2016). In plants, flavonoids play an important role as developmental regulators and signaling molecules (Lee et al. 2018). In addition, flavonoids enhance insect and disease resistance, as well as abiotic stress tolerance (Fukushima et al. 2017; Tu et al. 2018; Dai et al. 2019; Zhao et al. 2019). The brown seeds accumulated more flavonoids than the yellow seeds, and therefore the brown seeds may show higher resistance to insects, diseases, and other stresses than the yellow seeds.

In traditional forward genetics research, it is necessary to develop many markers and use a sufficiently large population to obtain quality experimental results (Zhu et al. 2017). In our study, the parents had a close affinity, which made it difficult to obtain enough polymorphic markers. Thus, we took advantage of the parents and the resequencing data of the two bulked populations to detect InDels and SNPs, from which we developed abundant markers. BSA-Seq was used to rapidly identify the preliminary region of CmBS-1. With the development of sequencing technology, resequencing and BSA-Seq have promoted gene mapping and cloning studies (Yang et al. 2017). For instance, using whole-genome resequencing BSA, Lv et al. (2018) fine-mapped the spotted to nonspotted trait gene in melon. Huang et al. (2017) used BSA and resequencing to fine-map the gene controlling nonspotted trait gene in melon. Huang et al. (2017) used BSA and resequencing to fine-map the gene controlling sparse panicle in Setaria viridis, while Zhu et al. (2017) performed BSA-Seq and virus-induced gene silencing strategies in cotton to fine-map and clone the virescent-1 gene. Song et al. (2017) used next-generation sequencing-based bulked segregant analysis to rapidly map two qualitative genes that control cotyledon color in soybean, and the result showed that BSA-Seq could also accelerate the mapping of more than one locus controlling a trait.

Based on BSA-Seq and genotyping, we mapped the CmBS-1 gene in a 233.98-kb candidate region on Chr 6, which contained 12 predicted genes. We detected a nonsynonymous SNP mutation that resulted in the change of an amino acid residue in the fifth exon region of the candidate gene MELO3C019554. Further gene function annotation and
relative expression analysis showed that MELO3C019554 encoded a PHD transcription factor, which may play a vital role in the seed coat color accumulation stage. We found that the relative expression of MELO3C019554 in the brown seed coat color was higher than in the yellow seed coat color using qRT-PCR. Although there were no mutations in the promoter of the candidate gene, we found two InDel mutations in the intron region of the candidate gene. There were certain cases revealed that introns had ability to regulate gene expression as internal promoters (Furger et al. 2002; Morello et al. 2002; Samadder et al. 2008). In addition, the stability of mRNA was related to introns. Introns give rise to the half-life of the transcript by enhancing the pre-mRNA 3’ end polyadenylation and processing (Lu and Cullen 2003; Millevoi et al. 2009).

In early research, genes encoding transcription factors were found to control the formation of seed coat color. For instance, Chandler et al. (1989) identified the first bHLH transcription factor regulating the anthocyanin biosynthesis pathway in maize seed. A further study then

Fig. 7 Phylogenetıc and alignment analysis of the candidate gene. a The phylogenetıc tree of the protein encoded by MELO3C019554. The protein is indicated by a red quadrate. b The sequences alignment analysis of the candidate gene and its homologs. The mutate location is highlight by a red box.
confirmed that the bHLH protein controls PA biosynthesis in the pericarp in rice (Sweeney et al. 2006). Nesi et al. (2000) revealed that the bHLH protein TT8 was essential for the expression of two flavonoid late-biosynthetic genes, including Dihydroflavonol 4-reductase (DFR) and BANYULS (BAN), and TT8, TT2, and Transparent TESTA GLABRA1 (TTG1) were also found to control flavonoid metabolism through forming a ternary complex in the Arabidopsis seed coat. In soybean, Glyma09g36990 encoding an R2R3 MYB transcription factor was the candidate gene for the brown seed coat phenotype, and its gene expression was closely related to an UDP-glucose: flavonoid 3-O-glucosyltransferase (UFGT) gene involved in the final step of anthocyanin biosynthesis (Gillman et al. 2011). Hong et al. (2017) identified one bHLH gene and two MYB-related genes as seed coat color candidate genes in Brassica napus. In cowpea, MYB domain proteins control the expression of genes that encode enzymes in anthocyanin biosynthesis, and the genes encoding MYB transcription factors were identified as candidate genes associated with a black seed coat (Herníter et al. 2018). The PHD transcription factor has been proved to be involved in abiotic stress, seed germination, and male sterility in different species. Wei et al. (2009) found six GmPHD genes that encode PHD finger proteins that respond to cold, salt, drought, and abscisic acid stress. PHD-domain proteins regulate seed germination and early seedling growth in Arabidopsis (Molitor et al. 2014). Ye and Zhao (2016) revealed that the gene encoding the PHD protein was responsible for seed germination inhibition. Thu et al. (2019) fine-mapped the male-sterile gene and identified Glyma.02G243200, which encodes a PHD protein, as the candidate gene in soybean. Currently, no research has suggested that the PHD transcription factor is responsible for seed coat color formation. In our study, the genes regulated by the PHD transcription factor and the mechanism by which the PHD transcription factor controls seed coat color were unclear and thus need further exploration.

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**Author contribution statement** HW and QF conceived and designed the research, ZH, JZ and AZ performed the experiment, ZH, XS and XC analyzed the data. ZH, HW and QF wrote the manuscript. All authors read and approved the manuscript.

**Declarations**

**Conflict of interest** The authors declare no conflict of interest.

**Ethical approval** The authors declare that the study complies with the current laws of China.

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