

Article

Bulked Segregant Analysis by Sequencing-Based Genetic Mapping of the Green Spotted Fruit Rind Regulating Locus in Wild Melon XNM020 Reveals Four Possible Candidate Genes

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Abstract: Fruit rind patterns are vital commercial quality traits in melon, in which the spotted or striped fruit rinds contribute to the commercial value of melon and can directly affect the choice of consumers. Although the spotted or non-spotted fruit rind pattern was studied in several cultivated melon accessions, the inheritance and regulating locus of this trait in wild melons are still unknown. Therefore, in this study, the inheritance and regulating loci of the green spotted fruit rind in a wild melon accession XNM020 were explored with F₂ segregating populations derived from crossing between XNM020 and a cultivated melon XNM125. Segregating ratios of phenotypic data indicated that the green spotted fruit rind in XNM020 has a monogenic dominant inheritance. BSA-Seq showed that two potential genomic regions on chromosomes 4 (from 0.00 to 2.97 Mb) and 5 (from 0.00 to 2.34 Mb) regulate the formation of the green spotted fruit rind in wild melon XNM020. According to the annotations of polymorphic SNPs (single-nucleotide polymorphisms) and small InDels (insertions and deletions) in target genomic regions and the predicted gene functions, four genes *MELO3C003316*, *MELO3C003375*, *MELO3C003388*, and *MELO3C014660* regulating chloroplast development or chlorophyll biosynthesis may be the best candidate genes. The results of this study enriched the inheritances of spotted fruit rinds in melon and also provided target genomic regions for marker-assisted selection breeding of melon focusing on fruit rinds.

Keywords: melon; spotted fruit rind; inheritance; BSA-Seq



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1. Introduction

Melon (*Cucumis melo* L., $2n = 2x = 24$), an important vegetable crop of the Cucurbitaceae family, is planted in over 100 countries for its nutrient-rich fruit. The total world production of melons was over 26 million tons in recent years and reached 28.56 million tons in 2022 (FAO, www.fao.org/faostat, accessed on 1 May 2024), of which China is the leading producer (14.25 million tons in 2022). Historical records indicate that melon has been cultivated for at least 4000 years [1]. During this long-term cultivation, melon evolves enormous genetic diversity for many horticultural traits such as fruit size and shape, fruit rind color and pattern, flesh thickness and aroma, and biotic and abiotic stress resistance [2]. According to the morphological differences in these traits, melons can be classified into different groups. For example, melon has been categorized into two subspecies, *C. melo* ssp. *melo* and *C. melo* ssp. *agrestis*, based on differences in ovary pubescence [3]. These things considered, compared with the ssp. *agrestis*, the ssp. *melo* fruits usually have thicker flesh and higher sugar content while its plants exhibit more vigorous vegetative growth [4].

Both the *ssp. melo* and *ssp. agrestis* melons can be further classified into several subgroups according to their geographic origins and morphological traits, of which fruit external appearances including rind colors and patterns play essential roles.

Melon fruit has abundant genetic diversity for its rind color and pattern [1]. In melon, the rind color can be light green, green, yellow, white, orange, and gray or a mixture of these colors, and the rind pattern can be striped or non-striped, spotted or non-spotted, mottled or non-mottled, and netted or non-netted. In addition, the colors of the rind pattern can also vary from light yellow, yellow, and dark yellow to light green, green, and dark green [5]. These diverse colors and patterns are determined mainly by the pigments such as chlorophylls, carotenoids, and flavonoids accumulated in melon fruit rinds at different development stages. For immature melon fruits, usually bearing either light or dark green rinds, the primary involved pigment is chlorophyll (both a and b); however, for those mature melon fruits, showing more diverse rind colors such as green–yellow, canary-yellow, and orange, the contributing pigments mainly are carotenoid and flavonoid [6–8]. For example, the yellow mature rind color of ‘Noy Amid’ melon is caused by the accumulation of naringenin chalcone (one type of flavonoid); the young ‘Dulce’ melon fruit rind accumulates both chlorophyll and carotenoids, but while the chlorophyll content decreases during fruit ripening, carotenoid level increases, resulting in a green–orange-colored mature fruit rind [6].

Given the importance of fruit rind color and pattern, numerous studies have been performed to reveal their genetic basis in melons. For fruit rind colors of melons, both monogenic and polygenic inheritances, depending on the used segregating populations and their parental lines, were reported by researchers [2,9–13]. For the monogenic inheritance, a single dominant gene determining dark young fruit exterior color in the segregating populations of ‘Krymka’ × ‘Eshkolit Ha’ Amaqim’ was reported [11]. In addition, Feder et al. [7] revealed that a single gene *CmKFB*, encoding Kelch domain-containing F-box protein, negatively regulates the naringenin chalcone accumulation, which results in the yellow fruit rind of casaba muskmelon [7]. Whole-genome linkage analysis using two segregating populations found that multiple independent single-nucleotide polymorphisms in the *CmAPRR2* gene encoding a two-component response regulator-like protein APRR2 are causative of the light rind in melons [13]. For the polygenic inheritance, Monforte et al. [10] proposed four loci (*ecol3.1*, *ecol7.1*, *ecol9.1*, and *ecol10.1*) with epistatic interactions that may control the external color of melons. Using the RIL population developed from parental lines ‘Védraçais’ and ‘Piel de Sapo’, two (*ECOLQU3.1* and *Wi*) and three loci (*YELLQU5.1*, *YELLQU10.1*, and *YELLQU12.1*) were, respectively, identified for the external color of immature fruit and yellowing of mature rind [12]. Considering this, Zhao et al. [2] identified a 12:3:1 segregation ratio for green, white, and yellow rinds in an F₂ segregating population developed from parental lines MS-723 (green rind) and B432 (yellow rind), indicating that green rind is dominant and epistatic to the non-green (white and yellow) rind.

Melon fruit rind patterns can be netted or non-netted, sutured or non-sutured, and spotted or non-spotted. Relatedly, these patterns are usually covered with different colors such as green, yellow, and orange. As one type of fruit rind pattern, the presence or absence of spots or stripes on fruit rinds is a vital fruit external trait that usually exists in commercial melons and some wild melon germplasm [1]. Inheritances and genetic regulating loci of spots or stripes on fruit rinds were explored by several studies with different bi-parental segregating populations, while some inconsistent results were obtained [8,11,12,14–19]. Both monogenic recessive and dominant loci or genes, depending on the genetic backgrounds of used parental lines, were found for the regulation of green spotted or striped fruit rinds in cultivated melons [11,12,14–16,18,19]. Considering this, the striped fruit rinds controlled by multiple genes were also reported by Harel-Beja et al. [17]. Furthermore, two dominant genes, *CmMt1* and *CmMt2*, with epistatic effect controlling the spotted fruit rinds were revealed in the segregating populations derived from the cross between SC (Songwhan Charmi) and MG (Mi Gua) [8]. In short, the inheritances of spotted or striped fruit rinds in cultivated melons are relatively complex and largely depend on the accessions

or varieties used. Other inherited patterns such as monogenic recessive gene [14–16], multiple genes [17], and two dominant and epistatic genes [8] were also found in some cultivated melons; certainly, new loci regulating spotted or striped fruit rinds in cultivated melons are also formed through different domestications, diversifying selections or genetic interchanges.

During the above genetic studies of fruit rind colors and patterns in melons, BSA-Seq has been used to identify regulated genes or loci, such as *CmAPRR2* identified in Zhao et al. [2] and *CmMt2* reported by Shen et al. [8]. BSA-Seq is a combination of bulked segregant analysis (BSA) and whole-genomic re-sequencing, which takes advantage of both techniques and has largely benefited from advances in next-generation sequencing technology [20]. BSA is a rapid approach for identifying a causal gene or locus underlying a target trait, which was initially proposed by Michelmore et al. [21] and Giovannoni et al. [22]. To quickly uncover the target genomic regions of a trait, BSA adopts the genotyping of two pooled individuals having extreme phenotypes within either segregating or natural populations [21]. As a helpful and powerful alternative to conventional gene mapping methods, BSA-Seq is more efficient and cost-effective, which requires genotyping (sequencing) of only two pooled DNA samples [23–25]. Recently, BSA-Seq has been successfully and widely used for the genetic mapping of several critical horticultural traits in melons, such as powdery mildew resistance [26], fruit pedicel length [27], fruit surface groove (suture or vein track) [2,28], fruit skin netting [29], mottled fruit rind [8], fruit rind color [2], fruit firmness [30], carpel number [31], and seed coat color [32].

Compared with cultivated melons, wild melons usually have small leaves and flowers and thin stems with numerous ramifications. Flowers are monoecious, and fruits are oval or egg-shaped, small (20–50 g) with a very thin, bitter, and inedible flesh surrounding tiny seeds [1]. Wild melon germplasms are valuable genetic resources for the genetic improvement of cultivated melons, especially for biotic and abiotic stress resistances, and are also essential materials to reveal the genetics of melon domestication [33,34]. For fruit rind colors and patterns, the wild melons at maturity are usually light green with dark green spots and have no ribs and vein tracts (also named suture) [1]. The accession XNM020 shows typical characteristics of a wild melon, in which its fruit rind is also covered with dark green spots. Although the spotted or non-spotted fruit rind pattern was studied in several populations derived from cultivated melons, the inheritance and regulating locus of this trait in wild melons have yet to be explored and reported. Therefore, in this study, the inheritance of the green spotted fruit rind was explored with an F_2 segregating population derived from the wild melon accession XNM020 and a cultivated melon XNM125. In addition, the green spotted fruit rind regulating locus *GS* in wild melon XNM020 was also genetically mapped with BSA-Seq.

2. Materials and Methods

2.1. Plant Materials and Morphological Data Collection

Two melon inbred lines, XNM125 and XNM020, were used as parental materials. Among them, XNM125 has uniformed light green and white fruit rinds at immature and mature fruit stages, respectively, while XNM020 is a wild melon and bears green spotted rinds for both immature and mature fruits (Figure 1). The F_1 generation was obtained by crossing XNM125 and XNM020 and has a similar fruit rind with its parental line XNM020 (Figure 1). And then, the F_2 segregating population was derived from the self-crossing of a single F_1 individual. Two parental lines, F_1 and F_2 plants, were grown in the plastic tunnel located in the Horticulture Farm of Northwest A&F University (HF-NWAFU), where cultivation management was performed based on standard practices. The plastic tunnel was maintained at a daily temperature between 15 °C and 33 °C, and the relative humidity of day/night was about 60%/85%. The two parents and F_1 plants were grown in a randomized complete block design consisting of three blocks with 8 plants per plot. Two F_2 populations 2021s_ F_2 with 94 plants and 2023S_ F_2 having 90 individuals were grown

randomly in the spring season (March to June) of 2021 and 2023, respectively. All plants were grown in rows with a 0.6 m row and 0.3 m plant spacing, respectively.

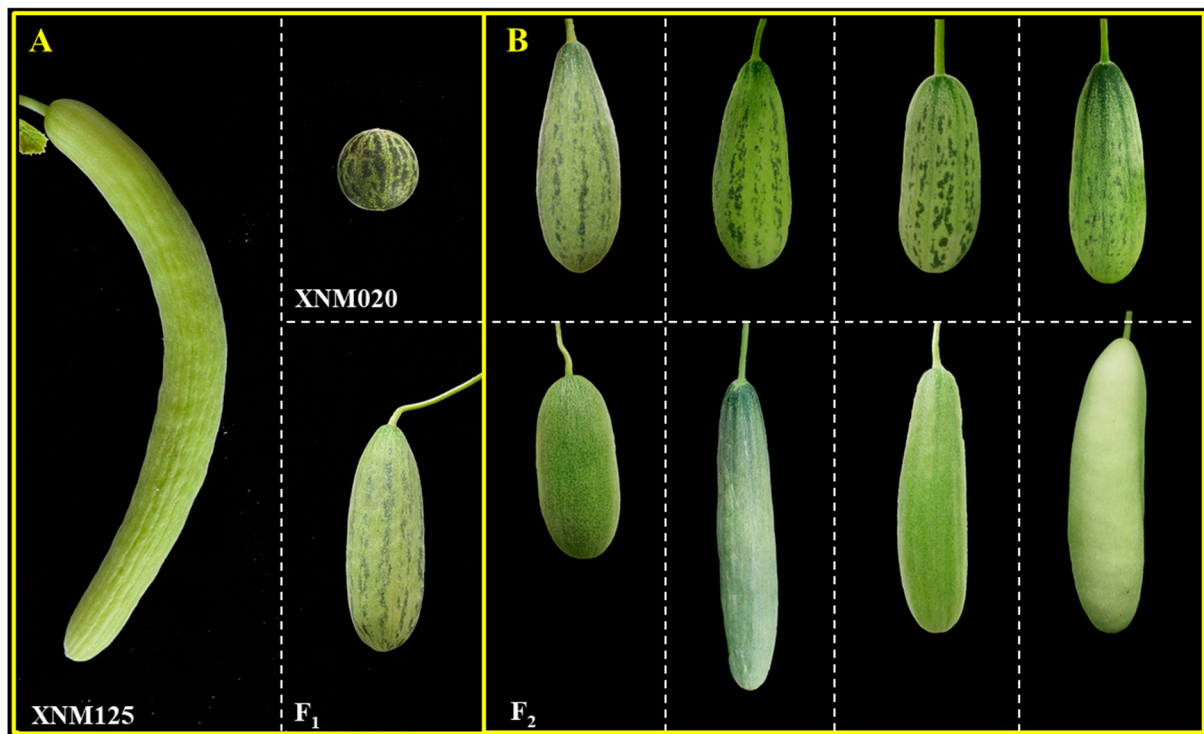


Figure 1. Fruit rinds of two parental lines, F_1 and F_2 individuals. (A) Fruit rind phenotypes of two parents (XNM125 and XNM020) and F_1 . (B) F_2 plants with typical phenotypes.

For each plant, hand pollinations were performed for its female flowers to ensure their good development and growth. The phenotypic data of fruit rinds with green spots or not were recorded at 10 to 15 days after pollination (DAP) by visual observation. Relatedly, each fruit was also photographed for reassessment. Segregation ratios of green spotted or non-spotted fruit rinds in F_2 populations were tested against the expected ratio with Chi-square (χ^2) tests.

2.2. DNA Extraction and Quality Detection

DNA extraction was performed with young leaf tissues of two parental lines, F_1 and F_2 individuals. Firstly, the leaf sample of each plant was collected into a 2.0 mL centrifuge tube, labeled with its plant number, and stored in a refrigerator at -20°C until use. Then, about 0.5 g leaf samples was frozen with liquid nitrogen and ground into fine powder in a high-throughput homogenizer (Tissuelyser-24 produced by Shanghai Jingxin Industrial Development Co., Ltd., Shanghai, China). Lastly, these fine powders were used for genomic DNA extraction following the CTAB method [35]. The quality and quantity of extracted DNA were checked by electrophoresis in 1% agarose gel and measured using NanoDrop 2000 spectrophotometers (Thermo Scientific, Wilmington, DE, USA), respectively. The high-quality DNA of each sample was diluted into 50 ng/ μL with ddH₂O and stored in a 4°C freezer, which was used to construct DNA bulks or as a DNA template for the amplification of polymerase chain reaction (PCR).

2.3. BSA-Seq Analysis

For bulked segregant analysis by sequencing (BSA-Seq), two bulked DNA samples (Spotted_bulk and Non-spotted_bulk) were constructed by mixing equal amounts of DNA from 20 individuals with representative spotted and non-spotted fruit rinds, respectively. Paired-end sequencing libraries (350 bp) were constructed for two parental lines and two

DNA pools following the manufacturer's instructions (Illumina, Hayward, CA, USA). These four DNA libraries were sequenced on an Illumina HiSeq 2500 platform in Biomarker Technologies (Beijing, China) with genome coverages over thirty-fold.

The obtained clean sequencing data were aligned against the reference genome of melon DHL92 (V3.6.1) using the Burrows–Wheeler aligner (BWA) [36]. The alignment results were sorted, and duplicate reads were marked using Samtools [37] and Picard (<http://broadinstitute.github.io/picard/>, accessed on 11 October 2021). Local realignment around InDel regions was performed by InDel-Realigner in GATK [38]. SNPs and InDels were called using the HaplotypeCaller module in GATK. Annotations of SNPs and InDels were performed based on the reference genome using snpEff software (Version 5.2c) [39], in which SNPs were categorized into intergenic regions, upstream or downstream regions, and exons or introns. SNPs in coding exons were further classified as synonymous SNPs or non-synonymous SNPs. InDels in exons were grouped according to whether they led to a frameshift.

To obtain the candidate mapping regions, the genomic regions related to spotted and non-spotted fruit rinds were calculated following Shen et al. [8], and the linkage analysis between phenotypic trait and variation sites was carried out based on the Euclidean distance (ED) algorithm [40], Δ SNP-index, and Δ InDel-index [24], of which, the average SNP-index and InDel-index for the spotted DNA pool and non-spotted DNA pool were calculated using a 1000 kb sliding window with a step size of 100 kb.

2.4. InDel Marker-Based Verification of the Candidate Mapping Region

To quickly verify the mapping regions revealed by BSA-Seq analysis, InDel marker-based traditional linkage analysis was conducted with the F₂-2021S population. Potential polymorphic InDel markers between two parental lines, XNM125 and XNM020, were selected according to the alignment of their re-sequencing data. Primers were designed with online software Primer3web (Version 4.1.0) (<https://bioinfo.ut.ee/primer3%E2%88%920.4.0/>, accessed on 25 December 2021). Sequence information of the used InDel markers is listed in Supplemental Table S1.

Genotyping of the InDel markers was performed with PCR and gel electrophoresis. Briefly, PCR amplifications were carried out in 10 μ L reactions, each containing 2 μ L template DNA (50 ng/ μ L), 0.5 μ L forward primers (5 μ mol/L), 0.5 μ L reverse primers (5 μ mol/L), 2 μ L ddH₂O, and 5 μ L 2 $\times$ Taq PCR Master mix (Tiangen Biotech Co., Ltd., Beijing, China). A touch-down PCR program was used, and the details are as follows: 2 min at 95 $^{\circ}$ C, 20 s denaturing at 94 $^{\circ}$ C, 20 s annealing at 68 $^{\circ}$ C, and 20 s elongation at 72 $^{\circ}$ C, followed by a 2 $^{\circ}$ C reduction in the annealing temperature per cycle for 6 cycles. Then, annealing temperature was reduced in each cycle by 1 $^{\circ}$ C for 8 cycles from 58 $^{\circ}$ C; the annealing temperature was maintained at 50 $^{\circ}$ C for the remaining 20 cycles, followed by a final step at 72 $^{\circ}$ C for 5 min. PCR products were size-fractionated in an 8% polyacrylamide gel at a constant 180 V for 1 h, visualized with silver staining, and photographed with a digital camera, following Li et al. [41]. Linkage analysis of the GS locus with molecular markers was performed with the Kosambi mapping function of JoinMap 4.0 following Qiao et al. [42].

3. Results

3.1. Genetic Analysis of the Green Spotted Fruit Rind in XNM020

The wild melon XNM020 and cultivated melon XNM125 set fruits with green spotted rinds and light green non-spotted rinds, respectively. To reveal the inheritance of green spotted fruit rinds in the wild melon XNM020, phenotypic data of fruit rind colors and patterns from two parental lines, F₁, and two small F₂ populations (2021S_F₂ and 2023S_F₂) are collected and listed in Table 1. All 24 F₁ plants showed green spotted fruit rinds, consistent with the parental line XNM020. For the 2021S_F₂ population with 94 plants, there were 70 and 24 individuals bearing fruits with green spotted rinds and non-spotted rinds, respectively. Similarly, among the 90 plants of the 2023S_F₂ population, 67 individuals

had green spotted fruit rinds and the remaining 23 plants showed non-spotted fruit rinds. Statistical evidence of deviation from the genetic 3:1 segregation ratio was measured with an χ^2 goodness-of-fit test in both F_2 populations (2021S_ F_2 and 2023S_ F_2). As shown in Table 1, the spotted-to-non-spotted-type plants fit well with the expected 3:1 segregation ratio.

Table 1. Genetic analysis of the green spotted fruit rind in XNM125- × XNM020-derived populations.

Population	Total Plants	Spotted Plants	Non–Spotted Plants	Expected Segregation Ratio	χ^2	<i>p</i>
XNM125	24	0	24	-	-	-
XNM020	24	24	0	-	-	-
F_1	24	24	0	-	-	-
2021S_ F_2	94	70	24	3:1	0.014	0.91
2023S_ F_2	90	67	23	3:1	0.015	0.90

Note: $\chi^2 > \chi^2_{0.05} = 3.841$ ($p < 0.05$) is considered significantly different from the expected segregation ratio.

3.2. BSA-Seq Identified Regulating Loci of the Green Spotted Fruit Rind in XNM020 Melon

BSA-Seq was performed to map the green spotted fruit rind in wild melon XNM020. Genomic DNA of two parental lines (XNM125 and XNM020) and two bulks (Spotted_bulk and Non-spotted_bulk) were re-sequenced with an Illumina HiSeq 2500 sequencer. Illumina high-throughput sequencing generated with 15.2, 15.1, 15.6, and 16.6 Gbp clean data for XNM020, XNM125, Spotted_bulk, and Non-spotted_bulk represented approximately 29-, 28-, 28-, and 30-fold genome coverage, respectively (Table 2). The Q30 values of four samples were all above 93.00%, indicating the high qualities of these obtained sequencing data (Table 2). All sequencing data were mapped to the reference genome of the DHL92 (Version 3.6.1), in which over 97.00% clean reads for each sample were successfully aligned. Sequence variant calling revealed that there were 2,246,184, 2,061,384, 2,784,981, and 2,766,081 polymorphic SNPs for XNM020, XNM125, Spotted_bulk, and Non-spotted_bulk, respectively; similarly, a total of 500,924, 450,791, 607,702, and 607,710 small InDels were also, respectively, identified for XNM020, XNM125, Spotted_bulk, and Non-spotted_bulk (Table 2). After filtering and polymorphic screening between parental lines, a total of 617,462 SNPs and 124,843 InDels with reliable qualities were obtained for the following linkage analysis. For linkage analysis, two approaches including ED algorithms and either Δ SNP-index or Δ InDel-index were performed to calculate the allele segregation of the SNPs and small InDels between the Spotted_bulk and Non-spotted_bulk. The ED algorithms of both SNPs and small InDels showed that two regions located on the beginning of both chromosomes 4 (from 0.00 to 2.97 Mb) and 5 (from 0.00 to 2.34 Mb) might be involved in the regulation of green spotted fruit rinds in XNM020 melon (Figure 2 and Table 3). Consistently, the Δ SNP-index of SNPs and the Δ InDel-index of small InDels also indicated two candidate regions at the beginning of both chromosomes 4 and 5 (Figure 3 and Table 3). Overall, BSA-Seq analysis suggested that two potential genomic regions regulate the formation of the green spotted fruit rind of wild melon XNM020.

Table 2. The quality, genome coverage, and identified variants of re-sequencing data.

Samples	Clean Reads	Clean Bases (Gbp)	Q30 (%)	Depth (×)	Mapped Reads	Mapping Ratio (%)	Number of SNPs	Number of InDels
XNM020	50,921,375	15.2	93.18	29	50,320,503	98.82	2,246,184	500,924
XNM125	50,422,593	15.1	93.29	28	49,772,142	98.71	2,061,384	450,791
Spotted_bulk	52,316,938	15.6	93.13	28	51,202,587	97.87	2,784,981	607,702
Non-spotted_bulk	55,391,437	16.6	93.00	30	54,255,913	97.95	2,766,081	607,710

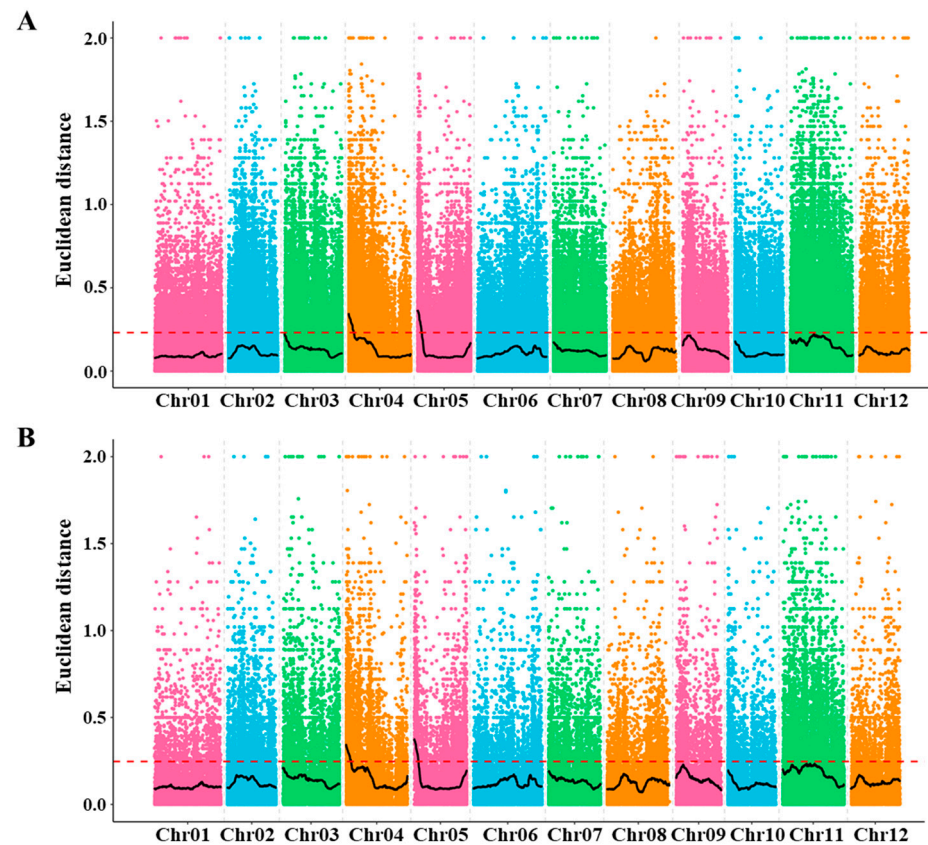


Figure 2. BSA-Seq-based mapping of the green spotted fruit rinds with the ED algorithms of SNPs (A) and small InDels (B).

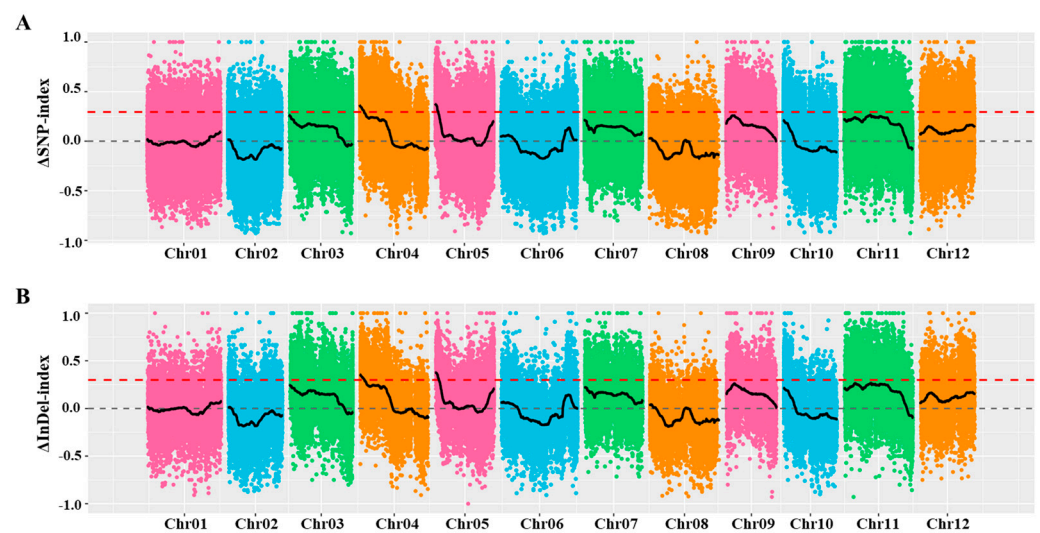


Figure 3. BSA-Seq-based mapping of the green spotted fruit rinds with the Δ SNP-index (A) and Δ InDel-index (B).

Table 3. Summary of candidate mapping regions for the green spotted fruit rind trait.

Chromosome ID	Sequence Variants	Linkage Analysis Method	Candidate Reigons (Mb)	
			Start	End
4	SNP	ED algorithm	0.00	2.97
4	InDel	ED algorithm	0.00	2.80

Table 3. Cont.

Chromosome ID	Sequence Variants	Linkage Analysis Method	Candidate Reigons (Mb)	
			Start	End
4	SNP	Δ SNP-index	0.00	2.23
4	InDel	Δ InDel-index	0.00	2.18
5	SNP	ED algorithm	0.00	2.30
5	InDel	ED algorithm	0.00	2.34
5	SNP	Δ SNP-index	0.00	1.51
5	InDel	Δ InDel-index	0.00	1.56

3.3. Verification of the Candidate Mapping Region on Chromosome 4 with InDel Markers

To verify the accuracy of results obtained with BSA-Seq analysis, InDel marker-based traditional linkage analysis was conducted with the F₂_2021S population for the target mapping region on chromosome 4. Four polymorphic InDel markers spanning the candidate region were designed and used for genotyping the individuals of F₂_2021S, in which genotypes same with XNM125, XNM020, and F₁ were recorded with ‘a’, ‘b’, and ‘h’, respectively. For the phenotype data of GS, the F₂ plants setting fruits with green spotted and non-spotted rinds were assigned with genotypes ‘c’ and ‘a’, respectively, following the instruction of JoinMap 4.0. The genetic linkage map of GS and four InDels (Mc4_indel13, Mc4_indel19, Mc4_indel24, and Mc4_indel25) was obtained and is shown in Figure 4. As depicted in the figure, the whole genetic distance of this linkage map is 29.6 cM, and the GS locus is flanked by markers Mc4_indel13 and Mc4_indel19 with genetic distances of 6.7 and 5.8 cM, respectively.

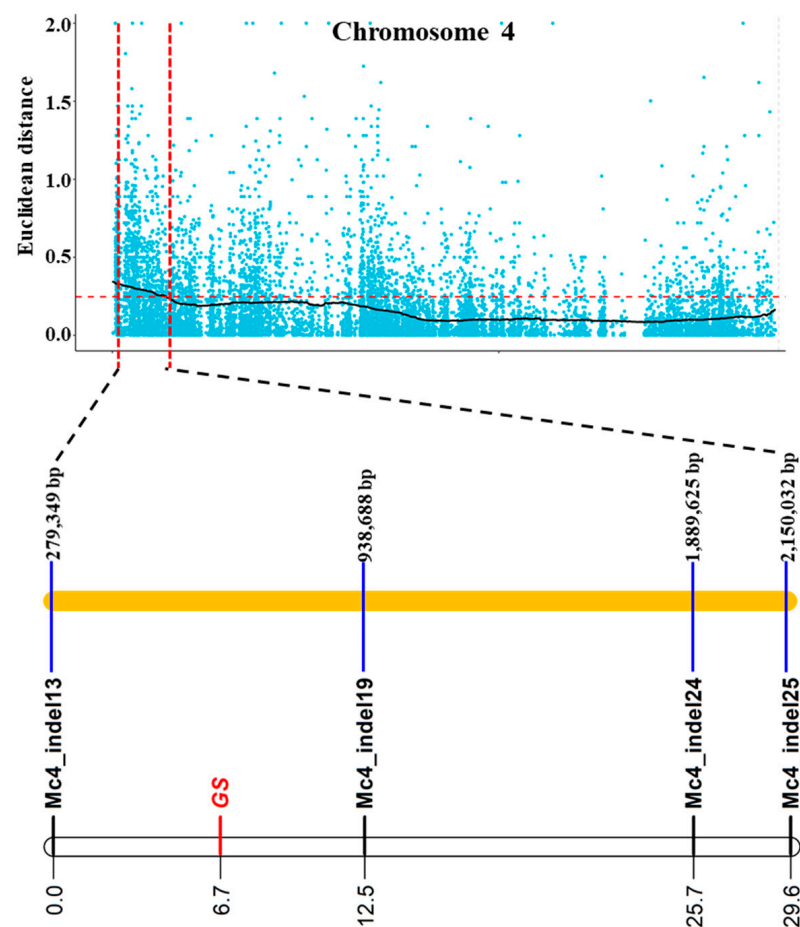


Figure 4. InDel marker-based verification of the target mapping region on chromosome 4.

3.4. Potential Candidate Genes Located in the Target Mapping Regions

To identify the potential candidate genes underlying green spotted fruit rinds of XNM020, both SNPs and small InDels in target genomic regions were firstly annotated based on the melon reference genome (DHL92 V3.6.1); and then, the SNPs or small InDels showing polymorphic between two bulks (the Spotted_bulk and Non-spotted_bulk) and located on gene coding regions were selected for further check and are listed in Table S2. As shown in Table S2, there are 387 SNPs and small InDels identified on chromosome 4, along with 158 on chromosome 5, respectively, causing coding sequence variations in 222 genes. Among them, 99 of the 152 genes on chromosome 4 exhibited non-synonymous or frameshift mutations. Similarly, within the corresponding region of chromosome 5, 34 of the 70 genes showed non-synonymous mutations. These 99 genes on chromosome 4 and 34 genes on chromosome 5, which have changed their encoded protein sequences, might be good candidate genes for regulating the green spotted fruit rinds in wild melon XNM020. These things considered, to further confirm the best candidate genes, we manually checked the annotated and predicated functions of these candidate genes. We found that three genes on chromosome 4, including *MELO3C003316*, *MELO3C003375*, and *MELO3C003388*, are involved in the biological processes of chloroplast or thylakoid organization and development or chlorophyll biosynthesis. There was one gene on chromosome 5 (*MELO3C014660*) that plays an essential role in chloroplast organization and differentiation. Considering the morphological features of the green spotted fruit rinds in wild melon XNM020, it is reasonable to infer that the above four genes regulating chloroplast development or chlorophyll biosynthesis are the best candidate genes.

4. Discussion

Fruit rind color and pattern, directly affecting the preferences of consumers, are the breeding targets that focus on the external qualities of melons. Uncovering the inheritances of both rind colors and patterns of melon fruits is essential for their genetic improvements. For fruit rind colors of melons, both monogenic and polygenic inheritances, depending on the used segregating populations and their parental lines, were reported by researchers [2,9–13]. Within bi-parental populations derived from crossing between ‘Krymka’ and ‘Eshkolit Ha’ Amaqim’, there is a single gene determining dark young fruit exterior color, which is dominant to light fruit color [11]. However, Monforte et al. [10] proposed four loci (*ecol3.1*, *ecol7.1*, *ecol9.1*, and *ecol10.1*) with epistatic interactions that may control the external color of melons. Feder et al. [7] found that the yellow fruit rinds of casaba muskmelons resulted from the accumulation of a yellow flavonoid pigment naringenin chalcone. Further fine genetic mapping reveals that a single gene *CmKFB* on chromosome 10 encoding Kelch domain-containing F-box protein negatively regulates the naringenin chalcone accumulation [7]. Using the RIL population developed from parental lines ‘Védraçais’ and ‘Piel de Sapo’, two (*ECOLQU3.1* and *Wi*) and three loci (*YELLQU5.1*, *YELLQU10.1*, and *YELLQU12.1*) were, respectively, identified for the external color of immature fruit and yellowing of mature rind [12]. The external color of immature fruit regulating loci *ECOLQU3.1* and *Wi* share identical chromosomes with the *ecol3.1* and *ecol7.1* detected by Monforte et al. [10], respectively, suggesting the immature fruit color in melons may be regulated by two conserved loci. The *YELLQU10.1* and *ecol10.1* loci on chromosome 10 are consistent with each other, and their shared genomic interval contains the gene *CmKFB* identified by Feder et al. [7]. Whole-genome linkage analysis using two segregating populations found that multiple independent single-nucleotide polymorphisms in the *CmAPRR2* gene encoding a two-component response regulator-like protein APRR2 are causative of the light rind phenotype in melons [13]. Meanwhile, Zhao et al. [2] identified a 12:3:1 segregation ratio for green, white, and yellow rinds in an F₂ segregating population developed from parental lines MS-723 (green rind) and B432 (yellow rind), indicating that green rind is dominant and epistatic to non-green (white and yellow) rind. Furthermore, BSA-Seq combined with GWAS analysis revealed two candidate genes related to fruit rind color (green, white, and yellow), which are *CmAPRR2* on chromosome 4 and the previously

identified *CmKFB* gene on chromosome 10, respectively [2]. Additionally, a minor effect gene *MELO3C003097*, an ortholog of Arabidopsis *SLOW GREEN1* (*SG1*), might also be involved in the formation of fruit rind color in melons [2].

Similar to fruit rind colors, inheritances of fruit rind patterns of cultivated melons were also explored by several researchers. In the early studies, Périn et al. [14,15] found that the dark green spots on fruit rinds are regulated by a monogenic recessive gene *mt-2*. A recessive gene regulating stripes on fruit rinds of melons was also found in segregating populations derived from a cross between ‘Dulce’ and ‘PI 414723-S5’ [16]. However, Burger et al. [11] found that the striped fruit rind in the American muskmelon ‘Dulce’ is controlled by a single dominant gene. Subsequently, Harel-Beja et al. [17] found that striped fruit rinds were controlled by multiple genes, of which the major-effect locus *str11.1* is located on chromosome 11. Pereira et al. [12] detected that the mottled (same as spotted) fruit rind was controlled by dominant gene *Mt-2*, which was identified on chromosome 2 by a genotyping-by-sequencing strategy. In the same genomic region of *Mt-2*, Lv et al. [18] also found dominant gene *CmSp-1* controlling the spotted fruit rinds in melons and narrowed this gene into a 280 kb region on chromosome 2. *Mt-2* and *CmSp-1* seem to be the same gene regulating spotted fruit rinds in melons. Interestingly, another dominant spotted or striped fruit rind controlling gene was also identified on chromosome 4 within a 172.8 kb region [19]. Recently, Shen et al. [8] reported that two dominant genes *CmMt1* and *CmMt2* with epistatic effect control the formation of spotted (or mottled) fruit rinds in melons. Overall, a relatively complex inheritance seems to exist for this essential fruit external trait. Nevertheless, the clear picture looks like two dominant genes with major effects on chromosomes 2 and 4 regulate spotted fruit rinds in cultivated melons.

Although previous genetic studies explored the inheritances of spotted or striped fruit rinds in cultivated melons, the inheritances of spotted or striped fruit rinds in wild melons are still unknown. In this study, F_1 and F_2 segregating populations were generated from the cross between wild melon germplasm XNM020 setting small spherical fruits with green spotted rinds and cultivated melon XNM125 bearing elongated fruits with white non-spotted rinds. All the fruits of F_1 plants showed green spotted rinds, same as the parental line XNM020, which indicated that the green spotted fruit rind in wild melon XNM020 has a dominant effect on the light green and non-spotted fruit rind. Considering this, phenotypic data of two small F_2 populations (2021S_ F_2 and 2023S_ F_2) showed that the green spotted fruit rind trait is controlled by a single gene (Table 1). Taken together, the green spotted fruit rind in wild melon XNM020 is regulated by a monogenic gene with dominance effect. This result is consistent with those in cultivated melons such as the American muskmelon ‘Dulce’ [11], the Spanish variety ‘Piel de Sapo’ [12], the thin-skinned Chinese melon ‘IM16553’ [18], and another thin-skinned Chinese melon ‘X010’ [19]. From the domestication perspective, the above results probably suggest the monogenic dominant locus controlling spotted or striped fruit rinds in melons may undergo non-domestications, at least for some or partially cultivated melons, and were kept by both ancestors and breeders for thousands of years of natural and artificial selections. Briefly, inheritances of green spotted or striped fruit rinds in cultivated or wild melons are relatively complex, but major-effect loci undergo their regulations.

In the present work, by using an F_2 segregating population and a BSA-Seq approach, we could quickly map the green spotted fruit rind regulating loci onto genomic regions of melons. As shown in Figure 2 and Table 3, two candidate regions at the beginning of both chromosomes 4 (from 0.00 to 2.97 Mb) and 5 (from 0.00 to 2.34 Mb) are involved in the regulation of green spotted fruit rinds in XNM020 melon. This result does not seem consistent with the monogenic dominant inheritance of green spotted fruit rinds in wild melon XNM020 that was revealed by phenotypic data from F_2 segregating populations. However, when we carefully re-checked the fruit rinds of each F_2 individual, we found that both green spotted or non-spotted rind patterns and green or light green rind colors were segregating. Totals of 68 and 22 plants were setting green and light green fruit rinds, respectively, for the 2023S_ F_2 population. Similarly, for the 2021S_ F_2 population, 70 plants

showed green fruit rinds, and 24 individuals had light green fruit rinds. The genetic segregating ratios of green to light green fruit rinds fit the expected 3:1 ratio for both two F_2 populations, in which 2023S_ F_2 had an $\chi^2 = 0.015$ ($<\chi^2_{0.05} = 3.841$) and the χ^2 value of 2021S_ F_2 was 0.014 ($<\chi^2_{0.05} = 3.841$). This suggests that the green fruit rind color shown in the wild melon XNM020 is also controlled by a single dominant gene. Actually, when the Spotted_bulk and Non-spotted_bulk were constructed, these two bulks were exactly bulks for green and light green rinds, too. Therefore, we can confidentially speculate that the two genomic regions on chromosomes 4 and 5 correspond either to the green spotted fruit rind locus or to the green fruit rind locus, respectively. Based on the verification result of four InDel markers on chromosome 4, as the GS locus is well linked, the green spotted fruit rind regulating locus located on chromosome 4 has a higher possibility. Simultaneously, the genomic region on chromosome 5 is likely regulating the variation in green or light green fruit rinds between XNM020 and XNM125. On the other hand, the above data also showed us the power of the BSA-Seq strategy on gene or major-effect locus mapping of essential traits in melons.

5. Conclusions

In summary, both the inheritance and regulating loci of the green spotted fruit rind in wild melon accession XNM020 were explored with F_2 segregating populations. Segregating ratios of phenotypic data indicated that the green spotted fruit rind in XNM020 has a monogenic dominant inheritance. BSA-Seq showed that two potential genomic regions on chromosomes 4 and 5 regulate the green spotted fruit rind in XNM020. Within the target genomic regions, four genes *MELO3C003316*, *MELO3C003375*, *MELO3C003388*, and *MELO3C014660* involved in chloroplast development or chlorophyll biosynthesis may be the candidates regulating the green spotted fruit rinds in melons. This study not only enriched the inheritances of spotted fruit rinds in melons but also provided target genomic regions for marker-assisted selection breeding of melons focusing on fruit rinds.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/agronomy14061106/s1>. Table S1: Sequence information of the used InDel markers; Table S2: Potential candidate genes located in the target mapping regions.

Author Contributions: Y.Z. (Yuqing Zhou) and Y.Y. conducted the majority of the reported research. Y.X. performed the phenotypic data collection. H.C. and Y.Z. (Yuan Zhou) helped with leaf sample collection and DNA extraction. Y.P., H.L. and H.Z. conceived and supervised the research. Y.P. wrote the manuscript with Y.Z. (Yuqing Zhou), Y.Y., H.L. and H.Z. All authors have read and agreed to the published version of the manuscript.

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