Bulked-segregant Analysis Identified a Putative Region Related to Short Internode Length in Melon

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Abstract. Short internode length (SIL) is one of the most commercially and important traits in melon varieties (Cucumis melo L.). SIL can result in a compact vining type that promotes concentrated fruit in high-density crops, leading to greater use of light resources for photosynthesis and greater yield per unit area. In our study, two parental melon lines ‘M1-32’ (P1, standard vine) and ‘X090’ (P2, short internodes), and their F1, F2, BC1P1, and BC1P2 progenies were evaluated after being grown in plastic greenhouse conditions in 2017 and 2018. Main stem length (MSL) and internode length (IL) of six melon generations indicated that a single recessive gene (MD7) controlled dwarfism in the ‘X090’ melon line. Whole-genome analysis revealed a genomic region harboring the candidate dwarfism gene on chromosome 7. Six polymorphic cleaved amplified polymorphic sequence (CAPS) markers from chromosome 7 were used to construct a genetic linkage that spanned 30.28 cM. The melon dwarfing locus MD7 responsible for SIL was positioned between markers M7-4 and M7-5, with 3.16 cM of flanking distance. The CAPS markers M7-4 and M7-5 developed have the potential to accelerate the development of dwarf melon varieties, especially in situations when dwarf genotypes are an important breeding goal using marker-assisted selection.

According to the Food and Agriculture Organization, China was the largest melon producer and consumer, accounting for 50.04% of the total production worldwide in 2014 [as cited in Sun et al. (2017)]. Compact or dwarf crops have great agricultural advantages, including greater planting density and resistance to storm and lodging, and reduced losses during harvesting operations (Amasino et al., 2003).

Many dwarf crop traits, including short internodes, short MSL, and bush-type growth habits, have been reported in watermelon (Citrus lanatus (Thumb.).). In watermelon, two allelic genes (dw-1 and dw-1') and three independent loci (dw-2, dw-3, and dw-4) have been shown to control dwarfism (Huang et al., 1998; Loy and Liu, 1972; Yang et al., 2010). The recessive gene dw-1 was discovered in the first dwarf watermelon that was mutated from the standard vining watermelon inbred line ‘WB-2’ (Mohr, 1956), which has short internodes formed by abnormal internode cells. The recessive gene dw-2 was identified in the short vining variety “Bush Desert King,” which has substandard internodes. Subsequently, the simply inherited recessive gene dw-1' was detected in the short vining watermelon variety “Somali Local” (Dyutin and Afanas’eva, 1987), whereas dw-4 in the short vining habit was identified in the self-pollinated population of the ‘5-6y’ watermelon (Yang et al., 2010). Furthermore, a potential dwarf candidate gene has been mapped to chromosome 7 and named Clal010726 (Dong et al., 2018). In cucumber, cp1 and cp2, and dw gene expression have been reported to be responsible for compact and dwarf-type cucumber, respectively (Robinson and Mishanec, 1965; Kaufman and Lower, 1976). A recent study revealed a gene association with short vining habit on chromosome 4 of the cucumber; this gene is highly homologous with cytokinin oxidase (CKX) (Li et al., 2014). A dwarf gene was mapped a dwarf gene to a 2.29 cM with interval using random amplified polymorphic DNA markers in near-isogenic lines (NILs) derived from the pumpkin Ait0 variety (Li et al., 2011). Inmelon, three recessive loci—si-1, si-2, and si-3—were reported to control compactness or dwarfism in ‘UC Top Mark bush’, ‘Persia202’, and ‘Maindwarf’, respectively (Knavel, 1988; 1990; Paris et al., 1984). Furthermore, Hwang et al. (2014) identified the dwarf gene mdw1 of the melon ‘PNU-WT1’ dwarf mutant on chromosome 7, which is highly homologous with CKX.

Bulked-segregant analysis (BSA) is an advanced method to identify markers rapidly that are linked tightly to causal genes underlying a given phenotype. In 1991, BSA was used for the first time to map disease-resistant genes (Michelmore et al., 1991). Since then, BSA has been widely used for rapid gene mapping and identification of molecular markers linked to a quantitative or qualitative trait of interest in horticultural crops, including watermelon (Dong et al., 2018), melon (Li et al., 2017), cucumber (Zhang et al., 2015), and tomato (Zhao et al., 2016). In our study, we used a combination of BSA and whole-genome resequencing to characterize the molecular inheritance of dwarfish genes associated with the diminutive plant habit of the melon line ‘X090’, and developed molecular markers for marker-assisted breeding of dwarf melon cultivars in China.

Materials and Methods

Plant materials. Two melon parental lines ‘M1-32’ (P1, female) and ‘X090’ (P2, male) were used to generate the F1, F2, BC1P1, and BC1P2 populations. ‘X090’ is a dwarf type with bush and short internodes, whereas ‘M1-32’ is a standard type. In 2017, parental lines ‘M1-32’ (n = 15) and ‘X090’ (n = 15), and their F2 progenies (n = 511), and backcross generations BC1P1 (n = 150) and BC1P2 (n = 150) were evaluated in a plastic greenhouse. A total of 985 F2 individual melons were planted in 2018. Irrigation, weeding, and pest control were conducted using standard horticultural procedures for typical Harbin climatic conditions.

Phenotypic data collection. MSL and IL were measured, and the number of internodes was recorded for individual plants at the adult stage. CM represents the height of the entire main vine and the length of the vine between the two adjacent leaves in the middle of the main vine. Plants were divided into two phenotypes—S (standard vine) and D (dwarf vine)—based on MSL and IL.

Genomic DNA extraction. Young leaves of P1, P2, F1, and F2 plants without any damage were collected and frozen rapidly at −80 °C for storage. Genomic DNA was extracted using the modified cetyltrimethylammonium bromide method (Allen et al., 2006).

Whole-genome resequencing of bulked DNA. DNA concentration was measured using an SMA3000 spectrophotometer (Plextech, Shenzhen, China) and was electrophoresed on a 1% agarose gel. Two bulk DNA samples were prepared by mixing an equal proportion of DNA from 30 standard-type and 30 dwarf-type F2 plants. Two bulk and two parental DNA samples were selected for sequencing. Briefly, DNA samples were disrupted using ultrasound, then were repaired distally, ligated with sequencing joints, and separated using 1% agarose gel electrophoresis to harvest target insert sizes ranging...
from 500 to 600 bp for sequencing. Polymerase chain reaction (PCR) amplification was performed to construct a paired-read sequencing library. The DNA libraries were sequenced using the Illumina HiSeqTM 2500 platform (Biomarker, Beijing, China) to generate 125 base paired-end reads.

**BSA analysis.** The reads of the dwarf parent (‘X090’) were first cleaned and then aligned with the melon reference genome (DHL92) using BWA (version 0.6.1-r104), from which merged alignments were sorted and read duplicates were removed using SAMTOOLS’s (version 0.1.19) rmdup command. The Unified Genotyper module of GATK3.3 was used to detect multiple samples of single nucleotide polymorphisms (SNPs) and insertions or deletions that were filtered with variant filtration and annotated with ANNOVAR (version Apr16). Associations among target sites were analyzed using the SNP index method to determine the genomic region of the dwarf gene.

\[
\text{SNP index (aa)} = \frac{Xaa}{Xaa + Maa}
\]

\[
\text{SNP index (bb)} = \frac{Xbb}{Xbb + Mbb}
\]

\[
\Delta (\text{SNP index}) = \text{SNP index (aa)} - \text{SNP index (bb)}
\]

where X represents ‘X090’; M represents ‘M1-32’; aa and bb are equal to the genotypes of the D and S bulks, respectively; Xaa and Maa represent the depth of the aa population derived from X and M, respectively; and Xbb and Mbb represent the depth of the bb population derived from X and M, respectively. The stronger the correlations between SNPs and dwarf traits, the closer \(\Delta (\text{SNP index})\) is to one. \(\Delta (\text{SNP index}) = 0\) represents no association of SNP with dwarf trait.

**CAPS marker development.** The dwarf gene was positioned on the target chromosome and mapped using the melon (DHL92) reference genome from the BSA results. Sequencing data underwent a series of quality control procedures using Perl scripts of Northeast Agricultural University to select different candidate sequences of \(\approx 500 \) bp carrying restriction site-specific SNPs using SNP2CAPS software. The SNP loci were converted to CAPS markers.

**Validation of CAPS markers by PCR.** The PCR product was cleaved by respective endonucleases to identify the polymorphism of the CAPS markers between two parental lines and their \(F_1\). PCR amplification was performed using the touchdown PCR reaction (Amanullah et al., 2018), which contained 2 \(\mu\)L template DNA, 0.4 \(\mu\)L Taq endonuclease, 1 \(\mu\)L of each forward and reverse primer, 2 \(\mu\)L Taq buffer, 0.6 \(\mu\)L dNTPs, and 13 \(\mu\)L double-distilled water in a total volume of 20 \(\mu\)L. The PCR amplification was programmed as follows: preheating at 94 °C for 7 min, followed by 30 cycles of heating at 94 °C for 1 min, 60 °C re-naturation for 30 s, cool-down at 0.5 °C and extension at 72 °C for 90 s, and finally extension at 72 °C for 10 min. The PCR product was separated using 1% agarose gel electrophoresis and digested with five restriction enzymes (PstI, MspI, MboI, HindIII, and SacI). The digestion reaction contained 5 \(\mu\)L PCR product, 0.3 \(\mu\)L restriction enzymes (concentration 10 U·mL\(^{-1}\)), 1.5 mol·L\(^{-1}\) enzyme corresponding buffer, and 8.2 \(\mu\)L double-distilled water in a total volume of 15 \(\mu\)L at 37 or 65 °C constant temperature in an incubator for 3 h depending on the endonuclease.

**Genetic linkage map construction.** Genetic linkage maps were constructed using quantitative trait loci (QTL) Ici mapping in which standard-vine F\(_2\) individuals were recorded as 2 and dwarf individuals were recorded as 1. The interval mapping analysis of gene loci was also performed to determine genetic distances using QTL Ici mapping (version 4.0).

**Statistical analysis.** Variance and difference analysis of field phenotypic data was performed using statistical software (SPSS 23.0) and Microsoft Excel 2007.

**Results**

**Inheritance of dwarf habit.** As shown in Fig. 1C and D, there were significant morphologic differences between the ‘X090’ and ‘M1-32’ plants during the third true-leaf stage. The average MSL of dwarf plants did not exceed 50 cm, whereas the average MSL

![Fig. 1. Differences in plant characteristics between the dwarf melon line ‘X090’ and the standard vining line ‘M1-32’ cultivated in a plastic greenhouse. (A) ‘M1-32’ mature plant with standard vine (behind) and ‘X090’ adult plant with short vine (front). (B) Segregation in F\(_2\) progenies, standard-type (left) and dwarf-type (right). (C) Standard-type F\(_2\) plant 23 to 25 d after seeding. (D) Dwarf-type F\(_2\) plant 23 to 25 d after seeding. (E) CM measurement in dwarf-type F\(_2\) plants. (F) CM measurement in standard-type F\(_2\) plants. CM represents the height of the entire main vine and the length of the vine between the two adjacent leaves in the middle of the main vine.](Image)
of standard plants exceeded 200 cm in the adult stage. Among the \( F_2 \) progenies examined (Fig. 1A and B), the average ILs of the seventh, eighth, and ninth internodes in dwarf plants were 1.8 cm, 2.1 cm, and 2.3 cm, respectively; whereas the average ILs in standard plants were 9.7 cm, 10.2 cm, and 11.1 cm, respectively. The average MSL in standard plants was 213.6 cm with an average of 24 internodes, but the average MSL in dwarf plants was 40.3 cm with an average of 21 internodes (Fig. 2).

Both the \( F_1 \) and \( BC_1P_1 \) generations behaved as standard plants whereas the \( BC_1P_2 \) generation exhibited a 1:1 ratio of standard (\( n = 78 \)) to dwarf (\( n = 76 \)) (\( \chi^2 = 0.24, P = 0.624 \)). Among the 511 \( F_2 \) progenies examined in 2017, the ratio of standard (\( n = 393 \)) to dwarf (\( n = 118 \)) conformed to Mendelian \( F_2 \) segregation ratios of 3:1 (\( \chi^2 = 0.992, P = 0.319 \)). Among the 985 \( F_2 \) progenies examined in 2018, 750 were segregated standard and 235 were dwarf, further supporting the previously observed 3:1 phenotypic segregation pattern for this trait (\( \chi^2 = 0.685, P = 0.408 \)) (Table 1). These results indicate that a single recessive gene controls dwarfism in ‘X090’, named \( MD7 \).

Table 1. Phenotypic performance of six generations from the 2017 and 2018 greenhouse evaluation.

| Yr    | Generation | S   | D   | Expected ratio | Actual ratio | \( \chi^2 \) value | \( P \) value |
|-------|------------|-----|-----|---------------|--------------|-------------------|--------------|
| 2017  | \( P_1 \) | 15  | 0   | —             | —            | —                 | —            |
| 2017  | \( P_2 \) | 0   | 15  | —             | —            | —                 | —            |
| 2017  | \( F_1 \) | 15  | 0   | —             | —            | —                 | —            |
| 2017  | \( BC_1P_1 \) | 150 | 0   | —             | —            | —                 | —            |
| 2017  | \( BC_1P_2 \) | 78  | 72  | 1:1           | 1.08:1       | 0.24              | 0.624        |
| 2017  | \( F_2 \) | 393 | 118 | 3:1           | 3.33:1       | 0.992             | 0.319        |
| 2018  | \( F_2 \) | 750 | 235 | 3:1           | 3.19:1       | 0.685             | 0.408        |

\( S = \) standard vine; \( D = \) dwarf vine.

BSA-identified candidate gene controlling dwarfism on chromosome 7 (Fig. 3). The DNA pool with mixed dwarf and standard vining plants was sequenced by the Beijing Genomics Institute to generate the preliminary BSA positioning results, which ranged from 0.35 to 1.8 Mb on chromosome 7, respectively (Fig. 4). As shown in Fig. 3, one significant peak fluctuation was located on chromosome 7, suggesting that genes controlling dwarf traits might be positioned on chromosome 7.

**CAPS primer screening and verification.** Resequencing results based on the parental

![Fig. 3. Calculated single nucleotide polymorphism (SNP) index plots across the whole genome. The x-axis indicates the position of 12 chromosomes in the melon; the y-axis represents the SNP index. The dashed line (green in online figure) is the threshold value calculated by Loess regression (0.5). The circle (red in online figure) on chromosome 7 represents a significant fluctuation peak.](image-url)

![Fig. 2. Comparisons of (A) internode length at the seventh, eighth, and ninth internodes; (B) main stem length, and (C) number of internodes between standard vines and dwarf vines.](image-url)
genome were used to design 10 pairs of CAPS primers for chromosome 7. PCR and restriction enzyme digestion were performed using DNA from both contrasted parents and F₁ plants. Among them, six pair of primers had a polymorphism between ‘X090’ and ‘M1-32’ accounting for 60% of the polymorphic rate.

**Genetic linkage map construction.** To map dwarf genes on chromosome 7, six pairs of polymorphic CAPS markers were used to construct a genetic linkage map using 1496 F₂ progenies (Fig. 5A). This unsaturated melon map spanned 30.28 cM with six CAPS markers; the melon dwarf locus MD7 was positioned between markers M7-4 and M7-5 (Fig. 5B), with 3.16 cM of flanking distance. The physical distance between the two markers M7-4 and M7-5 was 196 Kb, ranging from 1,059,752 to 1,255,618 bp. All six markers used for mapping are listed in Table 2.

**Candidate genes analysis.** There were 25 genes (MELO3C016908–MELO3C016932) found between the markers M7-4 and M7-5. Of these genes, eight contained 11 nonsynonymous SNPs (Table 3), seven genes have more detailed annotation when blasting them against *A. thaliana* genes, which from TAIR10 (https://www.arabidopsis.org/). MELO3C016912 encodes SLOMO (slow motion), an F-box protein required for auxin homeostasis and normal timing of lateral organ initiation at the shoot meristem. MELO3C016915 is a P-Type adenosine triphosphatase and mediates copper.

**Table 2.** CAPS markers used to map MD7.

| Marker | Primer sequence (5’→3’) | Enzyme | Annealing temp (°C) |
|--------|-------------------------|--------|--------------------|
| M7-1   | F:GGCATTCCACTCTCAATCAG R:TTCTTAGTCTCTACTCTGAATTTC | PstI | 55 |
| M7-2   | F:AGGTGCTATTATGTTGGAGGT R:ATACATCGCTCTCGTCACAGA | MspI | 55 |
| M7-3   | F:CCTACAAGGCTAAGTGGAATACC R:TCTCTATCTACTTCGTTGACAATGG | MboI | 55 |
| M7-4   | F:CGTCGCCAACCTTCCTCTAT R:CCAAAGGCTCGTGCTTATTA | HindIII | 55 |
| M7-5   | F:GACCTCTCAGCTCGCTATTA R:CGATCGCCCGTCGCTGAGTGTT | Sacl | 55 |
| M7-6   | F:GCATCAGTCTCAGGAGTAGTGAGTGTT | PstI | 55 |

CAPS = cleaved amplified polymorphic sequence.
transport to chloroplast thylakoid lumen. MELO3C016916 is homologous to receptor protein kinases and is involved in the specification of organs originating from the shoot apical meristem. MELO3C016918 encodes a plastid stroma localized fatty acid binding protein. MELO3C016923 is a tetratricopeptide repeatlike superfamily protein. MELO3C016926 is a sterile alpha motif domain-containing protein. MELO3C016932 is an initiation factor 4F subunit (DUF1350).

**Discussion**

Dwarfism is one of the most commercially important traits in melon breeding because of its positive effect on yield and the associated decreased labor required for cultivation and harvest. In China, two planting methods (creeping and hanging vine cultivation) are widely used for melon production in greenhouses, among which creeping vine cultivation is predominant to cover the production area. Hanging vine cultivation greatly reduces row and plant space because of the upward growth of the plants, leading to significant increases in yield. In greenhouse conditions, \( \approx 1200 \) melon plants/acre can be grown using creeping cultivation, whereas \( \approx 2100 \) plants, with 30 \( \times \) 35-cm plant spacing per acre, can be cultivated using hanging vines. Melon vining habit is a key determinant of cultivation methods. Diminutive dwarf-type melons provide an alternative to standard vining types for plastic tunnel production because of their amenability to low maintenance in upright cultivation. Therefore, there is a strong need to identify dwarfism genes and alleles to develop new dwarf melon varieties.
expression of \textit{ERECTA} in \textit{Arabidopsis} affected plant height and floral organ morphology (Torii, 1996), and BLAST alignment revealed high homology of \textit{MELO3C016916} to \textit{ERECTA}. Therefore, \textit{MELO3C016916} is hypothesized to affect IL in melon. Further study is needed to characterize the functions of these two genes. The CAPS markers M7-4 and M7-5 have the potential to accelerate the development of dwarf melon varieties using marker-assisted selection. ‘X090’ appears to be an important donor in melon improvement programs in which dwarf genotypes are an important breeding goal.

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