CmPMRl and *CmPMrs* Were Responsible for Resistance to Powdery Mildew Caused by *Podosphaera Xanthii* Race 1 in Melon

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Abstract

Cucumis melo L. is an economically important crop, the production of which is threatened by the prevalence of melon powdery mildew (PM) infections. We herein utilized the MR-1 (P_1; resistant to PM) and M4-7 (P_2; susceptible to PM) accessions to assess the heritability of PM (race 1) resistance in these melon plants. PM resistance in MR-1 leaves was linked to a dominant gene (*CmPMRl*), whereas stem resistance was under the control of a recessive gene (*CmPMrs*), with the dominant gene having an epistatic effect on the recessive gene. The *CmPMRl* gene was mapped to a 50 Kb interval on chromosome 12, while *CmPMrs* was mapped to an 89 Kb interval on chromosome 10. The *CmPMRl* candidate gene *MELO3C002441* and the *CmPMrs* candidate gene *MELO3C012438* were identified through sequence alignment, functional annotation, and expression pattern analyses of all genes within these respective intervals. *MELO3C002441* and *MELO3C012438* were both localized to the cellular membrane and were contained conserved NPR gene-like and MLO domains, respectively, which were linked to PM resistance. In summary, we identified patterns of PM resistance in the disease-resistant MR-1 melon cultivar, and conducted finally-mapping to identify two putative genes linked to resistance. Our results offer new genetic resources and markers guide the future molecular marker-assisted breeding of PM-resistant melon.

Key Message

Two genes for resistance to *Podosphaera xanthii* race 1 in melon were identified on chromosomes 10 and 12 of the *Cucumis melo* cultivar MR-1

Introduction

Powdery mildew (PM) is a disease that can adversely affect the production of economically important cucurbit crops including melons, cucumbers, and pumpkins. PM can develop in greenhouses and open fields, incurring substantial crop losses. In 2018, 358,900 hectares of melon crops were planted in China, accounting for 34.28% of the overall global melon crop area, with a total output of 12,788,200 tonnes, accounting for 46.76% of the overall global output, making China the leading global melon producer (FAO, 2018). Pathogens responsible for PM development include *Ascomycotina* fungal species, with *Golovinomyces cichoracearum* (formerly *Erysiphe cichoracearum*) and *Podosphaera xanthii* (formerly *Sphaerotheca fuliginea*) being the two best studied of these species (Křístková et al. 2009). *P. xanthii* is the only species that has been shown to cause PM in Israel, Turkey, Italy, Spain, Australia, South Africa, and Japan (Křístková et al. 2009), whereas both of these fungi have been identified as pathogenic agents in France, the USA, and the Czech Republic, although *P. xanthii* remains dominant in these regions (McCreight et al. 1987; Křístková et al. 2007). *G. cichoracerum* has also been detected as a cause of this disease in the Netherlands, the former Soviet Union, Germany, and the United Kingdom (Braun 1995). In China, *P. xanthii* races 1 and 2F are the dominant causes of PM, with other races being reported relatively infrequently.

In melon, resistant genes *Pm-1*, *Pm-2*, *Pm-3*, *Pm-4*, *Pm-5*, *Pm V.1*, *Pm XII.1*, *Pm-F*, *Pm-G*, *Pm-x*, *Pm-w*, *Pm-B*, *Pm-Edisto47-1*, *Pm-Edisto47-2* and *BPm12.1* have all been reported to promote resistance to infection with *P. xanthii* physiological race 1. Jagger et al. (1938) reported a dominant single gene associated with *P. xanthii* race 1 resistance in melons, and called this gene *Pm-1* (Teixeira et al. 2008). Harwood and Markarian (1968a) also identified *Pm-1* and detected another resistance gene (*Pm-2*) that together promoted resistance to *P. xanthii* infection. Harwood and Markarian (1968b) further identified the *Pm-3, Pm-4, and Pm-5* resistance genes in PI 124112 (Harwood and Markarian 1968b), the latter two of which were defined as *PmV.1* and *PmXII.1* by Perchepied et al (2005). These genes may be identical to *Pm-F* and *Pm-G*, which were previously identified in a study conducted by Epinat et al. (1992). The dominant *Pm-x* gene was initially reported in PI 414723 by Pitrat et al. (1991), who also reported the single dominant *Pm-w* gene in the disease-resistant WMR29 melon cultivar, with the location of this gene being consistent with the location of *PM-B* reported by Epinat et al. (1992). Ning et al. (2014) reported the disease resistance loci *Pm-Edisto47-1* and *Pm-Edisto47-2* on melon chromosomes 2 and 5, respectively. Li et al. (2017) identified a dominant disease resistance gene (*BPm12.1*) in the MR-1 melon strain which they localized to melon chromosome 12 (Table S1).

There have been relatively few studies of other physiological races of powdery mildew. The resistance gene *Pm-6* was identified as being related to *P.xanthii* race 2 resistance in PI 124112 (Kenigsbuch, 1989). In 2010, one study found that disease resistance in the PI 134198 melon variety was controlled by a single dominant gene (*Pm-8*), but the pathogenic physiological races included in that study were distinct from the current common races. McCreight and Coffey (2011) also reported a gene associated with race S resistance in 2011, but the genetic relationships among these genes remain unclear (Table S1).

In addition, it is likely that undiscovered disease resistance genes exist in other melon varieties or in wild and semi-wild resources, and the identification of these genes represents an important future direction for the breeding of resistant lines (Cui et al. 2018). The MLO gene is also a recessive resistance-related gene, and mutant MLO genes are associated with PM resistance in plants. Iovieno et al. (2015) systematically classified and predicted MLO genes in melon, cucumber, and zucchini plants. A series of studies performed by Cheng Hong et al. (2012, 2013, 2015) also found that mutant MLO genes in melon and wild melon plants play broad-spectrum roles in the context of PM resistance.

Herein, we used F_1, F_2, BC_1P_1, and BC_1P_2 plants prepared using the disease-resistant MR-1 and disease-sensitive M4-7 parental melon lines to evaluate patterns of disease resistance. Preliminary PM resistance-related gene mapping was conducted via QTL-seq analyses the F_2 population, while F_2 and F_3 populations were utilized to validate the results of such preliminary mapping. Finally mapping was performed using molecular markers in F_2 and F_3 populations, enabling us to identify candidate resistance genes within the mapped resistance-related intervals based upon the results of gene sequence alignment, functional annotation, and expression pattern analyses following PM infection. The functional roles of two identified candidate resistance genes were predicted, and subcellular localization analyses were conducted, providing a theoretical foundation for future studies of the functional roles of these and other genes associated with melon PM resistance. Our study also highlights valuable molecular markers and

safeguards that can aid in the molecular marker-assisted selective breeding of melon plants with the goal of developing PM-resistant cultivars.

Materials And Methods

Plant materials

The PM-resistant MR-1 melon line was selected as the female parent, while the susceptible M4-7 line was chosen as the male parent in the following breeding scheme: F_1(MR-1×M4-7), BC_1P_1(F_1×MR-1), BC_1P_2(F_1 ×M4-7), F_2 (F_1×F_1). In total, 13 differential lines (Iran H, TopMark, Védrantais, PMR 45, PMR 5, WMR 29, Edisto 47, PI 414723, MR-1, PI 124111, PI 124112, PMR 6, and Nantais Oblong) were utilized to assess *P. xanthii* race 1 susceptibility (Bardin et al. 1999; Hosoya et al. 1999, 2000; McCreight 2006).

In the summer of 2017, these materials were grown in a greenhouse at the Northeast Agricultural University Facility Horticulture Engineering Center (44°04'N, E125°42'). A total of 10 plants were planted for each of the 13 different host lines, while 30 plants each were grown for the P_1 (MR-1), P_2 (M_4-7), F_1, BC_1P_1, and BC_1P_2 lines. In addition, 396 total F_2 plants were grown to analyze patterns of genetic inheritance and to localize the resistance loci. In 2018, 1022 F_2 and 726 F_3 individuals were planted used for finally-mapping.

Microscopic Analyses Of Powdery Mildew Fungus

Melon leaves infected with powdery mildew were collected and treated with a decolorizing solution (0.15% trichloroethanol-trichloromethane) at 60°C for 1–2 h at a constant temperature until the leaves were completely decolorized. Leaves were then treated with a staining solution (0 15% trichloroacetic acid + 6% Cumas Brilliant Blue R-250 in 99% methanol) for 3–5 min, rinsed with sterile water, and observed for powdery mildew hyphae, haustorium, and conidiophore morphology using an ordinary light microscope.

Pm Resistance Phenotyping

PM fungi isolated from melon plants were collected in distilled water mixed with 1–2 drops of Tween, after which conidia suspensions were assessed via microscopy. Solutions were diluted until there were 20–30 conidia per microscopic field of view, at which time spore suspensions were sprayed over the entirety of melon plants (Ma et al. 2011). The leaves of 13 different melon lines were inoculated in this manner and the causative pathogen was identified as *P. xanthii* race 1 based upon the differential reactions of these 13 lines to the pathogen as determined based on the criteria previously detailed by McCreight (2006). In this study, plants without white spots were defined as resistant and those with white spots were defined as susceptible.

A total of 15 MR-1 and 15 M4-7 plants were then taken and inoculated with the above *P. xanthii* race 1 preparation under close management conditions, whereas the remaining 15 plants per group were inoculated in the same manner under field management conditions. Following inoculation, leaves and stems from these plants were collected at 24, 48, and 72 h post-inoculation, snap-frozen with liquid nitrogen, and stored at -80°C. Following F_3 plant inoculation, F_3 stem resistance was assessed, with F_3 individuals conforming to a 1:3 ratio being retained. Together with genotyping results, these F_3 plants were derived from the self-pollination of a single F_2 individual with the RlRl genotype and used as an F_3 population for *CmPMrs* finally mapping.

Dna Extraction And Pooling

Genomic DNA was extracted from leaves via a modified CTAB method (Allen et al. 2006). Plants with disease-free stems and leaves were recorded as L_RS_R (L: leaf, S: stem, $_R$: resistance, $_S$: susceptibility), whereas plants with visible lesions on stems but not leaves were recorded as L_RS_S, and those with lesions on both tissue types were recorded as L_SS_S. Following inoculation, these three susceptibility phenotypes were selected from among F_2 plants, and 20 plants per phenotype were combined to yield three pooled DNA samples.

Bulked Segregant Analysis

An Illumina HiSeq 2500 instrument was used to sequence DNA samples from two parents and three gene pools (BGI Bio). The genome-wide resequencing data from the two parents were aligned with the DHL92 v3.5.1 reference genome (Garcia-Mas et al. 2012) using a Burrows-Wheeler Aligner approach to detect single nucleotide polymorphisms (SNP), which were identified using samtools 1.0 (Li et al. 2009) from a BAM format file and annotated using the SnpEff tool (Cingolani et al. 2012). We excluded SNP positions with a SNP-index value of < 0.3 and a read depth < 6 from the two sequences. A sliding window analysis was applied to SNP-index plots, with a 2 Mb window size and 10 Kb increments. We calculated the average SNP-index values for the SNPs located within this window and used these for the sliding window plots (Takagi et al. 2013).

SNP-index values were estimated as follows (Zheng et al. 2018):

(1) SNP-index (xx) = Rxx / (Sxx + Rxx)

(2) SNP-index (yy) = Syy / (Ryy + Syy)

(3) Δ (SNP-index) = SNP-index (xx) - SNP-index (yy)

Where R corresponds to MR-1 and S corresponds to M4-7. xx and yy are the reads from different gene pools. Rxx indicates the depth of xx derived from R, Syy indicates the depth of yy derived from S, and Sxx

and Ryy indicate similarities.

Only regions with a fitting value greater than the 95% confidence interval threshold were considered for this analysis.

CAPS marker development and secondary mapping by QTL analysis based on BSA results

SNPs that differed between parental lines were identified via sequence comparisons, with the SNP2CAPS software being used to select SNPs on *AluI, BclI, DraI, EcoRI, BsaHI, HindIII, MboII, PstI, BamHI, MluI, ScaI, PvuI, XhoI, TaqI, KpnI*, and *MspI*. Primers specific for 500 bp regions before and after these SNPs (1000 bp total) were then designed using Primer Premier 6.0. Two parents and F_1 plants were used to screen for polymorphisms for use as CAPS markers for the genotyping of F_2 and F_3 populations. Genetic linkage map construction was performed using the IciMapping V3.3 software (Institute of Crop Science, Chinese Academy of Agricultural Sciences, Beijing, China) (Meng et al. 2015), and all the markers were grouped at a minimum logarithm of odds (LOD) score of 6.0. The QTL analysis was also performed using IciMapping V3.3 (Meng et al. 2015). Composite interval mapping (CIM) was used to search QTLs for PM resistance on one chromosome, with QTLs with an LOD score ≥ 5.0 being considered for detection.

Qrt-pcr

A Trizol approach was used to extract RNA from samples (Rio et al. 2010), after which cDNA was prepared using a Toyobo reverse transcription kit. All real-time qRT-PCR reactions were conducted using the Tiangen Biochemical Technology RealMaster Mix SYBR Green kit. Primers were synthesized by Sangon Biotech, and were designed with Primer Premier 5.0. The relative expression of all genes within the finally location was assessed, with vertical coordinates corresponding to relative expression and horizontal coordinates corresponding to the time of inoculation (0, 2, or 4 days post-inoculation). The $2^{-\Delta\Delta CT}$ approach was used to assess relative gene expression (Livak and Schmittgen, 2001), *MELO3C023264* (Actin) was utilized as a housekeeping gene. Differences in relative expression were analyzed using SPSS v23.0.

Candidate Gene Cloning And Subcellular Localization

To clone candidate genes, DNA recovery kits (Takara) were used to retrieve fragments of the appropriate size for cloning using the pEASY-T1 Cloning Kit (TransGen Biotech). The ligated product was then transformed into competent *E. coli* DH5α cells (TransGen Biotech), with an M13 primer being used for PCR analyses of bacterial solutions. Next, 0.5 mL of this bacterial solution was aspirated, mixed with 0.5 mL of 30% glycerol, and stored at -80°C. Bacterial sequencing was performed by Sangon Biotech.

MELO3C002441 and *MELO3C012438* ORF sequences from which the stop codon had been removed were fused to a GFP sequence to prepare an expression vector. Next, a tobacco leaf injection method was used

to assess the localization of these constructs within cells. At 3 days post-inoculation, tobacco leaves were taken from the injection site to prepare slides which were evaluated via confocal microscopy, with Agrobacterium transformed with an empty vector serving as a control. Chloroplast autofluorescence at a 640 nm excitation wavelength was assessed, while GFP signal was assessed at an excitation wavelength of 488 nm.

Phylogenetic Analysis Of Candidate Genes

Candidate genes were subjected to BLAST matching against the NCBI database (https://www.ncbi.nlm.nih.gov/) to identify candidate gene homologs and their amino acid sequences in other plants, and phylogenetic analysis was carried out using the MEGA-X software.

Results

Powdery mildew isolate classification

Leaves from PM-infected melon plants were stained using Coomassie Brilliant Blue, and the conidia therein appeared ovoid in shape. Fibrous bodies were clearly visible in the cytoplasm with a length of 57–76 μm and a width of 38–44 μm, consistent with the characteristics of *P. xanthii* (Fig. 1). Following inoculation, the susceptibility of 13 different host lines to this isolate was evaluated (Table 1), and resistance responses were consistent with the identity of this isolate as *P. xanthii* race 1, with no change in the dominant strain having occurred over the course of the year.

Table 1
The PM infection outcomes for 13 differential hosts

Differential hosts	Resistance evaluation in 2017	Resistance evaluation in 2018
IranH	S	S
Top Mark	S	S
Vedrantais	S	S
PMR 45	S	S
PMR 5	R	R
WMR 29	R	R
Edisto 47	R	R
PI 414723	R	R
MR-1	R	R
PI 124111	R	R
PI 124112	R	R
PMR 6	R	R
Nantais Oblong	S	S
Note: R: resistant, S: susceptible		

Genetic Analyses Of Pm-resistant And Pm-susceptible Melon Lines

As shown in Table 2, the stems and leaves of the female parental MR-1 line were disease resistant, whereas those of the male parental M4-7 were susceptible to PM. The leaves of F_1 plants were resistant to disease, whereas the stems of these plants were susceptible. F_2 plant isolation yielded plants conforming to three phenotypes: leaf and stem resistant (L_RS_R), leaf resistant stem susceptible (L_RS_S), and leaf and stem susceptible (L_SS_S). BC_1P_1 plants conformed to the L_RS_R and L_RS_S phenotypes, whereas BC_1P_2 plants conformed to the L_RS_S and L_SS_S phenotypes.

Table 2
Disease responses for P_1, P_2, F_1, F_2, BC_1P_1, and BC_1P_2 populations following artificial inoculation with *P. xanthii* race 1, with Chi-squared verification of the F_2, BC_1P_2 segregation ratios between resistant (R) and susceptible (S) individuals.

Year	Generation	L_RS_R	L_RS_S	L_SS_S	Excepted Ratio	χ^2	P Value
2017	P_1	30	-	-	-	-	-
	P_2	-	-	30	-	-	-
	F_1		30	-	-	-	-
	F_2	224	73	99	9:3:4	0.03	0.98
	BC_1P_1	14	16	-	1:1	0.13	0.72
	BC_1P_2	-	13	17	1:1	0.53	0.47
2018	P_1	30	-	-	-	-	-
	P_2	-	-	30	-	-	-
	F_1	-	30	-	-	-	-
	F_2	577	189	256	9:3:4	0.04	0.97

A dominant gene (*CmPMRl*) was found to control leaf resistance to powdery mildew, while a recessive gene (*CmPMrs*) was found to control resistance to powdery mildew on stems and vines. Analyses of inheritance patterns indicated that *CmPMRl* had a recessive epistasis effect on *CmPMrs*. In the F_2 population, the ratios of L_RS_R: L_RS_S: L_SS_S were consistent with a 9:3:4 ratio, as determined via χ2 test. In the BC_1P_1 population, the L_RS_S:L_RS_R ratio was consistent with a 1:1 ratio as determined by χ2 test. In the BC_1P_2 population, the L_RS_S: L_SS_S ratio was consistent with a 1:1 ratio as determined by χ2 test.

BSA and QTL analysis of the dominant resistance gene CmPMRl

MR-1 sequencing produced 134,014,482 reads, of which 95.0% could be mapped to the reference genome, M4-7 sequencing produced 122,001,928 reads, of which 93.8% could be mapped to the reference genome, and a total of 2,761,801 SNPs were identified between MR-1 and M4-7. In addition, 39,710,623 reads were sequenced in L_RS_R pool, with a reference genome mapped rate of 93.17%, 43,930,213 reads were sequenced in L_SS_S pool, with a reference genome mapped rate of 91.10%, and 45,151,799 reads were sequenced in L_RS_S pool, with a reference genome mapped rate of 92.82%.

SNP index values were calculated according to sequencing data from three gene pools with corresponding 95% confidence intervals. The L_RS_R and L_SS_S pool results revealed the candidate gene to be localized within the 21.7-23.3Mb interval on chromosome 12 (Fig. 2a). When the L_RS_S-pool and L_SS_S-

pool were calculated, the location was roughly the same as that calculated using the L_RS_R-pool and L_SS_S-pool, extending from 21.6 to 23.2 Mb (Fig. 2b). These results indicated that the dominant *CmPMRl* gene, which controls disease resistance in leaves and stems, is located in the 21.6–23.3 Mb interval on chromosome 12 (Fig. 2c). Based on the preliminary BSA localization results, 13 polymorphic markers (68.42% polymorphic) were selected within this initial *CmPMRl* location (Table S2). In total, 93 F_2 individuals were selected to construct a validation population, and 13 pairs of primers (*PM12-V1* to *PM12-V13*) were used for F_2 genotyping. Phenotypic leaf disease resistance data in the F_2 population was then used to localize the main effect locus associated with PM resistance in melon leaves to between *PM12-V4* and *PM12-V10* (21.59–23.30 Mb) on chromosome 12, with an LOD of 47.2 (Fig. 2d).

BSA and QTL analyses of the recessive resistance gene CmPMrs

Using a marker tightly linked to the *CmPMRl* gene (*PM12R-6*), we screened 726 plants with the same genotype and leaf phenotype as the disease-resistant parental MR-1 line. In the F_2 population, the stem disease-resistant phenotype separated at a 1:3 ratio, thus eliminating the effect of *CmPMRl* on *CmPMrs* (Table S3). Stem resistance was therefore considered to be a trait controlled by a recessive allele. The L_RS_R-pool and L_RS_S-pool results revealed the candidate gene to be located within the 0–2.03 Mb interval of chromosome 10 (Fig. 3a, b). Based on preliminary BSA localization results, 12 polymorphic markers were screened in the initial *CmPMrs* location (63.16% polymorphic) (Table S4), and 93 F_3 individuals were selected to construct a validation population, after which 12 primer pairs (*PM10-V1* - *PM12-V12*) were used for F_3 genotyping. With respect to stem resistance phenotype data in the F_3 population, the main effect loci related to PM resistance was localized between *PM10-V1* and PM10-V8 (0–2.04 Mb) on chromosome 10, and the LOD was 31.6 (Fig. 3c).

Finally mapping of the dominant resistance gene CMPMRl **and recessive resistance gene** CmPMrs

In this study, 1022 F_2 plants were genotyped with markers *PM12R-1* to *PM12R-12* (Table S2) and 20 recombinant plants were identified, further identifying the location of the dominant resistance gene *CmPMRl*. The *CmPMRl* was found to exist between markers *PM12R-5* and *PM12R-7* (22.78–22.91 Mb). Six new CAPS markers (Table S2) were developed and used to genotype the 5 recombinants (F_2-395, F_2-89, F_2-288, F_2-715, and F_2-1002). Gene recombination occurred between markers *PM12R-AD1*, *PM12R-AD4* and *PM12R-AD6*, enabling us to determine that the *R1* gene was localized between the *PM12R-AD1* and *PM12R-AD4* markers (Chr12: 22786222–22836405, about 50 Kb) (Fig. 4a, Table S5). No recombination was observed for the *PM12R-AD2* and *PM12R-AD3* markers, suggesting that this segment is the smallest segment localized to the *R1* gene interval in the F_2 population.

Genotyping of 726 F_3 plants using markers *PM10R-1* to *PM10R-12* (Table S4) identified 18 recombinant plants, leading to the location of the recessive resistance gene *CmPMrs*. The *CmPMrs* location was thus determined to be between markers *PM10R-4* and *PM10R-5* (0.47–0.59 Mb). Four new CAPS markers (Table S4) were developed and used to genotype the three recombinants (F_3-591, F_3-116, and F_3-106).

Recombination occurred only for labeled *PM10R-AD4*, with no recombination between labeled *PM10R-AD1* and *PM10R-AD4*. This led us to refine the *CmPMrs* location between markers *PM10R-AD1* and *PM10R-AD4* (Chr10:466642–552080, about 86 Kb) (Fig. 4b, Table S6), suggesting that this segment is the smallest that can be used to locate the *CmPMrs* gene interval.

Candidate Genes Functional Annotation And Sequence Alignment

When the *CmPMRl* finally-mapping interval was aligned to the DHL92 melon reference genome, we identified nine protein-coding genes (*MELO3C002441-MELO3C002449*) within this interval, of which six harbored nonsynonymous mutations between the two parental lines. Functional annotation of these genes revealed *MELO3C002445* to be unclassified, *MELO3C002441* and *MELO3C002443* to be involved in signal transduction, *MELO3C002442* and *MELO3C002444* to be involved in lipid and protein metabolism, *MELO3C002446* to play roles in unclear biological processes, *MELO3C002447* to be involved in redox reactions, *MELO3C002448* to be involved in the control of phosphate hydrolase activity, and *MELO3C002449* to be involved in glycolytic enzyme activity (Table 3).

Table 3
List of the predicted candidate genes in the *CmPMRl* candidate region

Gene ID	The number of nsSNPs	Description of gene function
MELO3C002441	1	Ankyrin repeat family protein
MELO3C002442	2	Aspartic proteinase
MELO3C002443	1	F-box plant-like protein, putative
MELO3C002444	0	Aminomethyltransferase
MELO3C002445	2	unkown protein
MELO3C002446	2	Glycine-Rich cell wall structural protein
MELO3C002447	0	L-ascorbate oxidase
MELO3C002448	0	phosphatase family protein
MELO3C002449	1	Glucan endo-13-beta-glucosidases
Total	9	

This same analytical approach led to the identification of 11 candidate genes within the *CmPMrs* finally-mapping interval, of which seven harbored nonsynonymous mutations between the two parental lines (*MELO3C012428-MELO3C012438*). Functional annotation of these genes revealed them to be primarily associated with plant physiological and biochemical responses (Table 4), including substance metabolism (*MELO3C012428, MELO3C012430, MELO3C0124233, MELO3C012434, MELO3C012435*), substance transport (*MELO3C012429*, *MELO3C012436*), redox reactions (*MELO3C012431*), pollen

budding and pollen tube elongation (*MELO3C012432*), ubiquitination (*MELO3C012437*), and PM resistance (*MELO3C012438*).

Table 4
List of the predicted candidate genes in the *CmPMrs* candidate region

Gene ID	The number of nsSNPs	Description of gene function
MELO3C012428	0	mRNA decapping protein 2 nuclear-transcribed mRNA
MELO3C012429	0	Aquaporin 2
MELO3C012430	1	Alpha/beta hydrolase family protein
MELO3C012431	9	Thioredoxin-like protein
MELO3C012432	0	Pollen-specific leucine-rich repeat extensin-like protein 1
MELO3C012433	2	Patatin
MELO3C012434	0	Anthranilate synthase component I
MELO3C012435	5	phosphatase family protein
MELO3C012436	1	Probable protein kinase
MELO3C012437	1	Ubiquitin-conjugating enzyme, E2
MELO3C012438	1	MLO-like protein
Total	20	

Assessment Of Candidate Gene Expression Patterns

A qRT-PCR analysis of genes expressed within the *CmPMRI* locus revealed that *MELO3C002441, MELO3C002444*, and *MELO3C002448* were significantly upregulated in disease-resistant MR-1 leaves following inoculation, whereas *MELO3C002446*, *MELO3C002447*, and *MELO3C002449* were downregulated, and the expression of the other three genes was unaffected. The expression of *MELO3C002441* was also significantly elevated in stem tissues, but was expressed at a lower level therein relative to leaves, consistent with the *CmPMRI* gene expression pattern (Fig. 5a). Following the inoculation of M4-7 plant materials, relative *MELO3C002441* expression was significantly reduced in leaves, whereas it was expressed at an at least three-fold higher level in MR-1 leaves relative to M4-7 leaves, suggesting that this gene is expressed at substantially different levels in PM-resistant and PM-susceptible plants (Fig. 5b). This gene encodes an anchor protein-containing repeat sequence harboring a nonsynonymous SNP that alters the identity of the amino acid in position 587 from a phenylalanine (Phe) in MR-1 plants to a cysteine (Cys) in M4-7 plants. Based on these findings, *MELO3C002441* was

identified as a *CmPMRl* candidate gene. For other details regarding expression levels of these genes, see Figures S1-S2.

A qRT-PCR analysis of genes in the *CmPMrs* locus revealed *MELO3C012429* and *MELO3C012438* to be significantly upregulated in stem tissues after susceptible M4-7 plant material inoculation, whereas *MELO3C012430*, *MELO3C012432*, *MELO3C012433*, and *MELO3C012436* were significantly downregulated, and the expression of the other five genes was unaffected. Of the two upregulated genes, *MELO3C012429* was significantly upregulated in stem tissues but did not exhibit altered expression levels in leaves. *MELO3C012438* expression rose significantly in the stem on day 4, with a > 2-fold increase in relative expression, whereas its expression levels in leaves were reduced such that this gene was expressed at significantly lower levels in leaf tissues relative to stem tissues at specific time points following inoculation (Fig. 5c). *MELO3C012429* was expressed at significantly higher levels in the leaves of disease-resistant MR-1 plants relative to stems, and such expression was not altered following inoculation. In contrast, *MELO3C012438* expression rose significantly in both leaf and stem tissues following inoculation, with significantly higher expression in stems relative to leaves at specific time points (Fig. 5d). *MELO3C012438* encodes an MLO-like protein harboring a nonsynonymous SNP that alters the identity of the amino acid in position 191 from an isoleucine (Ile) in MR-1 plants to a threonine (Thr) in M4-7 plants. Based on these results, we identified *MELO3C012438* as a candidate for the *CmPMrs* gene. For details regarding the expression levels of other genes, see Figure S3-S4.

Candidate Gene Subcellular Localization

We next evaluated the localization of *MELO3C002441* and *MELO3C012438* within cells (Fig. 6), with chloroplast autofluorescent signal being used to guide localization efforts. This analysis revealed that the MELO3C002441-GFP fusion protein primarily localized to the cell membrane (Fig. 6a-d). *MELO3C012438* exhibited localization largely identical to that of *MELO3C002441*, localizing to the cell membrane in a manner consistent with transmembrane domain predictions (Fig. 6e-h). The subcellular localization of these proteins may be linked to their functional role in the context of PM resistance, as they control melon epidermal cell resistance to PM infection.

Phylogenetic Analysis Of Candidate Genes

Phylogenetic analyses indicated that the MELO3C002441 protein clustered with soybean GmNPR4 and GmNRP4-like proteins, and was genetically distant from NPR proteins in other crops, suggesting that this gene is an anchor protein repeat sequence-containing gene that is functionally similar to the soybean NPR4 gene (Ankyrin repeat protein). Conservative structural domain analysis revealed that this gene harbored an N-terminal ANK-2 type anchor protein repeat sequence and a C-terminal PPG structural domain (Figure S5a, S6a).

The MELO3C012438 protein clustered with the cucumber CsaV35G036400 and watermelon Cla020573 proteins, which in turn clustered with the Arabidopsis AtMLO2, AtMLO6, and AtMLO12 proteins. These three proteins were genetically distant from the other MLO proteins, suggesting that this gene is an MLO family gene (Mildew Resistance Locus O) with functional similarity to Cucumber CsMLO8 and Arabidopsis AtMLO2, AtMLO6, and AtMLO12. Conserved structural domain analysis indicated that the gene encodes a conserved MLO structural domain (Figure S5b, S6b).

Discussion

In research on plant disease resistance, many *R* genes have now been cloned, and in 2018, researchers analysed 128 *R* genes and delineated nine different types based on their disease resistance mechanisms (Kourelis and van der Hoorn. 2018). In the study of plant resistance to powdery mildew, the clearer mechanisms of resistance are *RPW8.1* and *RPW8.2* found in *Arabidopsis*, which recognize the invasion of plant cells by haustorium and enhance the accumulation of hydrogen peroxide in the haustorium complex and the encapsulation of callus through the cytoskeletal function of actin towards the vicinity of the sucker membrane in elongation, thus enhancing (Orgil et al. 2007, Kim et al. 2014). In addition, in barley and *Arabidopsis*, the *MLO* gene was found to have a transcriptional regulatory function in the antifungal immune system, suggesting that this gene has a negative regulatory role in the plant defence response and suppresses the plant systemic immune response (Humphry et al. 2006, Humphry et al. 2010).

Breeding melon plants that are resistant to local PM strains is a key agricultural priority. Reports of PM-resistant melon germplasm resources were published as early as 1937, with over 30 types of PM-resistant melon materials having been described to date (de Oliveira Rabelo et al. 2017), encompassing over 20 resistance-associated genes including *Pm-1, Pm-2, Pm-3, Pm-4, Pm-5, Pm-6, Pm-R, Pm-W, Pm-X, Pm-Y, PmV.1, PmXII.1, Pm-pxA, Pm-pxB, Pm-An, pm-S, Pm-2F, Pm-x1, Pm-x3, Pm-x5, BPm12.1, Pm2.1*, and *pm12.1* (Cohen et al. 1990; Pitrat 1991; Périn et al. 2002; Perchepied et al. 2005; Fukino et al. 2008; Dogimont 2011; McCreight and Coffet 2011; Wang et al. 2011; Yuste-Lisbona et al. 2011; Zhang et al. 2012; Fazza et al. 2013; Li et al. 2017; Cui et al. 2020). This study is the first to have conducted the finally-mapping of two melon genes associated with the resistance to *P. xanthii* race 1 and to have identified proposed candidate genes associated with these phenotypes.

Herein, we identified *MELO3C002441* and *MELO3C012438* as candidate genes for the *CmPMRl* and *CmPMrs* associated with resistance to PM in the leave and stem of melon, respectively. The *MELO3C002441* gene was identified as an ANK protein family member that may be involved in systemic resistance to PM, while *MELO3C012438* was found to encode a conserved MLO domain, and may be a recessive gene linked to PM resistance.

A non-synonymous SNP in the *CmPMRl* candidate gene *MELO3C002441* may be linked to the functional variability associated with this gene in melon accessions. Disease resistance-related genes typically exhibit altered expression in response to disease. Román et al. (2019) leveraged this fact to identify

CmARP4 as one such candidate disease resistance gene. We similarly explored gene expression patterns and found *MELO3C002441* to be upregulated in plant leaves following PM grafting, whereas it was expressed at lower levels in stem tissues relative to leaves. This gene was expressed at significantly different levels in different tissue types. Ankyrin (ANK) repeat sequences were first detected in the context of the yeast cell cycle regulators *Swi6* and *Cdc10* and the Drosophila Notch signaling protein (Breeden and Nasmyth 1987). Several plant disease resistance-related ANK proteins have been reported to date including NPR1-4 (ANK-BTB), which are key regulators of plant SAR, as well as *AtPhos43* (ANK-M), which controls plant disease resistance-related signal transduction (Johal and Briggs 1992; Horvath et al. 2012). *OsXB25* (ANK-M) is an important regulator of rice *xa21* resistance to bacterial blight, while *OsPIANK1* (ANK-M) controls rice defense responses against rice blast (Schmidt et al. 2010). *ACD6* and *BDA1* (ANK-TM) respectively regulate apoptosis and disease resistance-related signal transduction in the context of disease-related stress in plants (Kim et al. 2010; Acevedo-Garcia et al. 2014). We thus hypothesize that *MELO3C002441* may play a role in resistance signaling and associated disease resistance responses. Further work, however, will be needed to clarify the functional role of this gene and to guide the development of PM-resistant melon cultivars.

The *MELO3C012438* resistance gene identified in this study, which was encoded on chromosome 10, was found to be 90% homologous to the cucumber *CsMLO8* gene that has previously been linked to partial PM resistance in cucumber stem, petiole, and hypocotyl tissues. *CsMLO8* is a recessive resistance gene and a member of the MLO gene family identified within the region of this chromosome identified via finally-mapping. Insertion of a transposon into the transmembrane domain of the gene resulted in gene loss of function (Berg et al. 2015). The *MELO3C012438* gene was found to harbor a non-synonymous SNP when comparing parental melon lines in this study. As such, our analysis was the first to identify a melon MLO gene via a forward genetics approach. Such MLO genes are widely known to govern plant biotic stress responses in other species. For example, *MdMLO* upregulation occurs in apples in response to exposure to exogenous salicylic acid and other stimuli (Pessina et al. 2014). MLO gene expression in barley has also been found to rise following wounding or exposure to *Magnaporthe grisea*, paraquat, and wheat powdery mildew exposure (Han et al. 2003). Overall, the PM resistance candidate genes identified herein offer important new insights into the mechanisms governing PM resistance in melon plants and may guide the generation of resistant germplasm resources.

Declarations

Author contribution statement

FL and PG conceived and designed the study. CF developed the population. XW, LT, and YB conducted the field experiments. HC performed sequencing data analysis. ZD wrote the manuscript. It is worth noting that HC, CF, and ZD are co-first authors.

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

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Figures

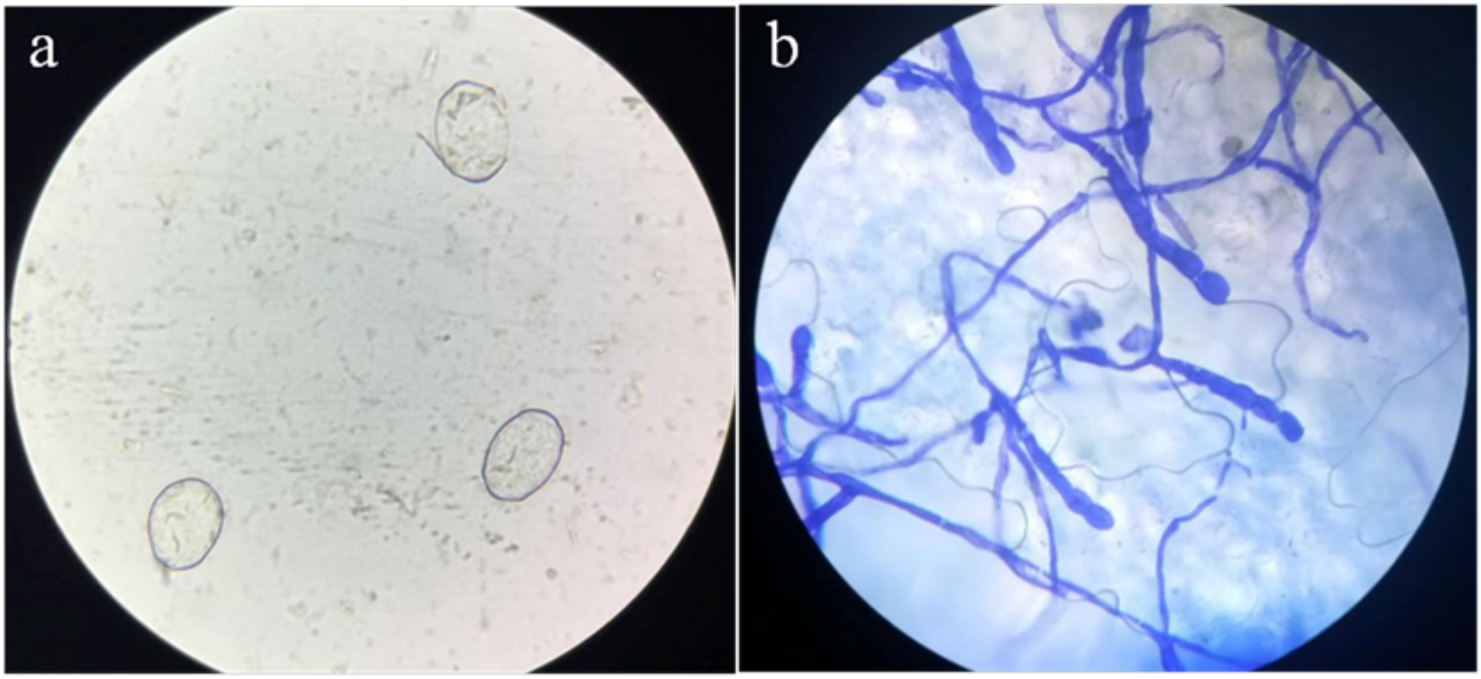

Figure 1

Representative spores isolated from melon leaves and colonies on melon leaves a: The spores isolated from melon leaves (10 × 40, Optical microscope) b: Colonies on melon leaves stained with Coomassie brilliant blue (10 × 25, Optical microscope).

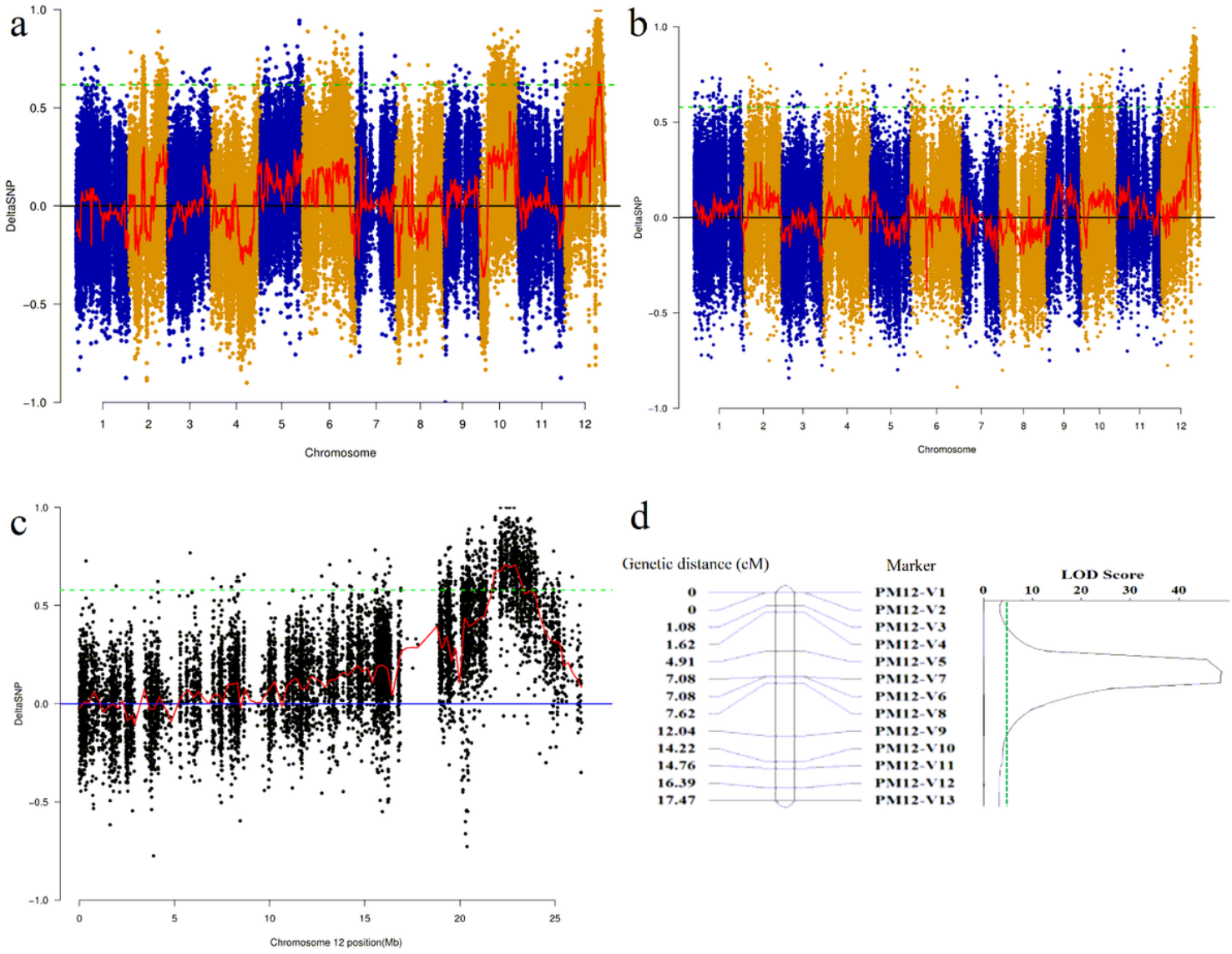

Figure 2

(a) The results of △SNP-index value comparisons for the LRSR-pool and the LSSS-pool. (b) The △SNP-index results when comparing the LRSS-pool and the LSSS-pool. (c) Preliminary mapping of dominant disease resistance genes on chromosome 12 in melon. (d) Verification of BSA localization results on chromosome 12. Note: The X-axis indicates the position on melonchromosome 12, and the Y-axis indicates △SNP-index values. The dotted green line is the threshold under the 95% confidence interval.

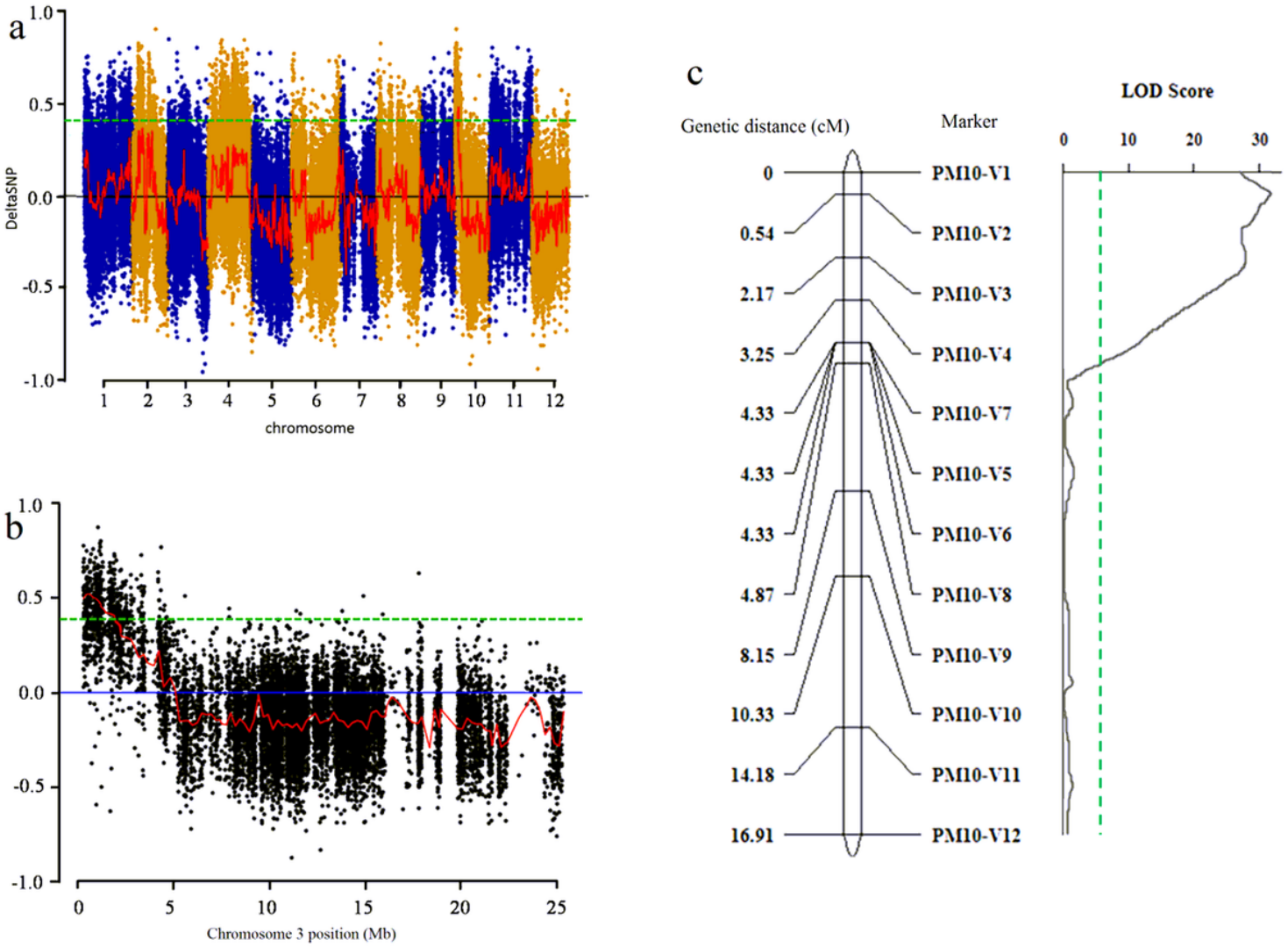

Figure 3

(a) △SNP-index results for the LRSR-pool and the LRSS-pool. (b) Preliminary mapping of recessive disease resistance genes onmelonchromosome 10. (c) Verification of BSA localization results on chromosome 10. Note: The X-axis indicates the position on chromosome 10 in melon, and the Y-axis indicates △SNP-index values. The dotted green line is the threshold under the 95% confidence interval.

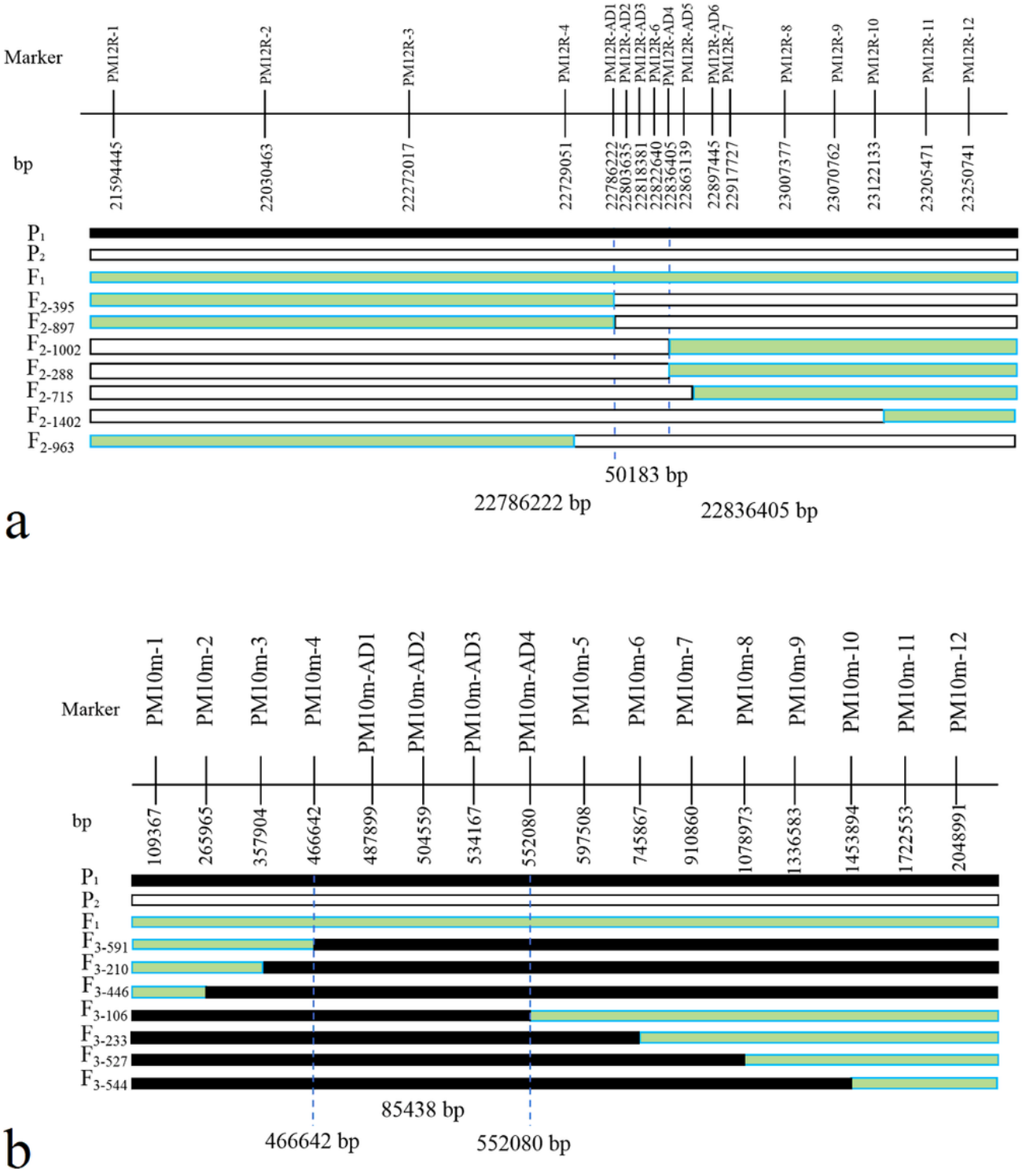

Figure 4

(a) Finally-mapping results forCmPMRl. (b) Finally-mapping resultsforCmPMrs.

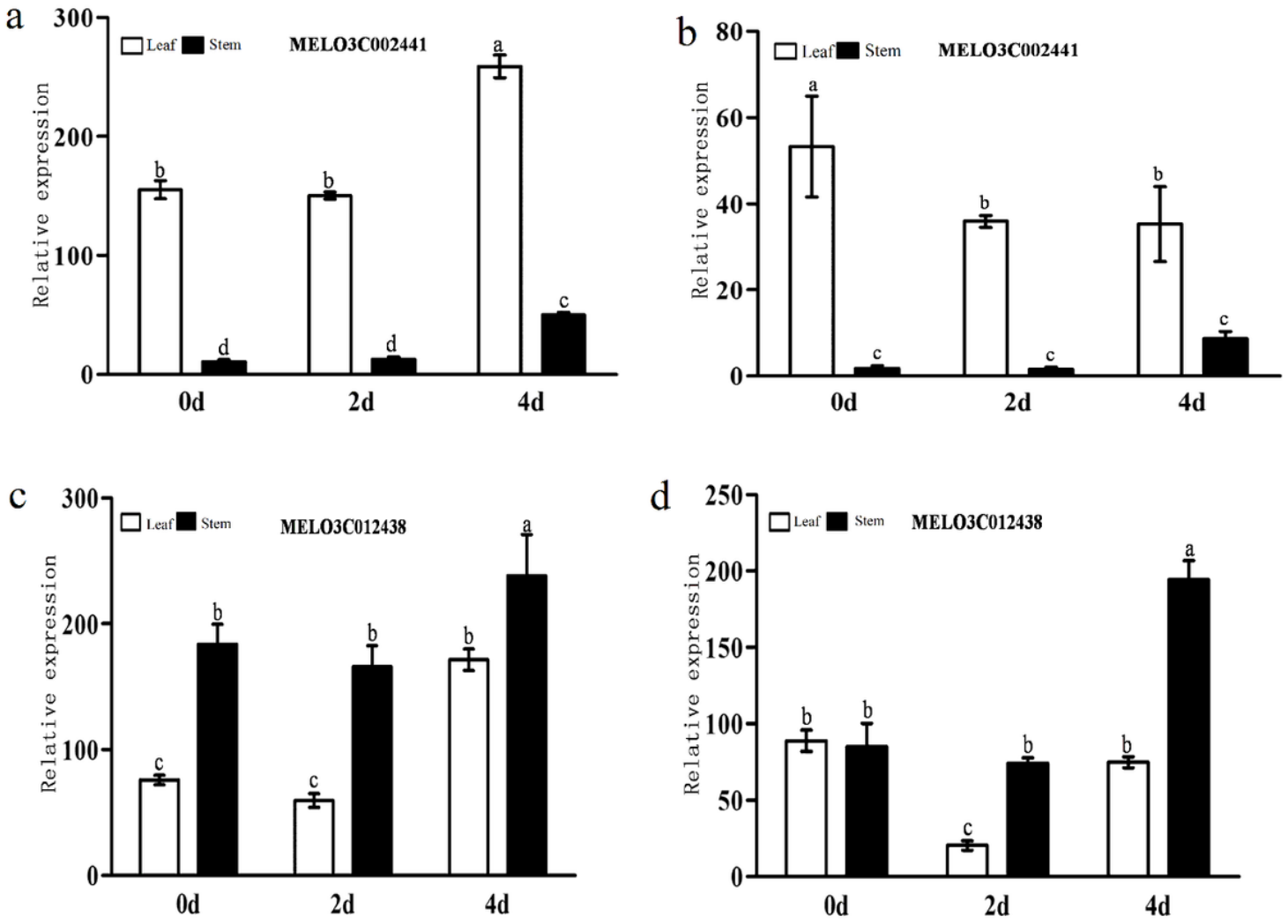

Figure 5

Relative MELO3C002441 expression in leaves and stems a: MR-1 c: M4-7. Relative MELO3C012438 expression in leaves and stems b: MR-1 d: M4-7.Identical letters on bars indicate a lack of any significant difference in expression between these bars, while different letters indicate significant differences in expression.

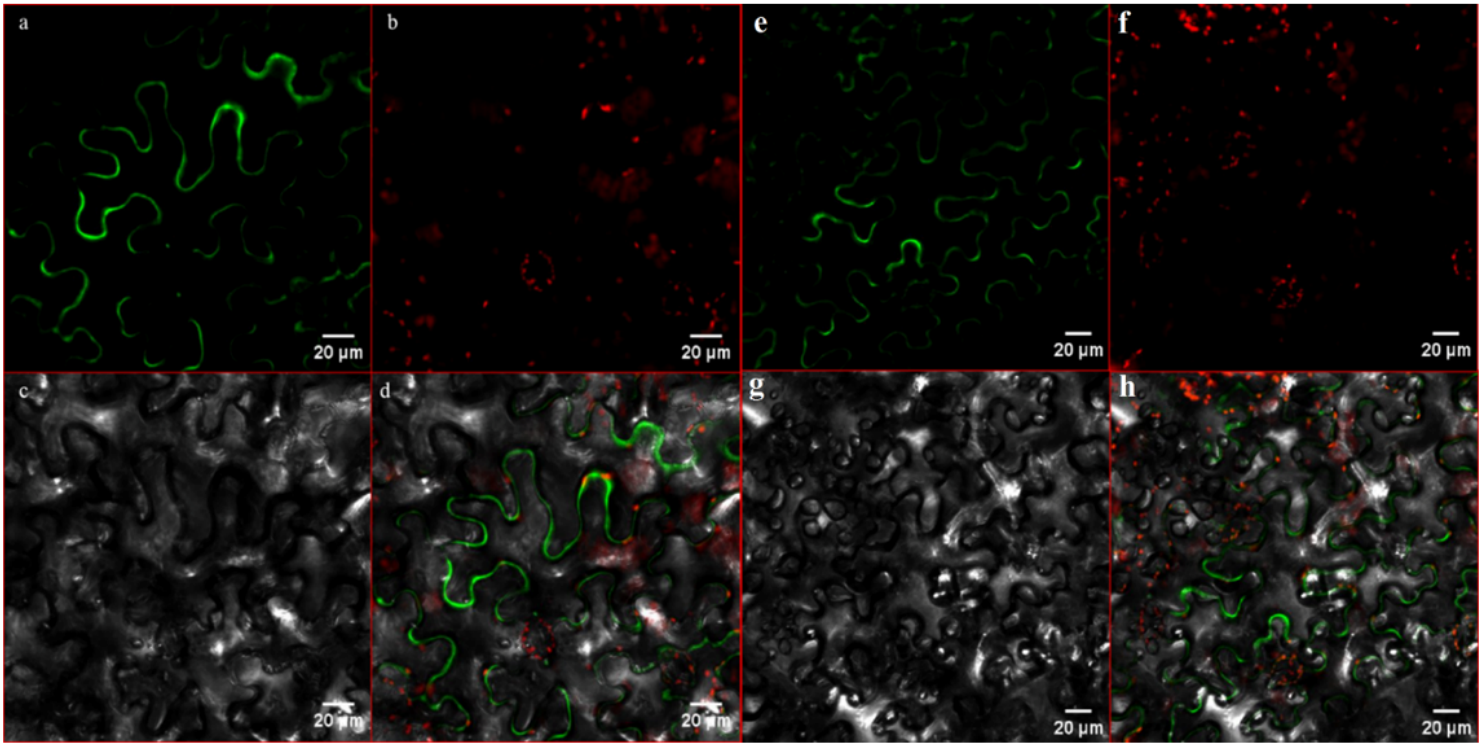

Figure 6

MELO3C002441 subcellular localization. a. MELO3C002441-GFP fluorescent signal. b. Chloroplast fluorescent signal. c. No FITC excitation. d. MELO3C002441-GFP and chloroplast merged image. MELO3C012438 subcellular localization. e. MELO3C012438- GFP fluorescent signal. f. Chloroplast fluorescent signal. g. No FITC excitation. h. MELO3C012438-GFP and chloroplast merged image.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigure.docx
- SupplementaryTable.docx