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Development of PCR-based Markers Associated with Powdery Mildew Resistance using Bulked Segregant Analysis (BSA-seq) in Melon

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Abstract

Powdery mildew (PM) is a fungus that causes disease in both the field and the greenhouse. Utilizing resistant cultivars is the most effective approach of disease management. To develop insertion-deletion (InDel) marker associated to this trait, whole genome of PM resistant line M17050 (P1) and PM-susceptible line 28-1-1 (P2) were sequenced. A total of 1,200 InDels, with an average of 100 markers per chromosome, were arbitrarily chosen from the sequencing data for experimental validation. One hundred InDel markers were ultimately selected due to their informative genetic bands. Further, an F2 segregating population of melons generated from these two parents was inoculated by PM pathogen. Based on bulk segregant analysis (BSA) using these 100 InDel markers, the powdery mildew resistance was associated with the genomic region *LVpm12.1* on melon chromosome12. This region overlapped the previously described QTL-hotspot area carrying multiple PM-resistance QTLs. Moreover, conventional QTL mapping analysis was done, which located *LVpm12.1* in the region between 22.72 Mb and 23.34 Mb, where three highly polymorphic InDel markers MInDel89, MInDel92, and MInDel93 were detected. Therefore these markers could be used to track this resistance locus in melon while the lines carrying this locus could be employed in PM melon resistance breeding programs after validation test.

Keywords: Powdery mildew; Resistance QTL; InDel marker; Whole genome re-sequencing; Melon

Introduction

Melon (*Cucumis melo* L.) is a member of the Cucurbitaceae family and an important horticultural economic crop with an annual global production of over 29 million tons [**1,2**]. Powdery mildew (PM) is a fungus that affects the leaves, petioles, and stems of most cucurbit crops in both field and greenhouse settings [**3**]. This disease can cause a decrease in weight-based productivity and a decrease in fruit quality [**4**], resulting in severe economic losses in many regions of the globe [**5**]. On melon, two biotrophic fungi typical of powdery mildew, *Podosphaera xanthii* and *Golovinomyces cicoriacearum*, were identified [**6, 7, 8, 9, 10, 11, 12**]. In *P. xanthii*, over 28 physiological races have been identified based on their responses to various melon varieties, and which are relatively dispersed across various geographical regions [**13, 14, 15**]. In Southern European regions, the most prevalent races are 1, 2, and 5 [**16, 17**]. In America, races 1, 2, and 3 are prevalent, while in France, races 0, 4, and 5 have been discovered [**13**]. In Japan, races 1, N1, N2, and 5 have been identified as the causal agents of powdery mildew in melon [**14**], while the other seven races 1, 2, 3, 4, 5, 6, and 7 have also been reported [**18**], followed by a new race N5, PxA and PxB [**19,20**]. The major Chinese races are pxCh1, 1, and 2F [**21, 22, 23, 24**]. In China, P. xanthii is generally considered to be the primary cause of PM in melon [**22,24**], and the recent reports on PM in Shanghai support this assertion [**25,26**]. Currently, the most common method for controlling powdery mildew is the application of chemical fungicides, which can be laborious and time-consuming [**27**]. Chemical control could result in the emergence of resistant pathogens, as well as increase the cost of melon production and pollute the environment [**28, 29**], rendering it ineffective. Searching for QTLs that confer PM resistance and introducing them into commercial varieties is an ideal strategy for PM control in cantaloupe.

Pm-w from WMR 29 [**30**], *Pm-x* from PI414723, and *Pm-y* from VA 435 [**31**] are examples of genes and QTLs in melon that confer resistance to powdery mildew. PI124112 was found to contain *PmV.1* and *PmXII.1* [**32**]. *Pm-1* was isolated from AF125Pm1, a Cantalupensis Charentais-type breeding line [**33**], while *Pm-pxA* and *Pm-pxB* were mapped from AR 5 [**33, 19**]. The *pm-8* QTL was carried by PI 134198 [**21**], *Pm-R* by TGR-1551 [**34**], and *pm-2F* by K7-1 [**35**]. [**36**] have identified an additional resistant QTL in PM5. The *pm-PMR6* QTL was identified by means of PMR6 [**37**], *qPM2* by means of TARI-08874 [**38**], and *BPm12.1* by means of MR-1 [**11**]. The QTLs *CmPMRI*, *CmPMrs*, *pm2.1*, and *pm12.1* were successively identified in PMR6 [**39**], whereas *qCmPMR-12* was isolated from wm-6 [**40**]. bHLH, ERF, and MYB families in PM-resistant TG-1 were found to play a crucial role in the interaction between melon and powdery mildew pathogens, as determined by transcriptomic analysis [**41**]. In addition, two genes, *MELO3C002434* and *MELO3C002393* (against race5), are associated with PM resistance in melon [**40, 42**]. More QTL and genes for resistance against PM in melon were also identified, including *Pm-2*, *Pm-3*, *Pm-4*, *Pm-5*, *Pm-6*, [**43, 44**], *Pm-x1*, *Pm-x3*, *Pm-x5* [**45**], *Pm-Edisto47-1*, *Pm-Edisto47-2* [**46**], *Pm-An* [**47**]. In addition, a number of these genes and QTLs have been identified on chromosomes 1, 2, 3, 4, 5, 10, and 12. Controversy surrounds the genetic basis of PM resistance in

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melons. Some studies suggest that PM resistance in melon is controlled by a single dominant gene **[48, 49, 21, 34, 38, 11, 40]**, whereas others suggest that it is controlled by a recessive gene. In addition, [**32**] state that the resistance of melon to PM is governed by various QTL.

Bulk segregant analysis (BSA) and conventional QTL analysis are two important QTL mapping techniques. BSA analysis **[19, 21, 38, 50]** and QTL mapping techniques **[51, 37, 11, 40]** were extensively utilized in melon. Due to the low cost of whole-genome re-sequencing (NGS), BSA has been combined with NGS to map genes of interest. With the release of the genome sequence, numerous markers for high-resolution genetic linkage and QTL mapping became accessible. BSA-Seq has been applied to cucumber **[52, 53, 54]**, rice **[55, 56],** soybean **[57, 58]**, and melon **[57,58, 40]**. Therefore, the combined use of BSA and next-generation sequencing (BSA-Seq) has accelerated the identification of quantitative trait loci (QTL) markers with strong links **[59]**. Marker-assisted selection (MAS) is a powerful genomic tool that aids phenotypic selection in the development of disease-resistant cultivars and can aid breeders in incorporating and pyramiding resistance genes, thereby reducing disease severity **[60, 61, 62]**. Currently, MAS is utilized extensively to identify the molecular markers associated with a specific trait during the development of disease-resistant cultivars **[33]**. Numerous molecular markers have been developed to detect allelic variation in different samples at the DNA level **[63]**. In breeding programs, the most prevalent markers are high-throughput genotyping markers like SNPs, KASP, and HRM markers, as well as PCR-based markers like InDels and SSR markers. In contrast to PCR markers that can be resolved on a gel, SNP and KASP genotyping require a relatively complex platform. Based on our knowledge and technical expertise, breeders readily accept the use of agarose gel electrophoresis for genotyping due to its simple requirements and straightforward laboratory operation. The development of PCR-based markers as a milestone in the development of breeding program markers was deemed significant by us. This will improve the efficiency and effectiveness of plant genotyping by accelerating the procedure during breeding programs. In plant genotyping, it has been reported that PCR-based InDel markers are reliable and effective **[64, 65, 66, 56, 67, 68]**. Identification of PCR-based Indel markers associated with PM resistance from PM-resistant melon resources may hasten the development of PM-resistant melon varieties **[19, 69]**. In this study, PCR-based InDel markers associated with PM resistance were developed using PM the whole genome sequencing data of PM resistant line and PM susceptible line in BSA analysis and QTL mapping *LVpm12.1*. These markers, in occurrence MInDel92, could be utilized to identify this resistant locus in melon.

Materials and Methods

Plant materials, plant growth conditions and inoculation with powdery Mildew fungus

Two melon breeding parents, M17050 (P1) and 28-1-1 (P2), which belongs to the Cantalupensis group of muskmelon species, *C. melo* ssp. *melo,* were used for whole genome re-sequencing. These two parents had different reactions to powdery mildew. Both these lines are lightly ribbed with sweet and flavorful flesh and have a reddish orange flesh color and a reticulated (net-like) peel. M17050 is a PM-resistant breeding line (R-line) with sutured and light gray-green peel, whereas 28-1-1 is a PM-susceptible breeding line (S-line) with no-sutured and dark gray-green peel (Figure 1 a-d). An F2 population consisting of 125 individuals, which was developed from F1-6 that was derived from crosses between M17050 and 28-1-1, has been subjected to artificial inoculation. The individuals from the segregant F2 population, which expressed different responses to PM disease, were used for BSA analysis and QTL mapping. All the plants were grown in the solar greenhouse of the Suqian Green Port Modern Agriculture Research Institute. For the QTL mapping of PM resistance in melon, M17050 (P1, resistant to PM) and 28-1-1 (P2, susceptible to PM) were used as parental lines to generate F2 populations. P1, P2, F1-6, and F2 individuals were all grown in a glasshouse with a temperature range of 22-28 degrees Celsius (day/night) and a relative humidity range of 60 to 75 percent. The PM fungus (P. xanthii) used in this study was isolated from the leaves of diseased melon plants according to the method of **[70]** with minor modifications (Figure 1e-f). In this experiment, the PM fungus was maintained on susceptible melon plants in the artificial growth chamber of the Suqian Greenport Modern Agriculture Research Institute (Figure 1g). The fungus was collected and suspended in sterile distilled water containing 0.02 percent Tween 20 before being used to inoculate plants at a concentration of 1 x 1^{06} as described by **[71]**.

Disease evaluation for resistance to Powdery Mildew

The plants were observed weekly from the fourteenth day after inoculation (dpi) until the flowering stage. Based on extremely high levels of resistance and susceptibility, the severity of powdery mildew disease was classified as follows: In Class 0, there is no infection on the leaf surface, whereas Class 1, there is infection and heavy sporulation on the entire leaf. For the inheritance study, lines without powdery mildew sporulation on the leaf were deemed highly resistant, while lines with powdery mildew sporulation on the entire leaf were deemed highly susceptible. Based on the same classification, resistant and susceptible plants in the F2 population were examined. Here, 30 plants from each

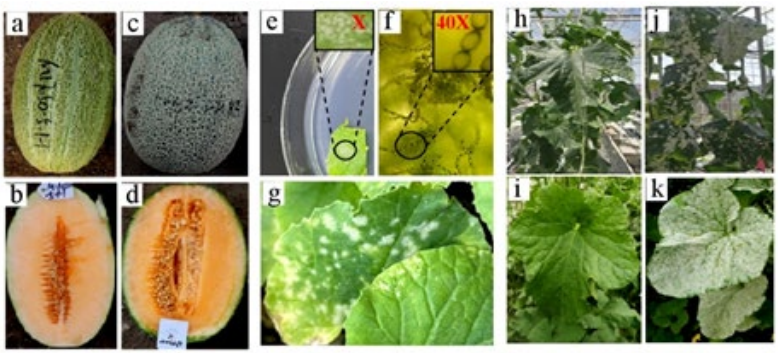

Figure 1: Fruits morphology and PM disease evaluation. Fruit appearance and fruit longitudinal section of P1 line (a-b) and P2 line (c-d) showing skin and flesh characteristics; Powdery mildew pathogen single spore isolation (e) followed by conidiophore and conidia observation under light microscope (f), Maintenance of PM disease on melon leaves using artificial inoculation in growth chamber (g); Leaves from P1 and resistant F2 plant (h-i) and that from P2 and susceptibility F2 plant (j-k); X and 40X, represents high magnification observation.

parental line, 50 plants from hybrid F1-6, and 125 plants from the F2 population were sampled and analyzed. Only plants from classes 0 and 1 were accounted for in the disease evaluation.

Library construction, sequencing, data filtering, alignment and variants calling

The CTAB extraction method was used to isolate genomic DNA from the fresh leaves of 30-day-old greenhouse-grown M17050 and 28-1-1 plants. Electrophoresis of a 5μL aliquot of extracted DNA on a 1% agarose gel and measurement of concentration with a Nanodrop spectrophotometer 2000 were used to determine the DNA's purity (Thermo Scientific, USA). For library construction, samples with more than 10 ng/μL of genomic DNA at OD260/280 values between 1.8 and 2.0 were considered. Initially, genomic DNA was sheared using an ultrasonic crusher (Ultrasonic Crusher Q800R3, Qsonica Co Ltd, USA) to produce DNA fragments averaging 350–500 base pairs in length (bp). According to the manufacturer's instructions, these fragmented DNA samples were purified using AMPure XP beads (http://www.beckm.com/ancou lter.cn) and freshly prepared 80 percent ethanol. The fragmented DNA was subsequently subjected to DNA end repair, library size selection, adenylation, and Illumina paired-end adapter ligation. The amplified ligated DNA products were selected. For each melon breeding line, two paired-end libraries with 15-fold depth were generated using the TruSeq DNA LT Sample Prep kit. On an Illumina Hiseq X Ten, PE150sequencer, the resultant libraries were sequenced (Shanghai OE Biotech. Co. Ltd., China). All procedures followed the OE Biotech Company's deep sequencing protocol (Shanghai OE Biotech. Co. Ltd, China). Version 3.6.1 of the Cucumis melo L. var. DHL92 genome sequence was obtained from the cucurbit genomics database (CuGenDB) (http://cucurbitgenomics.org/ftp/genome/melon/DHL92/v3.6.1/) and used as the reference sequence. A custom C program with the default parameters was used to eliminate low-quality reads data in order to recover clean reads data. The read data were aligned to the reference genome using the BWA (BWA0.7.10-r789) application. Using SAM Tools, the alignment output results in SAM format were converted into Binary Alignment Map (BAM) file format. The Mark Duplicates in Picard (v1.102) tool was used to eliminate duplicate reads, and the two BAM files were utilized for subsequent analyses. Using the bioinformatics program Genome Analysis Tool Kit (GATK) version 3.1 (https://gatk.broadinstitute.org/hc/en-us), local realignment, InDel filtering, and calling were accomplished.

InDels flanking sequences and primers designing

The polymorphism analysis was carried out in accordance with the protocol described by **[70]**, with minor modifications to identify InDel polymorphisms between the re-sequenced M17050 and 28-1-1. The reference genome sequence of melon variety DHL92 version 3.6.1 was examined to determine the InDel polymorphisms between the re-sequenced M17050 and 28-1-1. M17050 and 28-1-1 sequence reads were individually aligned to the reference sequence using the Short Oligo-nucleotide Alignment Program (SOAP) software, with no gaps counted. M17050 and 28-1-1 aligned reads datasets were compared, and polymorphic insertion/deletion sequences were detected, as described by **[68]**. 150-nucleotide sequences flanking both ends of an insertion/deletion site were extracted to create InDels markers. These sequences were extracted from the reference genome sequence using a simple Visual C++ script. The sequences were then used as templates for designing primers. Primer 5 (http://www.PromerBiosoft.com) was used to create PCR primers with a variety of properties (lengths ranging from 18 to 28 bp, Tms ranging from 57 to 63°C, and PCR products ranging from 80 to 300 bp).

DNA extraction and polymerase chain reaction

Total genomic DNA was extracted from fresh leaves of 35-day-old seedlings of 6 individuals each from the M17050, 28-1-1, 125 F2 mapping population, and a group of 192 melon breeding lines using the NuClear Plant Genomic DNA Kit (CWO531M) protocol (CWBiotech, Beijing, China). The DNA was adjusted to a final concentration of 50 ng/μL. In this study, two PCR reactions were carried out depending on the size of the PCR products, thereby determining the nature of the gel used for electrophoresis (with 160 volts). For agarose gel electrophoresis, 25μL of PCR reaction mixture containing 12.5μL 2xTaq MasterMix plus loading buffer (CWBiotech, Beijing, China), 1μL of each forward and reverse primer at 10μM, 1μL of DNA, and 9.5μL of nuclease free water was used. In the case of polyacrylamide gel analysis, a PCR tube was filled with a 10μL PCR reaction mixture containing 1μL of DNA extract, 3.4μL of 2xTaq Master Mix without loading buffer (CWBiotech, Beijing, China), 0.8μL of each forward and reverse primer at 10μM, and 4μL of nuclease free water. The following conditions were used for the amplification reaction: initial denaturation at 94 °C for 2 minutes, 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for 30 seconds followed by 72 °C for 2 minutes. PCR products from a 25μL reaction mix were separated on a 2 percent agarose gel in 0.5XTAE buffer and stained with ethidium bromide, whereas those from a 10μL reaction mix were separated on an 8 % polyacrylamide gel and stained with silver. The agarose and polyacrylamide gels were observed under UV and white light, respectively.

Bulked segregant analysis and QTL mapping

Four DNA pools were utilized for bulk segregant analysis, including two parent bulks and two F2 segregating bulks. The parent bulks were independently derived from the PM-resistant line M17050 and the PM-susceptible line 28-1-1. The two F2 segregating bulks were generated by combining an equal amount of DNA extracted from 30 extremely resistant (R-bulk) and 30 susceptible (S-bulk) F2 plants. Initially, polymorphism between the two re-sequenced lines was validated with 1200 arbitrarily chosen InDel markers using a PCR-based method. These validated markers were used to search for the polymorphism between two groups of DNA, such as group1 consisting of DNA from PM-resistant line M17050 and R-bulk and group2 consisting of DNA from PM-susceptible line 28-1-1 and S-bulk. On the other hand, the two parents and their derivative F2 population were genotyped using the informative InDel markers discovered on melon chromosome 12 for QTL analysis. Utilizing JoinMap 4.0 software **[72]**, a new genetic linkage map was generated. **[73]** mapping function was used to calculate map distances, and the genetic map was created using the MapChart 2.2 program **[74]**. The software WinQTLCart2.5 was used to detect QTL from genotypic and phenotypic data using composite interval mapping (CIM) **[36]**. WinQTLCart was executed using the CIM control parameters Model 6, forward stepwise regression, 10 cM window size, 2 cM step size, and 5 control markers. The threshold was determined through 1000 iterations of permutation tests.

Molecular screening validation assay

A panel of 192 melon breeding lines, including M17050 and 28-1-1, were molecularly screened using the newly developed markers and the six putative PM resistance-based markers MR-1, Mu7191, CMBR111, DM0191, SSR12407, SSR12202 Fang et al., unpublished; Syngenta, unpublished; [75, 37], which have been reported on melon chromosome 12 (Table S1). For the publicly available markers, genetic bands were denoted R for resistant allele and S for susceptible allele

Table S1: QTLs and genes reported in the QTL-hotspot region on melon chromosome12.

Markers/genes	Forward sequence (F)	Reverse sequence(R)	F Position	R Position	QTL/gene
CMBR111	TTTTCCTCCATTTTAACTTAGCC	AAGAGAGAAGCCATGGATGAA	22689820	22689911	*Pm-PMR6-1*
MIndel-89*	TGGAGAACTATGGAATATGGAGGA	GCTTACGGATGAATTTGGACTTGA	22717662	22717921	*LVpm12.1*
MIndel-92*	CTTGGGAGGATGAGTGAAGGTAAA	TTCGTACTTGAGTTGACTTACGGT	22778911	22779210	*LVpm12.1*
SSR12202	AGATTTGGAAGGATGTTAGA	AAGTCGGGTGGTAGTTGTTT	22799912	22800109	*Pm-PMR6-1*
MR-1	AATCTATCCCAAATCAAAGTC	AAGTTATATTGGTCTAGAAGTTT	22804440	22804747	*BPm12.1*
Mu7191	CTCTATCAGCTCAAAGGCCG	TTCGTCCTCGTTCTCTTGCT	22939658	22939784	*Pm-PMR6-1*
SSR12407	TAAAAATGACCATAGCACC	AAGTAAATGGCAGACAGAAC	23026248	23026229	*Pm-PMR6-1*
MIndel-93*	GGTCCTTTTGATGCCATTTGAAGA	TCGTATAGGTCGTTGATGCAAACT	23338823	23339049	*LVpm12.1*
DM0191	TTGAAGGTCTTTGCTGAAG	GCCTACCACATTTAATTCC	23563739	23563878	*Pm-PMR6-1*
MIndel-95	CCTTGTGTTGGATTGTGACATGAA	GTTTGTAGGTAGAGAGGGGAGTTG	23801350	23801608	*LVpm12.1*

* Markers developed in this study

while the two polymorphic DNA fragments amplified using markers MIndel89, MInDel92 and MInDel93 were labeled x and y.

Results

Inoculation and disease response in P1, P2, F1 and F2 population

Powdery milew disease happened naturally in a greenhouse while 20 melon hybrid combinations were being tested for resistance to fruit blotch disease. F1-6, which is a cross between M17050 and 28-1-1, has shown a high resistance to PM without sporulation on the leaf surface (Data not shown). To investigate the observed PM disease resistance, single spore isolation and the development of an F2 population were undertaken. To provide fresh spores for plant inoculation, the isolated spores were maintained on a highly susceptible melon variety (Figure 1e-g). Upon inoculation, P1 exhibited a resistant response with no sporulation on the leaf surface, while P1 was highly susceptible to powdery mildew disease (Figure 1h-i). The disease resistance in F1-6 confirmed the previous observation, whereas in the F2 population, all the resistant plants were categorized as class0 and all the susceptible plants as class1 (Figure 1j-k). PM response in the F2 population corresponded to Mendelian segregation with a ratio of 3:1 (resistant plants: susceptible plants), indicating that PM disease resistance in this study may be controlled by a single dominant gene.

Re-sequencing and InDel markers development from P1 and P2

The clean read quantity generated was 137,000,000 for P1 and 298,000,000 for P2 recordings, with an average of 217,500,000. The quality score of greater than 30 (>Q30) was 94.92 percent for P1 and 94.61 percent for P2 with an average of 94.77 percent, whereas the GC content was 36.87 percent for P1 and 36.61 percent for P2 with an average of 36.74 percent (Table 1). Using the Burrows–Wheeler Alignment (BWA), a total of 99,004,197 and 185,000,000 (average 142,022,098) reads from P1 and P2, respectively, were mapped at a depth of 10 to the reference genome sequence of *Cucumis melo* L. var. DHL92. (Table 2) displays that the overall genome coverage (10X) was 77.25 percent for P1 and 77.77 percent for P2, with a mean of 77.51 percent. Genome-wide insertion/deletion polymorphism created a total of 320,016 InDels between P1 and DHL92, with an InDels density of 853.38 InDels/Mb, and 324,885 InDels between P2 and DHL92, with an InDels density of 866.36 InDels/Mb. Between P1 and DHL92, the distribution of these InDels across the 12 melon chromosomes ranged from 15065 on chromosome09 to 40239 on chromosome04, whereas between P2 and DHL92, the range was 16372 on chromosome09 to 37300 on chromosome04 (Table S2 and Table S3). The alignment of reads from P1 and P2 yielded a total of 29393 InDels with a density of 78 InDels/Mb. These InDels are distributed across the 12 chromosomes of the melon, with chromosome09 containing the fewest (997), and chromosome06 containing the most (1,011). (5906). There was an equal

Table 1: Quality control summary of sequenced reads.

Samples	Raw_Reads	Clean_Reads	GC_Content	>Q30
P1	1.41E+08	1.37E+08	36.87%	94.92%
P2	3.08E+08	2.98E+08	36.61%	94.71%

Table 2: Mapping summary of sequenced reads.

Samples	Mapped reads	Mapped Rate	10X_coverage
P1	99004197	96.26%	77.25%
P2	1.85E+08	97.19%	77.77%

Table S2: Total InDels detected between P1 and DHL92.

chr	1-10bp	11-20bp	21-30bp	>30bp	total
chr00	14201	819	283	291	15594
chr01	20419	1199	384	509	22511
chr02	15019	780	288	309	16396
chr03	26440	1740	625	706	29511
chr04	35976	2395	852	1016	40239
chr05	22513	1430	527	608	25078
chr06	20770	1214	411	464	22859
chr07	25242	1648	601	633	28124
chr08	16732	819	281	300	18132
chr09	13801	714	257	293	15065
chr10	21727	1517	547	665	24456
chr11	32644	2332	816	902	36694
chr12	22775	1505	503	574	25357

Table S3: Total InDels detected between P2 and DHL92.

chr	1-10bp	11-20bp	21-30bp	>30bp	total
chr00	15446	919	326	339	17030
chr01	20914	1243	414	507	23078
chr02	18356	1051	404	451	20262
chr03	26037	1743	624	694	29098
chr04	33419	2162	762	957	37300
chr05	24144	1527	574	669	26914
chr06	20311	1160	375	437	22283
chr07	24385	1580	591	611	27167
chr08	21151	1243	454	475	23323
chr09	14889	825	301	357	16372
chr10	20112	1305	468	593	22478
chr11	29987	2104	708	867	33666
chr12	23209	1547	519	639	25914

variation in density ranging from 2.66 InDels/Mb on chromosome09 to 15.75 InDels/Mb on chromosome06 (Table 3). In relation to the length of the nucleotide sequence, three distinct types of insertions and deletions were identified and classified as small (1-10bp), medium (11-30bp), and large (>30bp). Large, medium, and small InDels represented 2.62 percent, 8.58 percent, and 88.80 percent, respectively, of the total genome-wide InDels annotated between P1 and P2.

Bulk segregant analysis, mapping of *LVpm12.1*

Bulked segregant analysis (BSA) is a technique used to identify genetic markers associated with a trait of interest. To discover genomic regions conferring PM disease resistance or susceptibility in this work, bulked segregant analysis was conducted using four DNA pools, consisting of two parent bulks and two F2 segregating bulks. One thousand two hundred InDel markers were selected for polymorphism analysis between the two parent bulk DNA using a PCR-based method. The result revealed 100 markers to be strongly polymorphic with clear band patterns between P1 and P2 (Table S4). Further, BSA analysis was performed on the four bulk DNA samples, including parent bulks and two F2 bulks, using the highly polymorphic markers. Here, clear, reproducible, and polymorphic genetic bands were produced by six InDel markers on melon chromosome 12 between the resistant DNA bulks (P1 and R-bulk) and susceptible DNA bulks (P2 and S-bulk) (data not shown). Graphical representation of parents, F1-6, and 3 recombinant individuals each from resistant and susceptible F2 plants has highlighted the suspected genomic region conferring PM-resistance (Fig.2 a). The markers MInDel76, MInDel81, MInDel89, MInDel92, MInDel93, and MInDel95 were found to be closely linked to this region. Named *LVpm12.1*, this QTL region was located between MinDel81 and MinDel93 (Fig.2 a). To confirm this finding, traditional QTL mapping was conducted through linkage map construction using JoinMap_4.0 software and QTL analysis using WinQTL software successively. The six markers detected in BSA analysis on melon chromosome 12 were used. Two QTL positions were obtained; one is non-significant and closed to MinDel81, while the second is highly significant and closed to MInDel93. The latter was located between both MInDel89 and MInDel92, and MInDel93 (Fig.2b). The BSA analysis and QTL mapping have placed *LVpm12.1* around markers MInDel89, MInDel92, and MInDel93. Genetically, the *LVpm12.1* was 8.7cM away from these markers, but it was in a region that went from 22.72 Mb to 23.34 Mb.

Table 3: InDels detected between P1 and P2.

chr	1-10bp	11-20bp	21-30bp	>30bp	total
chr00	942	73	21	20	1056
chr01	2467	147	46	71	2731
chr02	1578	105	38	48	1769
chr03	1491	137	34	57	1719
chr04	1681	113	34	53	1881
chr05	3570	256	99	113	4038
chr06	5236	403	137	130	5906
chr07	1080	70	22	34	1206
chr08	1524	95	38	35	1692
chr09	868	73	21	35	997
chr10	2265	159	59	67	2550
chr11	1620	121	52	49	1842
chr12	1780	119	51	56	2006

Characterization and validation of InDel markers associated to *LVpm12.1*

Among the six previously reported genetic markers utilized for molecular screening (MS) assay, only marker Mu7191 behaved appropriately under our imposed PCR conditions. The MS results of 192 melon breeding lines (96 melons with reticulated peel and 96 melons without reticulated peel) using the newly developed markers were compared to those of marker Mu7191 in order to validate their resistance diagnostic ability. Each marker produced polymorphic PCR products throughout the molecular screening. The consistency of

Table S4: Highly Polymorphic InDel markers experimentally validated between P1 and P2 on melon chromosome12.

Chr.	Marker name	Position	Forward primers	Reverse primers	Expected amplicons size
chr12	MInDel1	20008481	GGCAGGTCCCATACAGATTCT	ACCTTCAGAGTTTAGTCAGTTAGGT	299
chr12	MInDel2	20008481	GGCAGGTCCCATACAGATTCT	ACCTTCAGAGTTTAGTCAGTTAGGT	299
chr12	MInDel3	20018932	GAAGAGTGGTGAACGGAAAATAGC	CTACGGCCGGCATTTTAGTTATTT	269
chr12	MInDel4	20054385	GGGGAAGAGTTATTATTGGTAGTGT	CGTAATCTTTGCCCCATACAAGTT	268
chr12	MInDel5	20081744	ATACAATCGAACAACCCAACCCAA	GATCAAGCATCGAACAACACAAGA	299
chr12	MInDel6	20083684	TTTTGAGGAAGACGTGGAGAAAGA	CAAGTGGCTAATATCATTGGTCCT	167
chr12	MInDel7	20117354	GGATTTAACTACTACTCCCAGGCC	GAATGATTCTACACAGTAGGGGCT	277
chr12	MInDel8	20134533	GGTAAGGAATGCGCACTCTACTAT	CAGGAAATCTACCAATGCTTGACC	268
chr12	MInDel9	20141461	GAGCAAGGAATTTCGACCAATGAT	AGCTTTCGTTCTTCCTTTACTCCA	257
chr12	MInDel10	20155201	AAGAGCCCTAAACACAACTTCCTA	CGAACAGTTCTGGAGATACTACGA	293
chr12	MInDel11	20191207	GATCCGCCACTGATTCAAGT	TTAAACCCTAGCAAATTCACTCGC	289
chr12	MInDel12	20204301	AACCAAACCCATAAGACCTGAAGA	CAGGTTTGGATTGGATTGGATTGT	117
chr12	MInDel13	20209697	GAACGCACACATCAATCAGTCTAG	CGGGATTCCTCGACTGATATATGT	226
chr12	MInDel14	20218503	GACTGGCCAAATGTAAACGATTGT	TTTTGTTACACGATCGTTGAACCC	129
chr12	MInDel15	20620368	CTTCGAGATTGCACACTTTGTCTT	CCTATAGCTTCTTCACGCAAATGG	129
chr12	MInDel16	20694430	AAGAAGGAAATGGTTGCATTGGAG	TCTCTCCCTCTCTCTCACATTCTT	124
chr12	MInDel17	20740278	TTACCCCATTGATTTCACAGGTCT	CTTTCCCACCCTTTTCTCAGTTTT	280
chr12	MInDel18	20740352	TTACCCCATTGATTTCACAGGTCT	CTTTCCCACCCTTTTCTCAGTTTT	280
chr12	MInDel19	20740685	AAACAACCTAACATTGCTCACCTC	CTAATTGAGAAGGTGTTGCAGACC	203
chr12	MInDel20	20808160	AGTAGGCATTGTAGAGCTTTGTGA	CATTCCACCCATTGTCATTGTCAT	296
chr12	MInDel21	20983482	CTGCAACAATGTTTCACACTTTCC	TCTTCTTGAGTTGCTATCCTTCGT	235
chr12	MInDel22	20993210	TAGGGTTTGGGAGACTCATCATTC	AGGTCCATAAGTCATTTCCAACGA	286

chr12	MInDel23	20998229	GTGATTCGACCTTTAGTGGCAAAA	ATCTAAACTTGGCCAACTCACTTG	228
chr12	MInDel24	21001671	TGCTGGCGGAAGAAATTAAATGAG	GTCTAGAGCAGGGCAAAGATTTTG	204
chr12	MInDel25	21012231	CCTACAACTTCACCCACTTTTGAG	CGAGAATGTTTTACTAGCTCAACCA	263
chr12	MInDel26	21012734	ACGTGTTTACAATGTACCGTGA	TGGGGATAAAGTGAGGAAGAGTTG	173
chr12	MInDel27	21013801	ACTGGATTACATGACCACTAAGTG	CTAACAACTTATCACAAGTACTCGC	288
chr12	MInDel28	21014354	CGACTTGATAATAAGCACACGTGT	TGTGAACCTATTAGTCCACCAAGT	87
chr12	MInDel29	21016018	AAAATCGACCTACCTTCAACGA	TACTGTTGTTGTCCCACATATCGA	297
chr12	MInDel30	21202574	GTGGGGATTTGATTACGTGTTCAA	CAACTGGGAAGGATGACATGAAAA	273
chr12	MInDel31	21229417	GCGATCATGTTTTCCTCAACCTTC	CTACTTCGAGTGGCCTAAATGACT	250
chr12	MInDel32	21229663	AGTCATTTAGGCCACTCGAAGTAG	TTTCTCCACATGTAACTGTACCCA	174
chr12	MInDel33	21351602	AGCCATCAAAGCCCAAAGTCTATA	TGAATCCTTGAAAGTTTCCGACAC	100
chr12	MInDel34	21362591	CCAGACCCACCATGTAAAATACCA	AAAATCGATTAGCCAAACCTCGTG	217
chr12	MInDel35	21362703	CACGATTGTTTAGATTTGGACCCA	AAAATCGATTAGCCAAACCTCGTG	84
chr12	MInDel36	21363548	GGCCATTGAACCACACTTACAAAT	AATCCATAGACGACCACAAGAGTC	246
chr12	MInDel37	21374693	CAAACGAAGCCTAGACTCAAACTC	TTTTGTGCCGTGGAAAGATAGAGA	285
chr12	MInDel38	21377989	GTGATATGTACCCTTTCTGAACACA	AGTTCAACAATGTATGAGAGGGTGA	145
chr12	MInDel39	21378699	CCCCTTCTCCTTCTCACTTTATTT	TTAAAGGTATATCTAGGGAGGGCC	129
chr12	MInDel40	21382890	CGATTGCAAAACCTACAAGAGAGT	GGGCGTGTTTTAACCTCCTTTTAT	267
chr12	MInDel41	21383696	TGACACTACAAATACGACTCGCTT	AATAACTCTCATCCTCCACTGACG	189
chr12	MInDel42	21402647	CTGCCTAAGATTGCGCTACCTTTT	CAAATCTCGATTACTCTTGCACCC	263
chr12	MInDel43	21424800	ACTCAAAGTGGTGTTCAGATGTGA	GGAAGGTGTGTTGTTTTCTGAGTT	282
chr12	MInDel44	21426178	TGAAGTTGAAGAGGTAAGTTGGGT	CTGCCACGTGTAGAACATTTTAGA	232
chr12	MInDel45	21426418	CCACAAAAGCGGCTGATGATATTA	TCTCTCTCTCTATCTACCAGCTCA	130
chr12	MInDel46	21427266	AAAATACTCCGTCAACCATGCATC	TGACCGTGTACCAAAAGAATCTTG	107
chr12	MInDel47	21429242	GCAATGATCCTTTGAAACTCACATC	ATAAGGTTGTGGTTTGGGTTTGTC	270
chr12	MInDel48	21430150	TGATGGGGAAGAGATCAGAAAGTG	AACAAGAAACAAAATAGGTCGGCC	281
chr12	MInDel49	21430740	GAGCATTGAGGTTAACGTAAAGACA	GACTTTGCAAAAGAACGTGTAGAC	219
chr12	MInDel50	21440365	TGGTTAGAATAGTTTGTGTTGGAGG	TTGGGGCAAAGATTATCTAGGACT	270
chr12	MInDel51	21441123	CACAAAAGCGAAGATGACAACCTT	TTGGCTTGAATGATCTGGTTGTTC	139
chr12	MInDel52	21443795	AGAAGAAGGAGAAGAGAGAAGGGA	CCCTCCTTCTCTTGATCACTTTCA	113
chr12	MInDel53	21443959	TGAAAGTGATCAAGAGAAGGAGGG	TATTGGAAGAGGCATTAGAAGGGG	180
chr12	MInDel54	21445083	TGTACTTCCTTCTACCTAAAGCGA	CGTGGTTAGATTTGTTGTTGCA	248
chr12	MInDel55	21445095	TGTACTTCCTTCTACCTAAAGCGA	CGTGGTTAGATTTGTTGTTGCA	248
chr12	MInDel56	21445714	TGTAAAGAGAGGCACATGTGTTAGA	TGTCTATCATCTTGCCATCATAGTC	86
chr12	MInDel57	21446584	ATTGGGTTGGGCTCTTTTATTTGG	AAGAGACAGGCCGGAACTTCAATA	300
chr12	MInDel58	21449903	CCCCAACCTATAATGATCAACACC	ATATAGGTGGAAAGAAGAGGGCTA	191
chr12	MInDel59	21450126	TCTAGCCCTCTTCTTTCCACCTAT	CCAAAAGTGTTGCAATAGGAGTTCA	296
chr12	MInDel60	21453528	AGAGATCGCATGTGTGTATGGTTA	TAGACAAAGTTAACAGAACGCCCA	263
chr12	MInDel61	21453748	TGTCTATTAGAGCCGTACCACATG	AGGAAACACTAAGACAATCGAGCT	155
chr12	MInDel62	21459671	AAAGGCGATAAGTAGTGGTGAAGT	ATGTCCGTCGTCAAAACTTTCTTC	234
chr12	MInDel63	21459939	GAAGAAAGTTTTGACGACGGACAT	GGTTTGCCTTGTGAAAATGAGCTT	177
chr12	MInDel64	21460111	ACAACTACACTCCAAATTCCCCAT	TGAGAGAGAGAGAGAGAGAGAGAG	248
chr12	MInDel65	21468921	ACCCACGAGCATGTAGAAATAAGA	GCAAGGTGATATAAATGGTTGGAGC	258
chr12	MInDel66	21469302	ACACACACACACACACACTTTT	TGGCAATCAACAAAAGGGATGA	294
chr12	MInDel67	21471163	GCAGGCCTAACTTGGATATGTCTA	CGGTCACACGCGTATGTATAATTC	122
chr12	MInDel68	21471536	GTCATTGGATCATACACGTCACAA	GAAGGGAGAAGATAGAGCACAAGA	289
chr12	MInDel69	21475297	CTGTTTCTACTTCAGTTGCCAAGG	TTTCTTGCTCAATTCCTTCCAACG	235
chr12	MInDel70	21475417	TGTTAGATTTTATCGCCCTCACCC	CACAAGCAACTTACACTTCTCAGG	228
chr12	MInDel71	21475787	CCTGAGAAGTGTAAGTTGCTTGTG	TTTCCAAGCCATAAAGTCTGCGTA	286
chr12	MInDel72	21654289	GGGTGAGGTTGATATTAGATTCGA	CGAGAATTGAGAAACAGTTGCAAG	300
chr12	MInDel73	21654959	CGGAGTCGCATTTTATATTCGAAGT	AGACGAATGGAGGAGGAGAAATTC	260
chr12	MInDel74	21676618	AGTACTTACGTCCAAATCGAAGGA	TGTCATCATGGAGAGTTTAGCTTCA	298
chr12	MInDel75	21677490	CAAGCATACCTTTAACCGATGACA	GACCTCTAGACGATTGGATGTGAA	218
chr12	MInDel76	21769615	TCTGCATGAGATTTGGCTTAGAGA	GCATTGGGAATAGCAAGAGAAGAC	183
chr12	MInDel77	21771675	CATTCTACATGGGATCGCCTAGA	CAACTCTCTAGCAACCCCTCAATA	299
chr12	MInDel78	21771678	CATTCTACATGGGATCGCCTAGA	CAACTCTCTAGCAACCCCTCAATA	299
chr12	MInDel79	22046862	TTGAGGTGTGAGTAAGGTAGTTGG	ATGTCAAAACTCAACCTTACCCCT	274
chr12	MInDel80	22084487	TTATTGTGTCCTTTGGTTCGAGTG	CGTGACAAATTATGGTTTCGTGAC	289
chr12	MInDel81	22102962	GAGATTGTACGCGTTAAATAGAGCT	CCAAAGATTACAGAGTGCGAATCC	283
chr12	MInDel82	22718440	CAGTGCGAAATCTTCTCATTCCAA	GAGGAAATGCAACATATACCAGCC	262
chr12	MInDel83	23539027	AGTACCAATATAGGGGAAGTGTCA	ACCCTTTCTTGAGTTCCATCATCT	253

chr12	MInDel84	23539029	AGTACCAATATAGGGGAAGTGTCA	CAACAACCCTTTCTTGAGTTCCAT	258
chr12	MInDel85	23540675	AAGGCATACTGTACTAGCATCGAC	AATAGACTTTGGCTTTGAGTTCGC	258
chr12	MInDel86	23575605	AGAACAAGATGCAAGTCCTCTT	ACAGTCTATCTTGGTTTATCGCGA	260
chr12	MInDel87	23872301	ATGGAGGTGCTTATTCTTGATGGT	GAGGATGGGAAGAACTACAATGGT	258
chr12	MInDel88	23875771	CCAATCTCCTCTTCTCCTAACACC	GGCTCTGATCATCAACAAAACACA	290
chr12	MInDel89	23878792	TGGAGAACTATGGAATATGGAGGA	GCTTACGGATGAATTTGGACTTGA	283
chr12	MInDel90	23928923	CTCCAGAGCCAGTATCATAAAGCA	CCCTCCAACAAAGTGCAAAGATG	206
chr12	MInDel91	23931047	GAGAAAAGAGAGAATGAAGCACAGA	TTCCTCGTGTATCCATTCAAGGTT	238
chr12	MInDel92	23940777	CTTGGGAGGATGAGTGAAGGTAAA	TTCGTACTTGAGTTGACTTACGGT	300
chr12	MInDel93	24500866	GGTCCTTTTGATGCCATTTGAAGA	TCGTATAGGTCGTTGATGCAAACT	250
chr12	MInDel94	24728637	ATCATTGTAGACATGCTTGGGAGT	GGGAACTTGAAAGGAATTTTGGGT	296
chr12	MInDel95	24963555	CCTTGTGTTGGATTGTGACATGAA	GTTTGTAGGTAGAGAGGGGAGTTG	282
chr12	MInDel96	24963558	CCTTGTGTTGGATTGTGACATGAA	GTTTGTAGGTAGAGAGGGGAGTTG	282
chr12	MInDel97	25573886	TCCTTACACTTAGCTTGCTGGTAG	CCGGAAATCATCATGAGCTGTTTT	129
chr12	MInDel98	27318495	TCCTGAGGAACGGCTTGAATATAT	GCTGGCGATTTTGTGTGGATTATA	294
chr12	MInDel99	27473685	TCCCTTATCTTCAGACAACAGAACA	AGGTAATCAAGTTTGGGAAGACGA	290
chr12	MInDel100	27473687	TCCCTTATCTTCAGACAACAGAACA	AGGTAATCAAGTTTGGGAAGACGA	290

Table 4: Comparison of MS results of Mu7191 and that of the newly developed Markers.

MS of reference marker		MS consistency level of Candidate markers					
Mu7191		Mindel89		Mindel92		Mindel93	
Genotype	Individuals	Genotype	Individuals	Genotype	Individuals	Genotype	Individuals
R/R	12	x/x	5	x/x	6	x/x	1
R/S	1	x/y	0	x/y	1	x/y	0
S/S	179	y/y	163	y/y	167	y/y	167

R/R denotes homozygote resistant alleles, R/S denotes heterozygote alleles, and S/S denotes homozygote susceptible alleles. x/x and y/y denote the homozygosity of the two polymorphic alleles amplified by the newly developed markers, and x/y denotes their heterozygosity.

these polymorphic PCR products was examined after a comprehensive analysis. On the basis of Mu7191-derived PCR amplicons as references, it is evident that the MS analysis result obtained with marker MInDel 92 was more consistent than those obtained with markers MIndel89 and MIndel93 (Table 4). Consistency of their molecular screening results was stressed in seven melon breeding lines, including AM7, AM9, AM11, AM65, AM66, AM67, and AM68 (Table S4). The length of PCR products from this marker was determined based on a combination of expected amplicons size indicated by DNA sequencing data and DNA molecular weight marker (DL2000 DNA marker). The size of DNA fragment generated by MIndel92 from individuals displaying the R allele when using Mu7191 was between 200bp and 300bp while that of DNA fragments generated by MIndel92 from individuals with the S allele using Mu719 was 300bp (Fig S1). In order to adapt these markers to various electrophoresis platforms, polyacrylamide and agarose gels were loaded with derivative PCR solutions. Using these two electrophoresis platforms, the markers have generated distinct and informative patterns of genetic bands.

Discussion

Powdery mildew (PM) is a fungus that induce disease in both the field and the greenhouse. One of the pragmatic approaches to control this disease is the use of resistant cultivars. Since MAS has been extensively used to search for molecular markers that are linked to a specific trait during the breeding of disease-resistant cultivars, the development of molecular markers that are highly linked to disease-resistant genes is viewed as an effective method for enhancing disease resistance in plants. On the basis of our knowledge and technical expertise, the use of PCR-based markers that can be separated on both Agarose and polyacrylamide gel electrophoresis could be readily accepted by breeders due to its simple requirements and straightforward laboratory operation. In this study, PM-resistant line M17050 (P1) and the PM-susceptible line 28-1-1 (P2) were re-sequenced to generate PCR-based InDel markers for BSA analysis and QTL mapping using the F2 population. The QTL named *LVpm12.1* was identified in a region containing the InDel markers InDel89, MInDel92, and MInDel93. The screening results of marker Mu7191 and MInDel92 were more consistent based on the molecular analysis of a panel of melon breeding lines with varying susceptibilities to powdery mildew. Moreover, the PCR products of this marker could be easily separated using either agarose or polyacrylamide gels. Therefore, MInDel92 appears to be closer to Mu7191 on chromosome 12 of the melon than MInDel89 and MInDel93. Marker Mu7191 is linked to PM-resistance QTL *Pm-PMR6-1*, so it is more obvious that MInDel92 can identify melon varieties carrying this QTL. The possibility of developing InDel markers for both polyacrylamide and agarose gel electrophoresis with dependency on the size of insertion/deletion is mentioned **[65]**. However, high-density insertion and deletion is needed, which could be exploited for the discovery of valuable InDel markers for genotype screening through agarose and polyacrylamide gels. Thus, the availability of a large number of genome-wide InDel makers is essential to achieve this goal. Whole genome re-sequencing has helped to generate numerous InDel markers in crops due to its cost-effectiveness **[65, 68]**. Moreover, the reliability and efficiency of PCR-based InDel markers in plant genotyping **[66, 56, 67, 68]** as well as their strongest amplification aptitude **[56]** are reported. PCR-based Indel markers associated with PM resistance from PM-resistant melon resources are developed to accelerate resistance gene introgression in melon **[19, 69]**. The region harboring *LVpm12.1* and its closely linked InDel markers is found on melon chromosome 12, where some other QTLs with PM-resistance are mapped. The locus containing *LVpm12.1* is positioned from 22.72Mb to 23.34Mb. The QTL *pm-PMR6* from PM-resistant variety PMR6 **[37]** ranged from 22.69 to 23.56Mb, while *BPm12.1* from PM-resistant variety MR-1 spanned from 22.80 to 22.88Mb **[11]**, both of which are mapped on melon in the same chromosomal region.

The locus of *qCmPMR-12* from wm-6 is extended from 22.00 to 22.90 Mb [**40**], while *pm12.1* derived from PMR6 is located from 22.36 to 24.25 Mb [**39**]. This indicates that the physical chromosomal position of *LVpm12.1* overlaps that of *pm-PMR6*, *BPm12.1*, *qCmPMR-12*, and *pm12.1*.The fact that these five QTLs are inherited in the same manner despite having different parental origins suggests that the resistance to PM disease in this chromosomal region is conferred by the same QTL or a cluster of QTLs. This indicates the existence of a QTL-hotspot on melon chromosome 12 that confers resistance to PM disease. It also demonstrates that the methods used to map it are trustworthy, thus strengthening the credibility of the InDels markers for PM resistance breeding. In our upcoming research, we intend to evaluate an extensive screening ability of these markers through molecular and field resistance screening of a panel of melon genotypes, which will compose of commercial varieties, breeding lines, landraces, and cultivars. The PM resistance of the seven melon breeding lines selected in this study based on the molecular screening results of both markers Mu7191 and MInDel92 should be confirmed using field resistance screening.

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Author contributions

YMNA designed the experiment, analyzed the data, drafted the manuscript and supervised the work; XL, XY, and YMNA performed all the laboratory experiments, collected the molecular data, WYF, WTZ, ZJD, GLX, and WHL proceed the phenotypic evaluation experiments; Md.AA, LHZ and GLX gave critical revision of the manuscript.

Competing interests

The authors declare no competing interests.

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