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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) markers associated to this trait, the whole genomes of the PM resistant line M17050 (P1) and the 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 F_2 segregating population of melons generated from these two parents was inoculated by the 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 the melon chromosome 12. This region overlapped the previously described quantitative trait locus (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 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 tests.

Keywords: InDel marker; mapping; whole genome re-sequencing; resistance QTL

Powdery mildew (PM) is a fungus that affects the leaves, petioles, and stems of most cucurbit crops in both field and greenhouse conditions (Perez-Garcia et al. 2009). This disease can cause a decrease in weight-based productivity and a decrease in fruit quality (Candido et al. 2014), resulting in severe economic losses in many regions of the globe (Romero et al. 2008). Two biotrophic fungi of powdery mildew, *Podosphaera*

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xanthii (Castagne) Braun & Shishkoff *(P. xanthii)* and *Golovinomyces cichoracearum* (de Candolle) Heluta (*G. cichoracearum)*, were identified in melon (Kuzuya et al. 2006; Kristkova et al. 2009; Li et al. 2017; Hong et al. 2018). 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 worldwide (Bardin et al. 1999; Hosoya et al. 1999; McCreight 2006). The major Chinese races are pxCh1, race1, and 2F (Wang et al. 2006; Liu et al. 2010; Cheng et al. 2011; Ma et al. 2011; Zhang et al. 2011a). In China, *P. xanthii* is generally considered to be the primary cause of PM in melon (Cheng et al. 2011; Zhang et al. 2013), and the recent reports on PM in Shanghai support this assertion (Gu et al. 2010; Li et al. 2015). Since chemical control remains ineffective (McGrath 2001; Hollomon et al. 2002), the most appropriate strategy of PM controlling is the detection of resistant quantitative trait loci (QTLs) and their introgression into commercial varieties.

Multiple genes or QTLs have been isolated from different sources for PM resistant varieties development in melon (Cohen et al. 1990; Pitrat 1991; Perchepied et al. 2005; Fukino et al. 2008; Teixeira et al. 2008; Liu et al. 2010; McCreight & Coffey 2011; Yuste-Lisbona et al. 2011; Wang et al. 2012a; Zhang et al. 2013; Wang et al. 2016; Li et al. 2017; Cao et al. 2021; Cui et al. 2020, 2022). As a consequence, controversy has arisen over the genetic basis of PM resistance in melons. Some studies suggested that PM resistance is governed by a single dominant gene, while others suggested that it is governed by a recessive gene, and still others suggested that it is governed by multiple QTL.

Bulk segregant analysis (BSA) and conventional QTL analysis are two important QTL mapping techniques extensively used in melon breeding (Fukino et al. 2008; Liu et al. 2010; Wang et al. 2012a, 2016; Li et al. 2017; Branham et al. 2021; Cao et al. 2021). Due to the low cost of whole-genome re-sequencing (WGS), BSA has been combined with WGS to map genes of interest in many crops such as cucumber (Lu et al. 2014; Win et al. 2017), rice (Yang et al. 2013; Zheng et al. 2016); soybean (Dobbels et al. 2017; Song et al. 2017), and melon (Dobbels et al. 2017; Song et al. 2017; Cao et al. 2021). In breeding programs, the most prevalent markers are high-throughput genotyping markers like single-nucleotide polymorphism (SNP), kompetitive allele specific PCR (KASP), and high-resolution melting (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. To discover PCR-based InDel markers associated with PM resistance to speed up melon resistance breeding, BSA analysis and the QTL mapping method using whole genome re-sequencing data were applied simultaneously to an F_2 population derived from a PM resistant parent M17050 and PM susceptible parent 28-1-1.

MATERIAL AND METHODS

Plant material. The materials used consist of a PM resistant parent M17050 named P1 and a PM susceptible parent 28-1-1 denoted P2, both of which belong to the Cantalupensis group of muskmelon species, *Cucumis melo* L. subsp. *melo*. Both these parents are lightly ribbed with sweet and flavourful flesh and have a reddish orange flesh colour and a reticulated (net-like) peel. However, M17050 has sutured and light grey-green peel, whereas 28-1-1 is not sutured but dark grey-green peel (Figure 1A–D). These two parents were used to produced F_1 hybrid designated as F_{1-6} and an F_2 population.

Plant growth and PM inoculation. All the materials, including the two parents, F_{1-6} and F_2 population were grown in a greenhouse with a temperature range of 22–28 °C (day/night) and a relative humidity range of 60 to 75%. The PM fungus (*P. xanthii*) used in this study was isolated from the leaves of diseased melon plants according to the method of Kuzuya et al. (2006) with minor modifications (Figure 1E–F). In this experiment, the PM fungus was maintained on susceptible melon plants (Figure 1G) in the artificial growth chamber in Suqian Greenport Modern Agriculture Research Institute. The fungus was collected and suspended in sterile distilled water containing 0.02% Tween 20 before being used to inoculate plants at a concentration of 1×10^6 as described by Zhang et al. (2011a).

Disease scoring and plant resistance evaluation. The plants were observed weekly from the 14th 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: class 0 is denoted as no infection on the leaf surface and class 1 as infection and heavy sporulation on the entire leaf. For the inheritance

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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 F_2 population were examined. Here, 30 plants from each parent, 50 plant from F_{1-6} individuals, and 125 plants from the F_2 population were sampled and analysed for field resistance to PM disease. Only plants ranked in class 0 and 1 were taken into account for subsequent analysis.

Whole genome re-sequencing and InDel markers development. Melon breeding parents, P1 and P2 were used for whole genome re-sequencing. Library construction, sequencing, data filtering, alignment and variants calling were performed exactly as described by Adedze et al. (2021). The polymorphic InDel between P1 and P2 were detected following the protocol described by Guo et al. (2019). The reference genome sequence of melon variety DHL92 version 3.6.1 was examined to determine the InDel polymorphisms between the re-sequenced P1 and P2. Primer 5 (http://www.PromerBiosoft.com) was used to create PCR primers with a variety of properties (lengths ranging from 18 to 28 bp, primer melting temperature (Tm) ranging from 57 to 63 °C, and PCR products ranging from 80 to 300 bp).

Polymerase chain reaction and electrophoresis. Total genomic DNA was extracted from fresh leaves of 35-day-old seedlings of 6 individuals each from P1, P2, and 125 individuals of F_2 population, as well as a panel of 192 melon breeding lines using the Nu-Clear Plant Genomic DNA Kit (CWO531M) protocol (CWBiotech, Beijing, China). Two PCR reactions were set based on the size of the PCR amplicons and the nature of the gel used for electrophoresis, such as agarose gel or polyacrylamide gel as described by Adedze et al. (2021)

Bulked segregant analysis and QTL mapping. To investigate the bulked segregant analysis, four DNA bulks, including two parent bulks and two F_2 bulks were used. The parent bulks were independently constructed by pooling an equal DNA amount of 12 plants from P1 and P2. The two F_2 segregating bulks were generated by combining an equal amount of DNA extracted from 30 extremely resistant (R-bulk) and 30 susceptible (S-bulk) F_2 plants. Initially, polymorphism between the two re-sequenced lines was validated with 1 200 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 group 1 consisting of DNA from P1 and R-bulk and group 2 consisting of DNA from P2 and S-bulk. On the other hand, the two parents and their offspring F_2 population were genotyped using the informative InDel markers discovered on melon chromosome 12 for QTL analysis. Utilizing JoinMap 4.0 software (Van Ooijen 2006),

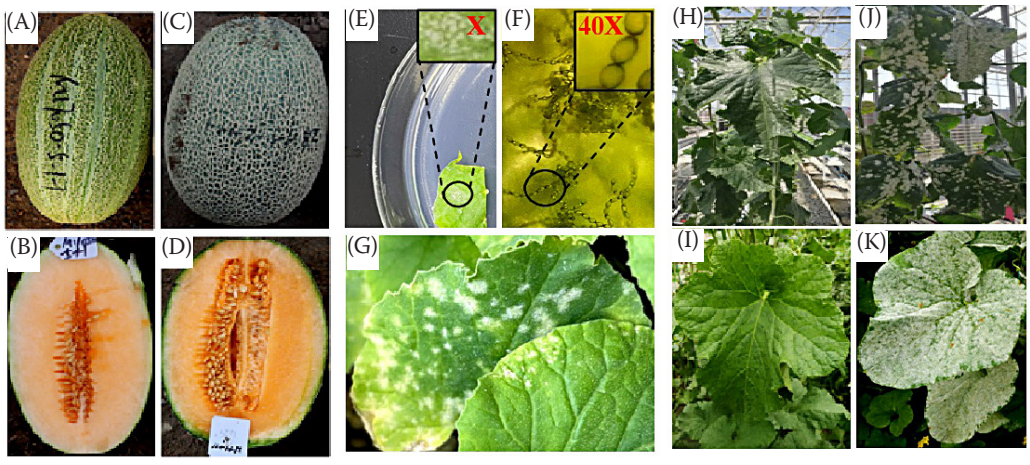

Figure 1. Fruits morphology and powdery mildew (PM) disease evaluation: fruit appearance and fruit longitudinal section of P1 line (A–B) and P2 line (C–D) showing skin and flesh characteristics; powdery mildew pathogen single spore isolation (E) followed by conidiophore and conidia observation under light microscope (F); maintenance of PM disease on melon leaves using artificial inoculation in growth chamber (G); leaves from P1 and resistant F_2 plant (H–I) and that from P2 and susceptibility F_2 plant (J–K)

X, 40X – high magnification observation

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a new genetic linkage map was generated. Kosambi's (1943) mapping function was used to calculate map distances, and the genetic map was created using the MapChart 2.2 program (Voorrips 2002). The software WinQTLCart 2.5 was used to detect QTL from genotypic and phenotypic data using composite interval mapping (CIM) (Wang et al. 2012b). 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 1 000 iterations of permutation tests.

Molecular screening validation assay. A panel of 192 melon breeding lines, including P1 and P2, were 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; Ritschel et al. 2004; Liu et al. 2015; Choi et al. 2020), which have been reported on melon chromosome 12 (Table S1 in Electronic Supplementary Material (ESM)). For the publicly available markers, genetic bands were denoted R for resistant allele and S for susceptible allele while the two polymorphic DNA fragments amplified using markers MInDel89, MInDel92 and MInDel93 were labelled x and y.

RESULTS

Inoculation and disease response in P1, P2, F_1 and F_2 population. Powdery mildew disease happened naturally in a greenhouse while 20 melon hybrid combinations were being tested for resistance to fruit blotch disease. F_{1-6}, which is a F_1 cross from P1 and P2, 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 F_2 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 P2 was highly susceptible to powdery mildew disease (Figure 1H–I). The disease resistance in F_{1-6} confirmed the previous observation, while the segregation between the plants in class 0 (89 plants) and those in class 1 (36 plants) in the F_2 mapping population was consistent with a 3 : 1 ratio (Figure 1J–K). Using Chi-square test ($\chi^2 = 0.963 < 3.84$, $P = 0.05$), there is no statistical significance between the observed ratio (2.47 : 1) and predicted Mendelian ratio (3 : 1). Consequently, resistance to PM disease in this investigation may be governed by QTLs with a dominant effect.

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% for P1 and 94.61% for P2 with an average of 94.77%, whereas the GC content was 36.87% for P1 and 36.61% for P2 with an average of 36.74% (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% for P1 and 77.77% for P2, with a mean of 77.51%. 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 15 065 on chromosome 9 to 40 239 on chromosome 4, whereas between P2 and DHL92, the range was 16 372 on chromosome 9 to 37 300 on chromosome 4 (data not shown). The alignment of reads from P1 and P2 yielded a total of 29 393 InDels with a density of 78 InDels/Mb. These InDels are distributed across the 12 chromosomes of the melon, with chromosome 9 containing the lowest number of 997, and chromosome 6 containing the highest number of 5 906. There was an equal variation in density ranging from 2.66 InDels/Mb on chromosome 9 to 15.75 InDels/Mb on chromo-

Table1. 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	99 004 197	96.26	77.25
P2	1.85E+08	97.19	77.77

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Table 3. InDels detected between P1 and P2

Chr	1–10	11–20	21–30	> 30	Total
	(bp)				
chr00	942	73	21	20	1 056
chr01	2 467	147	46	71	2 731
chr02	1 578	105	38	48	1 769
chr03	1 491	137	34	57	1 719
chr04	1 681	113	34	53	1 881
chr05	3 570	256	99	113	4 038
chr06	5 236	403	137	130	5 906
chr07	1 080	70	22	34	1 206
chr08	1 524	95	38	35	1 692
chr09	868	73	21	35	997
chr10	2 265	159	59	67	2 550
chr11	1 620	121	52	49	1 842
chr12	1 780	119	51	56	2 006

some 6 (Table 3). In relation to the length of the nucleotide sequence, three distinct types of insertions and deletions were identified and classified as small (1–10 bp), medium (11–30 bp), and large (> 30 bp). Large, medium, and small InDels represented 2.62, 8.58, and 88.80%, respectively, of the total genome-wide InDels annotated between P1 and P2.

Bulk segregant analysis and mapping of *LVpm12.1*. To discover genomic regions conferring PM disease resistance in this work, BSA was conducted using four DNA pools, consisting of two parent bulks and two F_2 segregating bulks. Polymorphism analysis was first performed with 1 200 InDel markers between the two parent bulks. The result revealed 100 markers to be strongly polymorphic with clear band patterns between P1 and P2 (data not shown). Further, BSA analysis was performed on the four bulk DNA samples, including parent bulks and two F_2 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, F_{1-6}, and 3 recombinant individuals from F_2 highlighting the genomic region, *LVpm12.1* that control PM-resistance in this study (Figure 2A). The markers MInDel76, MInDel81, MInDel89, MInDel92, MInDel93, and MInDel95 were close to this region, which was finally located between MInDel81 and MInDel93 (Figure 2A). Linkage map was constructed using JoinMap_4.0 software, and *LVpm12.1* mapping was executed using WinQTL software. Two QTL positions were detected; one is non-significant and close to MInDel81, while the second is highly significant and linked to MInDel93 (Figure 2B). The BSA and QTL analyses results revealed that markers MInDel89, MInDel92, and MInDel93the were linked to *LVpm12.1* and it was genetically located at 8.7cM from them in a region ranging from 22.72 to 23.34 Mb.

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

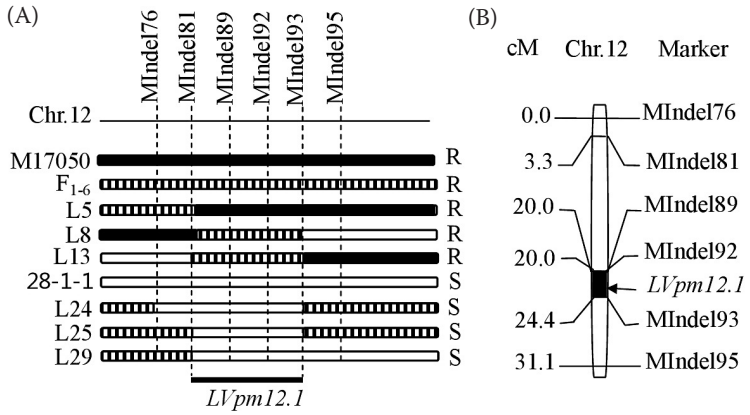

Figure 2. Mapping of powdery mildew (PM) resistance QTL *LVpm12.1* and identification of closely linked InDel markers using bulk segregant analysis (BSA) and QTL analyses: BSA analysis has located *LVpm12.1* between MInDel81 and MInDel93 (A); QTL analysis has mapped *LVpm12.1* between MInDel92 and MInDel93 (B)

M17950, 28-1-1 – PM-resistant and PM-susceptible breeding lines; L5, L8, L13 – the recombinant resistant F_2 individuals; L24, L25, L29 – the susceptible F_2 individuals; R, S – resistant and susceptible phenotype; F_1 – hybrid from cross between the two breeding parents; Chr.12 – chromosome; cM – centimorgan; black-squared shape in (B) indicates *LVpm12.1* position

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Table 4. Comparison of molecular screening (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 – homozygote resistant alleles; R/S – heterozygote alleles; S/S – homozygote susceptible alleles; x/x, y/y – the homozygosity of the two polymorphic alleles amplified by the newly developed markers; x/y – their heterozygosity

markers used in molecular screening (MS), only marker Mu7191 has behaved well under the imposed PCR conditions. PCR products pattern from the 192 melon breeding lines using the newly developed markers and Mu7191 were examined to validate their detection accuracy. Mu7191-derived PCR product was used as a reference. The result obtained using marker MInDel 92 was more consistent with that of Mu7191 than other markers (Table 4, Figure S1 in ESM). Consistency of their molecular screening results was emphasized in seven melon breeding lines, such as AM7, AM9, AM11, AM65, AM66, AM67, and AM68 (data not shown). The length of PCR products generated by this marker was determined based on the expected amplicons size of WGS data and DNA molecular weight marker (DL2000 DNA marker). 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

The pragmatic solution to control PM is the use of resistant cultivars. The development of molecular markers that are highly linked to disease-resistance 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 and simple electrophoresis platforms using agarose and polyacrylamide gels could be readily accepted by breeders due to its low requirements and straightforward laboratory operation. In this study, PM-resistant line P1 and susceptible line P2 were re-sequenced to generate PCR-based InDel markers for BSA and QTL analyses using F_2 population. The QTL named *LVpm12.1* was identified in a region containing the InDel markers InDel89, MInDel92, and MInDel93. The results of marker Mu7191 and MInDel92 were found 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 MInDel92 could be easily separated using either agarose or polyacrylamide gel electrophoresis. 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 gene *Pm-PMR6-1*, so it seems that MInDel92 can identify melon varieties carrying the same QTL. The possibility of developing InDel markers for both polyacrylamide and agarose gel electrophoresis with dependency on the size of insertion/deletion is mentioned (Liu et al. 2015). 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 (Liu et al. 2015; Adedze et al. 2021). Moreover, the reliability and efficiency of PCR-based InDel markers in plant genotyping (Song et al. 2015; Yang et al. 2016; Hu et al. 2020; Adedze et al. 2021) as well as their strongest amplification aptitude (Yang et al. 2016) are reported. PCR-based InDel markers associated with PM resistance from PM-resistant melon resources are developed to accelerate resistance gene introgression in melon (Fukino et al. 2008; He et al. 2013). The region harbouring *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 *pm-PMR6* gene from PM-resistant variety PMR6

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(Lu et al. 2014) ranged from 22.69 to 23.56 Mb, while *BPm12.1* from PM-resistant variety MR-1 spanned from 22.80 to 22.88Mb (Li et al. 2017), 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 (Cao et al. 2021), while *pm12.1* derived from PMR6 is located from 22.36 to 24.25 Mb (Cui et al. 2022). This indicates that the physical chromosomal position of *LVpm12.1* overlaps that of *pm-PMR6*, *BPm12.1*, *qCmPMR-12*, and *pm12.1*. It is possible that the resistance to PM disease in this chromosomal region is conferred by a single locus or a cluster of loci. Notably, the majority of these QTLs and genes were dominantly inherited. This confirms the inheritance pattern of *LVpm12.1* as experimentally demonstrated by the Mendelian segregation of the F_2 population following inoculation. On the other hand, it is believable that the methods used to map this QTL are trustworthy, thus strengthening the credibility of the newly developed InDels markers for PM resistance breeding. In conclusion, PCR-based InDel markers firmly linked to PM resistance *LVpm12.1* on melon chromosome12 were developed using WGS data from PM resistant and susceptible lines in conjunction with BSA and QTL analyses. The markers disclosed here were viewed as tools for monitoring the introgression of resistance in new varieties. According to Zhang et al. (2013), *P. xanthii* is the most common cause of PM disease in China. In addition, some closely related *P. xanthii* races, including pxCh1, race1, and 2F, have been identified in China (Wang et al. 2006; Liu et al. 2010; Ma et al. 2011). Therefore, it is likely that any of these races could be liable for the PM infestation observed in this study; however, further research implicating race identification is necessary to specify the PM resistance reported in this study.

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