

Molecular mapping and candidate gene analysis for resistance to powdery mildew in *Cucumis sativus* stem

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ABSTRACT. Powdery mildew (PM) of cucumber (*Cucumis sativus*), caused by *Podosphaera xanthii*, is a major foliar disease worldwide and resistance is one of the main objectives in cucumber breeding programs. The resistance to PM in cucumber stem is important to the resistance for the whole plant. In this study, genetic analysis and gene mapping were implemented with cucumber inbred lines NCG-122 (with resistance to PM in the stem) and NCG-121 (with susceptibility in the stem). Genetic analysis showed that resistance to PM in the stem of NCG-122 was qualitative and controlled by a single-recessive nuclear gene (*pm-s*). Susceptibility was dominant to resistance. In the initial genetic mapping of the *pm-s* gene, 10 SSR markers were discovered to be linked to *pm-s*, which was mapped to chromosome 5 (Chr.5) of cucumber. The *pm-s* gene's closest flanking markers were SSR20486 and SSR06184/SSR13237 with genetic distances of 0.9 and 1.8 cM, respectively. One

hundred and fifty-seven pairs of new SSR primers were exploited by the sequence information in the initial mapping region of *pm-s*. The analysis on the F₂ mapping population using the new molecular markers showed that 17 SSR markers were confirmed to be linked to the *pm-s* gene. The two closest flanking markers, pmSSR27 and pmSSR17, were 0.1 and 0.7 cM from *pm-s*, respectively, confirming the location of this gene on Chr.5. The physical length of the genomic region containing *pm-s* was 135.7 kb harboring 21 predicted genes. Among these genes, the gene Csa5G623470 annotated as encoding Mlo-related protein was defined as the most probable candidate gene for the *pm-s*. The results of this study will provide a basis for marker-assisted selection, and make the benefit for the cloning of the resistance gene.

Key words: *Cucumis sativus* L.; *Podosphaera xanthii*; SSR marker; gene prediction

INTRODUCTION

Powdery mildew (PM) of cucumber (*Cucumis sativus* L.), caused by *Podosphaera xanthii*, is a major foliar disease worldwide (Barnes and Epps, 1956; Thomas, 1986). PM, downy mildew, and Fusarium wilt are the most significant diseases of cucumber in China (Cao et al., 2007). Powdery mildew occurs with small spots appearing on the foliage, and then foliage and stems were covered with a white, powdery mass of spores and hyphae successively (de Ruyter et al., 2008). The symptoms of PM are characterized by a short latent period, high secondary infection frequency, strong epidemic ability, and year-round presence. Besides, it develops and spreads rapidly in the middle and later phases of the growth period, and results in significant reductions in yields (Zhang et al., 2011). The most efficient method for controlling PM in cucumber is the use of resistant cultivars, and thus understanding the molecular biology of PM resistance plays a key role in cucumber breeding programs.

Reports about the inheritance of PM resistance genes in cucumber are various. Some reports state that resistance is a quantitative trait controlled by multiple recessive genes (Smith, 1948; Barnes and Epps, 1956; Fugieda and Akiya, 1962; Kooistra, 1968; Kooistm, 1971; Zhang et al., 2005; Sakata et al., 2006). However, Shanmugasundarum et al. (1971) differentiated between hypocotyl and leaf resistance and concluded that a recessive gene for hypocotyl resistance (*pm-h*) is essential for overall resistance. He et al. (2013) and Walters et al. (2001) also showed that a single-recessive gene (*pm-h*) controls hypocotyl resistance, and the role of a single gene in resistance is supported by other studies (Zhang et al., 2004; Liu et al., 2008a). By contrast, some studies reported that resistance is controlled by a pair of incomplete recessive genes and two pairs of epistatic genes (Shanmugasundarum et al., 1971; Mao et al., 2005). Moreover, Morishita et al. (2003) suggested that resistance to PM is controlled by a pair of recessive genes and a pair of incompletely dominant genes and that resistance is temperature-dependent. There is no report of inheritance for the PM resistance in cucumber stem at the adult stage. The goal of this research was to determine the inheritance of resistance in the stem of inbred line NCG122.

The method used to determine resistance to PM in regular cucumber breeding programs requires evaluations in multiple locations, with replication to control environmental

variation, including exposure to different regions of the pathogen, and to make valid selections for resistance over a period of several years of crossing and inbreeding. In contrast to disease ratings, it may be probable to select for resistance more efficiently using molecular marker-assisted selection (MAS) (Zhang et al., 2013a). In cucumber, there are various studies about the molecular biology of PM resistance. Sakata et al. (2006) detected three quantitative trait loci (QTL) for PM resistance at 20°C and two at 26°C. Of these, one major QTL was found to confer resistance at both 20° and 26°C. Liu et al. (2008b) identified five QTLs for PM resistance in three linkage groups in two environments. Zhang et al. (2011) reported four QTLs (*pm5.1*, *pm5.2*, *pm5.3*, and *pm6.1*) associated with PM resistance and *pm5.2* on chromosome 5 (Chr.5) was a major QTL. Hofstede et al. (2008) and de Ruiter et al. (2008) identified two linked PM resistance QTLs, *pm-l* and *pm-h*, conferring leaf resistance and hypocotyl resistance, respectively. He et al. (2013) defined six genomic regions in four chromosomes that harbored QTLs for PM resistance, and among them was QTL *pm5.2* for hypocotyl resistance. Zhang (2006) and Zhang et al. (2004, 2007) reported AFLP markers linked to both resistant and susceptible genes for PM in cucumber, at genetic distances of 5.6 and 7.0cM, respectively. Zhang et al. (2008) and He et al. (2013) reported SSR markers linked to resistant genes for powdery mildew in cucumber, at genetic distances of 2.6 and 13.0cM. However, there are no reports on chromosomal mapping for PM resistance in the cucumber stem (*pm-s*, a single-recessive nuclear gene), and no reports on any molecular markers tightly linked to *pm-s*.

In the present study, inbred lines NCG-122 and NCG-121, with resistance and susceptibility to PM in cucumber stem, respectively, were applied to construct a genetic population for analyzing inheritance and mapping the *pm-s* gene. The results will lay the foundation for future fine-mapping, gene cloning, and MAS breeding.

MATERIAL AND METHODS

Experimental materials

European greenhouse-type cucumber inbred lines NCG-122 (P_1 , Figure 1A, resistant to PM in the stem) and NCG-121 (P_2 , Figure 1B, susceptible to PM in the stem) were used as the parents for the F_1 , reciprocal F_1 (F_1'), and F_2 populations. The two inbreds were preserved by T.C. Wehner at North Carolina State University, USA.

Experiment design

All plants were grown under a clear plastic tunnel at Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, during 2013 and 2014. Ten plants each of P_1 , P_2 , F_1 , F_1' were planted in the spring of 2014 in a randomized complete block with three replications. A total of 181 plants of F_2 were also planted. The distances between plants and lines were 25 and 55 cm, respectively.

Disease investigation and data statistics

The symptoms of PM disease occurred naturally in the disease nursery and were evaluated at the later stage of life (10-12 weeks after planting), as either resistant (no PM symptoms at all on the stem) or susceptible (sporulating PM colonies visible on the stem).

Each plant was assessed by two investigators. The segregation ratio was analyzed using the SAS 9.2 and Microsoft Excel 2003 software.

Construction of SSR linkage groups and initial chromosomal mapping of the *pm-s* gene

SSR primers (2112) from cucumber genetic map by Ren et al. (2009) were used to map the *pm-s* gene initially. The modified CTAB method was used for the isolation of genomic DNA from young leaf tissue from each of the 30 plants used for P₁, P₂, F₁, F₁' , and for each plant of the F₂ population. The SSR reaction system was the same as that used by Zhang et al. (2014). Join Map 4.0 (Van Ooijen, 2006) was used to construct a linkage map.

The linkage map was constructed by screening SSR markers that showed polymorphisms between paternal and maternal parents and then using BSA method (Michelmore et al., 1991) to obtain DNA from either seven resistant or seven susceptible plants in the F₂ generation to form the gene pool for primer screening. Finally, primers that had been screened for polymorphisms were used to analyze the genotypes of each plant in the F₂ population, and construct the linkage map using Join Map 4.0.

The linkage map obtained in this study was compared with a previous integration map (Zhang et al., 2012), and the linkage groups and chromosomal location of the *pm-s* gene identified.

Development of new molecular markers and second mapping of the *pm-s* gene

In the initial mapping region harboring the *pm-s* gene, new SSR primers were designed based on the full-sequence information of the cucumber genome (Huang et al., 2009). The new primers and 10 pairs of SSR primers published by He et al. (2013) were first used to screen for polymorphisms in the parents. Then, selected primers were used in the F₂ population to determine whether or not they were linked to the *pm-s* gene.

Sequence annotation and gene prediction in the genomic region containing the *pm-s* gene

The sequences were aligned with the cucumber genome sequences (Huang et al., 2009) using BLASTn with an E-value cutoff of 1×10^{-20} . Only matches with an identity of more than 95% were retained. Gene prediction and annotation was accomplished just as Zhang et al. (2013b).

RESULTS

Inheritance of the resistance to PM in the NCG122 cucumber stem

The parental lines P₁ (NCG-122) and P₂ (NCG-121) were resistant and susceptible, respectively (Figure 1A and B), and the F₁ and reciprocal F₁ of NCG-122 x NCG-121 were all susceptible. In the F₂ population, there were 133 plants susceptible and 48 plants resistant. The chi-square analysis ($\chi^2 = 0.27 < 3.841$) showed a segregation ratio of 3:1 (Table 1). In the backcross population of BC₁P₁, there were 43 plants resistant and 45 plants susceptible. The chi-square analysis ($\chi^2 = 0.05 < 3.841$) showed a segregation ratio of 1:1 (Table 1). In the backcross population of BC₁P₂, all the plants (87 plants) were susceptible. Therefore, the resistance to PM in the NCG-122 stem was consistent with the control by the *pm-s* gene. The susceptibility was dominant over the resistance.

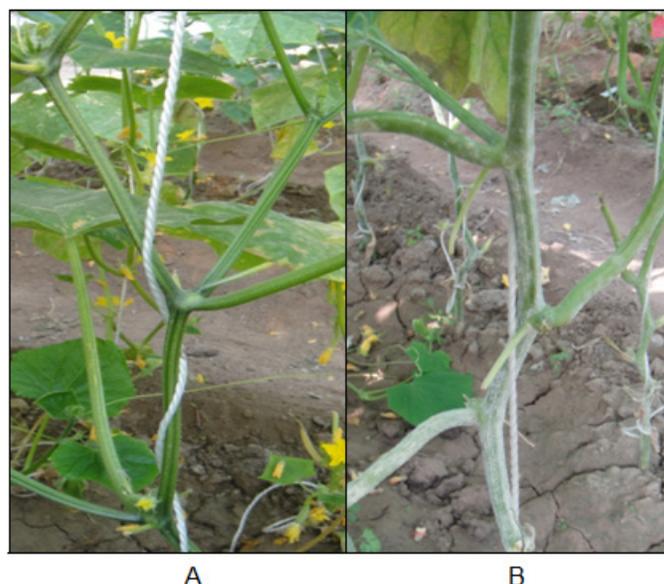


Figure 1. Phenotype pictures of the parental lines. **A.** P₁ (NCG-122 with high resistance to powdery mildew in cucumber stem). **B.** P₂ (NCG-121 with high susceptibility to powdery mildew in cucumber stem).

Table 1. Segregation ratios of disease-resistant and disease-susceptible plants in NCG122 x NCG121 genetic populations.

Populations	No. of total plants	No. of disease-resistant plants (R)	No. of disease-susceptible plants (S)	Expected ratio (R:S)	Goodness of fit χ^2
P ₁ (NCG122)	10	10	0	-	
P ₂ (NCG121)	10	0	10	-	
F ₁	10	0	10	-	
BC ₁ P ₁	88	43	45	1:1	0.05
BC ₁ P ₂	87	0	87	-	
F ₂	181	48	133	1:3	0.27

Construction of SSR linkage groups and initial chromosomal mapping of the *pm-s* gene

Of the 2112 pairs of SSR primers tested, 282 (13.4%) showed obvious polymorphisms between the parental lines NCG-122 and NCG-121. These primers were used to look for polymorphisms in the genomic DNA bulks prepared from the resistant and susceptible plants. Ten pairs (3.5%) were chosen to analyze the DNA from 181 plants in the F₂ population. The resulting data were used to construct a linkage group using Join Map 4.0 (LOD = 10). The total length of the linkage group was 16.3cM, and the average genetic distance was 1.6cM. The *pm-s* gene was located between SSR20486 and SSR06184/SSR13237 with genetic distances of 0.9 and 1.8cM, respectively (Figure 2B).

Second mapping of the *pm-s* gene using new molecular markers

One hundred and forty-seven pairs of new SSR primers were designed by the genome sequence of the initial mapping region of the *pm-s* gene. Four of these and three of 10 pairs of SSR primers published by He et al. (2013) showed polymorphisms among the parents and

were used to analyze the F_2 mapping population. Then, a new linkage group was constructed with 17 SSR primers, and a total length of 20.4cM (see Table 2 for names and sequences of the primers). Among these, the markers most closely linked to the *pm-s* gene were pmSSR27 and pmSSR17, with genetic distances of 0.1 and 0.7cM, respectively (Figure 2C).

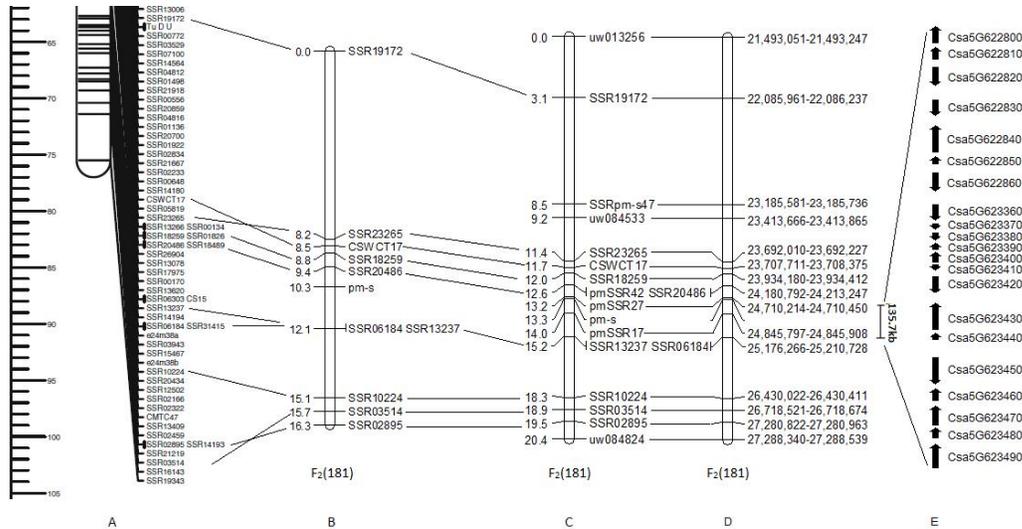


Figure 2. Molecular marker linkage and chromosomal mapping of the *pm-s* gene in cucumber. **A.** Chromosome 5 of cucumber. **B.** SSR linkage of the *pm-s* gene for preliminary mapping. **C.** Linkage of molecular markers to the *pm-s* gene for second mapping. **D.** Physical map of molecular markers. **E.** Predicted genes among the flanking markers.

Table 2. Sequence of SSR for the genetic linkage map of the *pm-s* gene in cucumber.

Primer name	Forward primer	Reverse primer	Motif	Fragment size
UW013256	CGACGTCGTGTTTCTCTGG	GCCCCAAAAGGATGGATTAT	(CT) ₃	197
SSR19172	CGAGGAGAAGGACTACGACG	CGTGTGGAGCTTTCTCACAA	(CAC) ₁₁	277
UW084533	TTCCCTCTATCAACAATGTCCA	TTTCCCTCTAAAAATTGAACATCAC	(AG) ₁₀	200
SSRpm-s47	CGGAAGTAGCATTGTTAGAGGA	GAAGCTCCATTATCACCACAA	(AG) ₃	156
SSR23265	CCTCCCTCCATTTTCCACTA	TGAGCCAGTTGGGGTTTITAG	(CT) ₁₉	218
CSWCT17	TGAAATTATGGGTTTCATTTT	GACAATGATAAACTCCCTGA	(TG) ₁₁	180
SSR18259	CGGTTTCTTCTGGCAAATGT	TTCCCTCAGCAGTCTCCAAT	(TC) ₁₇	233
SSR20486	ATAAACAGGGATCACGGCTG	CAGTTCAGTGAATCACC	(CACTTC) ₈	154
pmSSR42	CAGCCACACTGACCTTTGGA	GCTCAGTGGGTTTGATCCTCT	(AATTC A) ₄	224
pmSSR27	CCCTATGCGTGAAAAGCCACT	CGCCTCAAACCCATACCCAA	(AAT) ₇	237
pmSSR17	CGGAGACAGCTGTTGGTCAA	GAGTGAGGTAGAGGTGAGGGT	(TGGGTA) ₄	112
SSR13237	AGGGAGTTGGAAGAGGTGGT	AGTGAAAACAGTCAGGAGGTGA	(A) ₅₆	205
SSR06184	ACTAACCAAAACCAACGTC	CAGGCTTAATTGTTAAGAAAACAGAA	(TCATC) ₆	192
SSR10224	AAGTGAGTTGCAATGGCTGA	TCCATTGAGGTGATCTGAAAAA	(TC) ₁₉	390
SSR03514	TAGGGTCCCCTTCCCTCAT	GGGTACCAAAAAGCAAGTGA	(TC) ₁₄	154
SSR02895	GAGTTGGCAAGTCACGTTGT	TTTCCCTCATTATGCCATCC	(AT) ₁₉	142
UW084824	AAGTACTGCCTCAATCTCTAT	CTCACGTGCCGAGATTAAGAA	(AT) ₁₂	200

Sequence annotation and gene prediction in the genomic region harboring the *pm-s* gene

The physical distance between pmSSR27 and pmSSR17, the two flanking markers tightly linked to the *pm-s* gene, was about 135.7 kb (Figure 2D). A total of 21 predicted candidate genes

were located in the region, and their distribution on the chromosome is shown in Figure 2E. Fifteen of these predicted candidate genes have functional annotation, and these annotated genes include BTB/POZ fold, Ribophorin, zinc finger, peptidase family, NUDIX hydrolase conserved site, major intrinsic protein, alpha/beta hydrolase N-terminal, transferase, acyl hydrolase, Mlo-related protein, Tetraspanin, and serine carboxypeptidase (Table 3). *Csa5G623470* predicted to encode Mlo-related protein is the most likely candidate gene for the *pm-s*. The functional annotation of this gene showed organized defense response to fungus and participated in cell death.

Table 3. Annotation of genes located in the *pm-s* genetic mapping region.

Candidate genes	Position	Function
<i>Csa5G622800</i>	24,708,800-24,712,077	-
<i>Csa5G622810</i>	24,713,278-24,716,307	IPR000210 BTB/POZ-like; IPR004249 NPH3; IPR011333 BTB/POZ fold; IPR013069 BTB/POZ
<i>Csa5G622820</i>	24,717,496-24,721,550	IPR007676 Ribophorin I
<i>Csa5G622830</i>	24,735,193-24,738,505	IPR000679 Zinc finger GATA-type; IPR013088 Zinc finger NHR/GATA-type; IPR016679 Transcription factor GATA plant
<i>Csa5G622840</i>	24,741,435-24,750,050	IPR007484 Peptidase M28
<i>Csa5G622850</i>	24,750,072-24,750,730	-
<i>Csa5G622860</i>	24,752,324-24,756,109	IPR000086 NUDIX hydrolase domain; IPR007722 mRNA decapping protein 2 box A; IPR015797 NUDIX hydrolase domain-like; IPR020084 NUDIX hydrolase conserved site
<i>Csa5G623360</i>	24,762,529-24,766,481	IPR000425 Major intrinsic protein; IPR012269 Aquaporin; IPR022357 Major intrinsic protein conserved site; IPR023271 Aquaporin-like
<i>Csa5G623370</i>	24,766,751-24,767,082	-
<i>Csa5G623380</i>	24,769,944-24,771,510	IPR022742 Alpha/beta hydrolase N-terminal
<i>Csa5G623390</i>	24,773,390-24,773,916	-
<i>Csa5G623400</i>	24,774,031-24,776,029	-
<i>Csa5G623410</i>	24,777,415-24,777,798	-
<i>Csa5G623420</i>	24,781,456-24,785,158	IPR002641 Patatin/Phospholipase A2-related; IPR016035 Acyl transferase/acyl hydrolase/lysophospholipase
<i>Csa5G623430</i>	24,790,747-24,798,753	IPR000991 Glutamine amidotransferase class-I C-terminal; IPR001317 Carbamoyl-phosphate synthase GATase domain; IPR005801 ADC synthase; IPR005802 Para-aminobenzoate synthase component I; IPR006220 Anthranilate synthase component II/delta crystallin; IPR006221 Anthranilate synthase glutamine amidotransferase; IPR006805 Anthranilate synthase component I N-terminal; IPR011702 Glutamine amidotransferase superfamily; IPR015890 Chorismate binding C-terminal; IPR017926 Glutamine amidotransferase type 1
<i>Csa5G623440</i>	24,798,793-24,799,324	-
<i>Csa5G623450</i>	24,815,415-24,823,422	IPR000719 Protein kinase catalytic domain; IPR002575 Aminoglycoside phosphotransferase; IPR004147 ABC-1; IPR011009 Protein kinase-like domain
<i>Csa5G623460</i>	24,823,711-24,825,973	IPR000608 Ubiquitin-conjugating enzyme E2; IPR015582 Ubiquitin-conjugating enzyme E2 H10; IPR016135 Ubiquitin-conjugating enzyme/RWD-like; IPR023313 Ubiquitin-conjugating enzyme active site
<i>Csa5G623470</i>	24,827,408-24,831,456	IPR004326 Mlo-related protein
<i>Csa5G623480</i>	24,835,878-24,838,039	IPR018499 Tetraspanin
<i>Csa5G623490</i>	24,840,363-24,845,726	IPR001563 Peptidase S10 serine carboxypeptidase; IPR018202 Peptidase S10 serine carboxypeptidase active site

DISCUSSION

Several reports about the inheritance of resistance to PM in cucumber are available, but the conclusions often disagree (Barnes and Epps, 1956; Fugieda and Akiya, 1962; Kooistm, 1971; Shanmugasundaram, 1971; Morishita et al., 2003). The reasons for this include factors such as 1) differences in pathogenicity and race differentiation within PM; 2) differences in cultivars and breeding lines of cucumber; 3) differences in methods of disease evaluation methods and rating scales; 4) differences in the environmental conditions used during testing for PM resistance; 5) possible complexity in the inheritance of PM resistance; and 6) whether PM resistance is treated as a quantitative trait or targeted in different parts of the plant. This study focused on the inheritance of resistance to PM in the cucumber stem, thus eliminating the influence of other genes. It was determined that the *pm-s* gene controls the resistance to PM in the NCG-122 cucumber stem, and that the *pm-s* gene is located within a genetic distance of 0.8cM on Chr.5. The tightly linked SSR markers to the *pm-s* resistance gene found in this research will be applied in marker-assisted selection to improve the quality and marketability of cucumbers.

An *MLO-like* gene (*Csa5G623470*) was identified in the genomic region harboring the *pm-s* gene after sequence annotation and gene prediction. The gene belonged to *MLO* homology protein family with seven transmembrane domains. In barley and *Arabidopsis thaliana*, the *mlo* gene was a kind of recessive gene with positive defense reaction to PM. The function incapacitation of the susceptible gene caused durable and broad-spectrum resistance to PM (Büschges et al., 1997; Consonni et al., 2006). In cucumber, the gene was identified as

the resistance gene to PM in cucumber hypocotyl by Berg et al. (2015). In the same physical region harboring the *pm-s*, Nie et al. (2015) also found an *MLO-like* gene. So, *Csa5G623470* was speculated as the resistance gene for powdery mildew in cucumber stem in the present study. To prove, this guess will be the next focus of our study. We will narrow down the physical distance of the *pm-s* gene by fine-mapping technique, and clone the gene using map-based cloning method.

So far, there are no reports of chromosomal mapping for PM resistance in cucumber stem (*pm-s*), and no reports of any molecular markers tightly linked to *pm-s*. SSR markers (2112 pairs) were first applied to map the resistance to PM in cucumber stem in our study. The molecular markers defined in the present research will be good for MAS breeding. Moreover, the results of this study will lay the good foundation for cloning the resistance gene. An *MLO-like* gene, which is important to the resistance to PM in cucumber, identified on Chr.5 in our study was also reported by Nie et al. (2015). In this point, the results of the two studies could corroborate and support each other. The differences between the two types of research are as follows. Firstly, the plant materials were different. Cucumber inbred line S1003 was used as plant material in the study of Nie et al. (2015). S1003 belonged to East Asian type cucumber. The resistance to PM in S1003 was quantitative and conferred by multiple genes. The European greenhouse-type cucumber inbred line NCG122 was used in our study. The resistance to PM in the NCG122 stem was qualitative and conferred by the single recessive gene. Secondly, the disease rating methods were different. Nie et al. (2015) screened the whole seedling (including leaves) for disease rating, while we just focused on the stem (no leaves) for disease rating. Thirdly, the technique for molecular marker analysis was different. Windows QTL Cartographer 2.5 was used for QTL detecting in the previous study, while the bulked segregant analysis technology was adopted for gene mapping in the present study.

CONCLUSION

Genetic analysis revealed that resistance to PM in the stem of NCG-122 was qualitative and controlled by the *pm-s* gene. The *pm-s* gene was mapped between pmSSR27 and pmSSR17, with genetic distances of 0.1 and 0.7cM, respectively, confirming the location of this gene on Chr.5. The physical length of the genomic region harboring *pm-s* was 135.7 kb containing 21 predicted genes. Among these genes, the gene *Csa5G623470* annotated as encoding Mlo-related protein was identified as the most likely candidate gene for the *pm-s*.

Conflicts of interest

The authors declare no conflict of interest.

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