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ORIGINAL ARTICLE

QTL-seq analysis of powdery mildew resistance in a Korean cucumber inbred line

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Abstract

Key message **QTL mapping and RT-PCR analyses identified the *CsGy5G015660* as a strong powdery mildew resistance candidate gene and natural variation of *CsGy5G015660* allele was observed using 115 core germplasm.**

Abstract Powdery mildew (PM) is among the most serious fungal diseases encountered in the cultivation of cucurbits. The development of PM-resistant inbred lines is thus of considerable significance for cucumber breeding programs. In this study, we applied bulked segregant analysis combined with QTL-seq to identify PM resistance loci using F_2 population derived from a cross between two Korean cucumber inbred lines, PM-R (resistant) and PM-S (susceptible). Genome-wide SNP profiling using bulks of the two extreme phenotypes identified two QTLs on chromosomes 5 and 6, designated *pm5.2* and *pm6.1*, respectively. The two PM resistance loci were validated using molecular marker-based classical QTL analysis: *pm5.2* (30% R^2 at LOD 11) and *pm6.1* (11% R^2 at LOD 3.2). Furthermore, reverse transcriptase-PCR analyses, using genes found to be polymorphic between PM-R and PM-S, were conducted to identify the candidate gene(s) responsible for PM resistance. We found that transcripts of the gene *CsGy5G015660*, encoding a putative leucine-rich repeat receptor-like serine/threonine-protein kinase (RPK2), showed specific accumulation in PM-R prior to the appearance of disease symptoms, and was accordingly considered a strong candidate gene for PM resistance. In addition, cleaved amplified polymorphic sequence markers from *CsGy5G015660* were developed and used to screen 35 inbred lines. Natural variation in the *CsGy5G015660* allele was also observed based on analysis of a core collection of 115 cucumber accessions. Our results provide new genetic insights for gaining a better understanding of the genetic basis of PM resistance in cucumber, and pave the way for further utilization in cucumber PM resistance breeding programs.

Introduction

Cucumber (*Cucumis sativus* L.) is an economically important and widely cultivated vegetable crop worldwide. Powdery mildew (PM), which is primarily caused by the obligate biotrophic ectoparasite *Podosphaera xanthii* (formerly known as *Sphaerotheca fuliginea* Schlech ex Fr. Poll.), is probably the most common, conspicuous, and widespread fungal disease of cucurbits, including cucumber, and affects cucumber production worldwide through causing serious losses in yield and quality. PM is readily recognized by the presence of a visual white powdery fungal mass on leaf surfaces, petioles, and young stems (Zitter et al. 1996), and typically induces a reduction in leaf photosynthetic capacity and fruit quality (Nail and Howell 2004). In most cucumber production areas, PM is typically controlled by the application of fungicides (Xu et al. 2016b); however, in addition to being harmful to the environment and human health, excessive fungicide usage can increase selection pressure on *P.*

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xanthii populations to acquire increasing levels of fungicide resistance (Rubio et al. 2015). Accordingly, the development of resistant cultivars represents the most efficient approach to controlling PM disease and is a primary focus of cucumber breeding programs.

To enable the breeding of PM-resistant cucumber cultivars, it is essential to gain a better understanding of the genetic and molecular defense mechanisms plants deploy against PM. Identification of PM-resistant lines and modes of resistance inheritance commenced in the 1960s for a number of cucumber lines: 'Puerto Rico 37' (Kooistra 1968), PI 197087 (Barnes 1961), Natsufushinari (PI 279465) (Fugieda and Akiya 1962), PI 200815 and PI 200818 (Kooistra 1968). More recently, a number of studies have focused on detecting quantitative trait loci (QTLs) in cucumber. For example, Sakata et al. (2006) identified six temperature-dependent QTLs for PM resistance in cucumber using a population of F_7 recombinant inbred lines (RILs) derived from the susceptible Santou and resistant PI 197088–1. Similarly, five PM resistance QTLs in three linkage groups were identified from S06 line-derived $F_{2:3}$ populations (Liu et al. 2008), and two QTLs originating from PI 250147 were identified using an F_2 population (de Ruiter et al. 2008). Furthermore, Zhang et al. (2011) identified four QTLs (*pm5.1*, *pm5.2*, *pm5.3,* and *pm6.1*) from the cucumber inbred line K8 using F_2 and $F_{2:3}$ populations, whereas six QTLs on four chromosomes and two major QTLs (*pm5.1* and *pm5.2*) on chromosome 5 were identified from WI 2757 using $F_{2:3}$ families (He et al. 2013). A total of nine QTLs were identified from the RILs derived from a cross between CS-PMR1 and Santou, four of which (*pm3.1*, *pm5.1*, *pm5.2,* and *pm5.3*) were successfully validated (Fukino et al. 2013). Moreover, two main hotspots for PM resistance were identified on chromosomes 1 and 6 using the F_2 population derived from a cross between BK2 and H136 (Zhang et al. 2015), and recently, Wang et al. (2018) identified four QTLs that explained 63.0% of the PM resistance in an RIL of PI 197088.

In addition to the identification of PM resistance QTLs, the selection of key genes that control PM resistance has recently been actively pursued. Xu et al. (2016b) delimited a dominantly inherited major QTL for PM resistance in a 41.1-kb region and identified two cysteine-rich receptor-like kinase genes (*Csa1M064780* and *Csa1M064790*) as candidate genes from the Jin5-508-derived SSSL0.7 line. In addition, a single-recessive gene (*CsaMLO8* or *CsMLO1, Csa5M623470*) was identified as a strong candidate for PM susceptibility using PI 197088 and *C. sativus* cv. Anaxo (Berg et al. 2015, 2017; Nie et al. 2015a). Two candidate PM resistance genes, *Csa2M435460* and *Csa5M579560*, have also been identified from Jin5-508-derived SSL508-28 via whole-genome re-sequencing followed by qRT-PCR analysis (Xu et al. 2016a). Furthermore, Xu et al. (2017) identified eight potential candidate genes in a ~ 6.8-Mb substituted segment derived from the resistant donor Jin5-508 in SSL508-28. Most recently, three plausible candidate genes, *Csa5M622800*, *Csa5M622830,* and *Csa5M623490*, identified from *Cucumis hystrix* introgression lines and a single recessive gene (*Csa5M622830*), which encode GATA transcription factors, have been proposed as strong candidates for genes playing roles in PM resistance (Zhang et al. 2018).

Considering the complexity of the regulatory mechanisms underlying PM resistance, the identification of PM resistance QTLs and dissection of QTLs to facilitate the detection of PM resistance (or susceptibility) gene(s) from inbred line(s) are of fundamental importance for cucumber breeding programs. Nevertheless, even though extensive studies have been conducted and considerable data have accumulated, details of the molecular defense mechanisms involved in PM resistance have yet to be sufficiently elucidated, and at present few research findings have found practical application in marker-assisted selection (MAS). QTL analysis is a fundamental approach for genetic dissection of quantitative traits and is useful for map-based cloning of target genes. The rapid advances in high-throughput sequencing methods have facilitated the efficient identification of QTLs based on the rapid and massive identification of polymorphic sequences (Salvi and Tuberosa 2005). The combination of bulked segregant analysis (BSA) and high-throughput whole-genome re-sequencing (QTL-seq) has been successfully employed in the identification of QTLs for important agronomic traits, such as rice blast disease resistance and seedling vigor, using RILs and F_2 populations (Takagi et al. 2013). Subsequently, the QTL-seq approach has been widely applied in cucumber (Lu et al. 2014; Wei et al. 2016; Win et al. 2019), tomato (Illa-Berenguer et al. 2015), chickpea (Das et al. 2015; Singh et al. 2016; Srivastava et al. 2017), oilseed rape (Wang et al. 2016), barley (Hisano et al. 2017), groundnut (Kumar et al. 2019), and peanut (Zhao et al. 2020).

The main purpose of this study was to clarify the genetic architecture of PM resistance in a Korean cucumber inbred line (PM-R) by using a combination of the BSA and QTL-seq approaches. The PM-R was generated from the Nebakja, one of the Korean commercial cultivars. PM-resistance of Nebakja is not related to *CsaMLO8* gene, which was identified by genotyping analysis. In this study, we conducted whole-genome re-sequencing of two parental lines [PM-resistant (PM-R) and PM susceptible (PM-S)] and two DNA bulks (PM-R and PM-S bulks). We accordingly identified two PM resistance QTLs on chromosome 5 (*pm5.2*) and chromosome 6 (*pm6.1*). Furthermore, we applied classical QTL analysis using molecular markers to validate the genomic region of QTLs and estimate the effects on PM resistance. In silico sequence comparison and RT-PCR assays were also applied to identify potential candidate genes conferring PM resistance to the Korean PM-R inbred

line. Finally, we succeeded in identifying the putative gene *CsGy5G015660*, encoding a leucine-rich repeat (LRR) receptor-like serine/threonine-protein kinase (RPK2) as a plausible PM resistance candidate gene. We also revealed the natural occurrence of *CsGy5G015660* alleles and examined the applicability of this gene for use in MAS in cucumber breeding programs.

Materials and methods

Plant materials and growth conditions

In this study, we used two Korean cucumber inbred lines, PM-R and PM-S, as parental lines to generate F_1, F_2, and F_2-derived F_3 populations. The seeds of both parental lines, F_1 and F_2 generation progeny were kindly supplied by Dongbu Seed Company (Ansung, South Korea). Populations of the parental lines and F_1 and F_2 generations consisting of 132 individuals were grown in the greenhouse of the Asia Seed Company (Icheon, South Korea) in the summer of 2016. The $F_{2:3}$ families (8 plants per family) were grown in the greenhouse of Sejong University (Gonjiam, South Korea) in the summer of 2017. To minimize the effects of environmental factor(s) for accurate disease screening, we performed analyses at different locations and seasons. Tissue samples of 35 cucumber inbred lines were kindly supplied by Dongoh Seed Company (Icheon, South Korea).

Evaluation of powdery mildew responses in greenhouses

Two parental lines, PM-R and PM-S, their F_1 progeny, F_2 population, and $F_{2:3}$ families were screened for PM resistance. The development of naturally occurring PM disease under greenhouse conditions became apparent at approximately 60 days after planting when PM disease symptoms was clearly distinguishable and continued for a 2-week interval until disease symptoms had spread to all parts of the plants. For the evaluation of disease symptoms in each F_2 individual, severity was classified based on the percentage of infected area and the density of PM spore coverage of each leaf, as determined using the following visual rating scores: 0 = absence of symptoms (spores), 1 = symptoms on 1–10% of the surface area of infected leaves, 2 = 11–30%, 3 = 31–60%, 4 = 61–80%, 5 = 81–99%, and 6 = thick mat of powdery mildew spores (Fig. 1a). Mean disease scores were calculated as an average of the disease rating scores of three

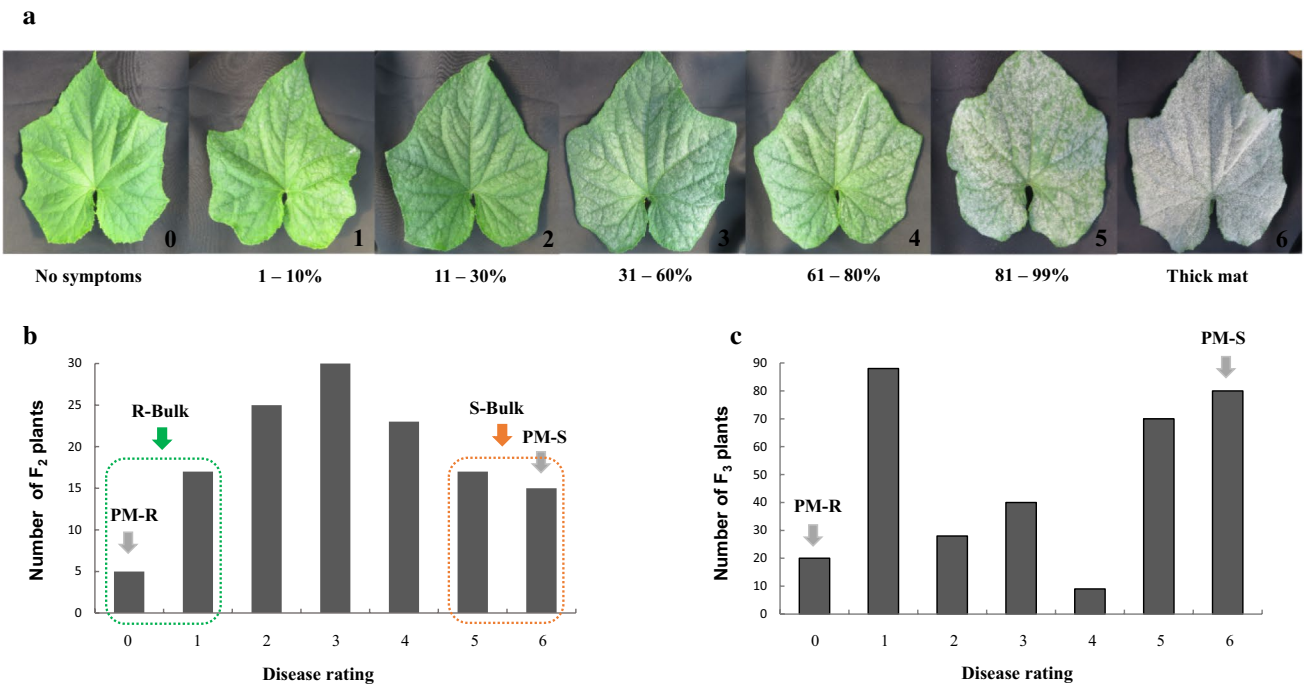

Fig. 1 Classification of powdery mildew (PM) disease symptoms in terms of the surface area of cucumber leaves affected (**a**). Disease severity was classified using the following rating scale: 0 = no symptoms, 1 = 1–10% of leaf area affected, 2 = 11–30%, 3 = 31–60%, 4 = 61–80%, 5 = 81–99%, and 6 = a thick mat of PM or completely senesced leaf. Frequency distribution of PM disease ratings of the F_2 (**b**) and F_3 (**c**) individuals comprising PM-resistant and PM-susceptible bulks. Gray arrows indicate the PM disease rating of the parental lines. Green and orange arrows indicate the DNA samples of the PM-resistant and PM-susceptible lines that were bulked to generate resistant and susceptible bulks, respectively. The *X* and *Y* axes represent the PM disease rating and the number of individuals corresponding to each disease rating, respectively (color figure online)

different individuals. For each $F_{2:3}$ family, the disease rating scores of each F_3 individual were obtained during the summer of 2017. The average disease score of each $F_{2:3}$ family was determined as the average of eight F_3 plants per family.

Whole-genome re-sequencing and construction of PM-resistant and PM-susceptible bulks

After confirmation of F_2 disease scores using the $F_{2:3}$ families consisting of eight individuals per family, 15 F_2 lines showing consistently extreme resistance and susceptibility to PM were selected as the PM-resistant bulk and PM-susceptible bulk, respectively, (Table S1; Fig. 1b). Genomic DNA (gDNA) was isolated from the fresh young leaves of the two parental lines and F_2 individuals of both bulks using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The quality and quantity of gDNA was measured using a Nanodrop ND-2000 spectrophotometer following the manufacturer's instructions (Thermo Fisher Scientific, USA). Equal amounts of gDNA obtained for each individual were mixed to construct PM-resistant and PM-susceptible bulks, respectively. Both the parental lines and the PM-resistant and PM-susceptible bulks were subjected to whole-genome re-sequencing using an Illumina HiSeq 2000 sequencer. A library of ~ 280-bp insert size was constructed at the Theragen BiO Institute (TBI, TheragenEtex, Korea) and pair-end sequenced (2 × 100 bp) in an Illumina HiSeq 2000 sequencer using HiSeq Sequencing Kits. Raw paired-end reads were generated by applying a base-calling pipeline [Sequencing Control Software (SCS), Illumina].

QTL-seq analysis by BSA sequencing

For the purposes of this study, we used the BSA-assisted QTL-seq approach described by Win et al. (2019), which is outlined briefly below. The raw paired-end reads of both parental lines were aligned to the Gy14 cucumber genome sequence (version 2.0) (Yang et al. 2012) using the program Bowtie 2 (Langmead and Salzberg 2012). SAMtools software was applied to identify single-nucleotide polymorphism (SNP) and insertion–deletion (InDel) differences between the two parental lines (Li et al. 2009). The sequence data of the resistant and susceptible bulks were equalized as described by Takagi et al. (2013), after which, the short reads obtained from the resistant and susceptible bulks were aligned to the PM-S genome sequence using the program BWA (Li and Durbin 2009). The Illumina phred-like quality and mapping scores were set to values greater than 30. Thereafter, SNP and InDel calling was carried out between resistant and susceptible bulks using the program SAMtools (Li et al. 2009). The SNP-index, calculated for each bulk by QTL-seq and MutMap (Abe et al. 2012; Takagi et al. 2013), was determined from the reads of all the resistance genotypes in the parental lines. PM resistance was distinguished according to the total number of short reads for this SNP locus. In both bulks, loci with an SNP-index < 0.3, and a read depth < 7 were filtered out to avoid errors during sequencing or alignment. However, the loci with an SNP-index < 0.3 in one bulk and ≥ 0.3 in the other bulk were considered to be true SNPs according to Takagi et al. (2013). An SNP-index value of 0 indicates that all short reads harboring the SNP are from PM-S, whereas an SNP-index value 1 indicated that all short reads harboring that SNP are from PM-R. Differences in the SNP indices of the two bulks were calculated as Δ (SNP-index) = SNP-index (PM-R) − SNP-index (PM-S). The average SNP-index of the SNPs was measured at a given genomic interval with 2-Mb window size and 10-kb increment by using the sliding window method. The statistical confidence intervals of Δ (SNP-index) with a given read depth under the null hypothesis of no QTLs were generated to ensure the accuracy of QTL identification by QTL-seq, according to the analytical process described by Takagi et al. (2013).

Classical QTL analysis

For further confirmation of the QTLs associated with PM resistance, we performed classical QTL analysis, using simple sequence repeat (SSR), InDel, cleaved amplified polymorphic sequence (CAPS), and derived cleaved amplified polymorphic sequence (dCAPS) markers to screen 132 individuals of the F_2 population. The gDNA used in these analyses was isolated using a DNeasy Plant Mini Kit (Qiagen), the quality and quantity of which was measured using a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific, USA). A total of 35 markers [10 SSR (Ren et al. 2009; Zhang et al. 2012), 20 InDel, 2 CAPS, and 3 dCAPS markers] flanking the QTL-seq-derived genomic regions of chromosomes 5 and 6 were identified. Geneious Pro 9.1.8 (Biomatters, Auckland, NZ) was used to identify polymorphic sequences, design primer sets, and select the appropriate restriction enzymes. The markers used for classical QTL analysis in the present study are shown in Table S2. Polymerase chain reactions (PCR) were performed according to Win et al. (2019). Amplified DNA fragments were visualized on 2% agarose gels prepared with 0.5 × TBE buffer and stained with Red-Safe (iNtRON, South Korea). For the application of CAPS and dCAPS markers, appropriate restriction enzymes were used to cut the PCR amplicons according to the manufacturer's instructions (New England BioLabs, MA, USA), as shown in Table S2. Linkage analysis was performed using the MAPMAKER 3.0 program (Lander and Green 1987) at a high logarithm of the odds (LOD) threshold (> 5.0) with the Kosambi mapping function. The composite interval mapping (CIM) function of the QGENE program (Nelson

1997) at a significant ($P < 0.01$) LOD score threshold under 1000 permutations was used to estimate the phenotypic variance explained by the QTLs.

Podosphaera xanthii (P. xanthii) inoculation, RNA isolation, and expression analysis

Both parental lines (PM-R and PM-S) were grown in a growth chamber (Daihan Scientific, Wonju, South Korea) at 23 °C under long-day conditions (16/8-h light/dark photoperiod) with a light intensity of 120 µmol m^{-2} s^{-1}. PM conidia were harvested from the naturally infected leaves of PM-S in the greenhouse, and a suspension of spores was diluted to a concentration of 10^6 spores/mL^{-1} using in 0.01% Tween-20. Seedlings of both PM-R and PM-S lines at the two-leaf stage were artificially inoculated by evenly spraying leaf surfaces with the PM spore suspension. The inoculated cucumbers were covered with polythene to maintain a high level of humidity until samples were collected. We collected leaf samples at 0, 0.5, 1, 2, 5, and 7 days post-inoculation (dpi) to determine the expression of selected candidate genes located in the two PM resistance QTL regions. Visible symptoms of PM disease, the appearance of white spores of *P. xanthii*, were observed at 7 dpi. At each time point, we collected five leaves per plant from three independent biological replicates. The collected samples were immediately frozen in liquid nitrogen and maintained at 80 °C until used for RNA isolation.

Total RNA was isolated from leaf samples using Plant RNA Purification Reagent (Invitrogen, MA, USA), and the quality of the isolated RNA quality was determined using a Nanodrop ND-2000 spectrophotometer (Thermo Fisher, MA, USA). Only high-quality RNA samples (A260/A230 > 2.0 and A260/A280 > 1.8) were used in subsequent experiments. First-strand complementary DNA (cDNA) was synthesized from 5 µg of total RNA according to the instructions of a ReverTra Ace qPCR RT Master Mix kit (Toyobo, Osaka, Japan). The gene-specific primers used for expression analysis were designed using Geneious Pro 9.1.8 (Biomatters, Auckland, New Zealand) and are shown in Table 1. RT-PCR reaction mixtures (20 µL) were prepared using EmeraldAmp GT PCR Master Mix (TaKaRa, Seoul, Korea) and amplification was performed in a Bio-Rad T100™ Thermocycler (BioRad, CA, USA). The amplification program used was as follows: 94 °C for 5 min, followed by 30–36 cycles (based on the primer set used) at 94 °C for 20 s, 56–63 °C (based on primer set) for 10 s, and 72 °C for 20 s, with a final cycle at 72 °C for 5 min. The PCR amplicons were visualized on 2% agarose gels prepared with 0.5 × TBE buffer and stained using RedSafe. The *ACTIN* gene was used as a reference control (Migocka and Papierniak, 2011).

Annotation of two QTLs and comparison with previously identified PM resistance QTLs

The Gy14 genome sequence and annotation file (version 2.0; https://cucurbitgenomics.org/) were downloaded and used to identify the genic and intergenic sequences of *pm5.2* and *pm6.1*. Geneious Pro 9.1.8 (Biomatters, Auckland, New Zealand) was used to identify the SNPs and InDels from the genic (exon and intron) sequences of both genic regions (*pm5.2* and *pm6.1*) following the manufacturer's instructions. For the comparison of *pm5.2* and *pm6.1* with previously identified QTLs associated with PM resistance, all marker sequences flanking the previously identified QTLs were used for BlastN analysis using Geneious Pro 9.1.8 (Biomatters, Auckland, New Zealand). On the basis of this comparison, all the previously identified PM resistance QTLs were physically mapped to the Gy14 genome (version 2.0) and compared with *pm5.2* and *pm6.1*.

Identification and confirmation of candidate PM resistance gene(s)

The sequences of PM-R and PM-S encompassing the two genomic regions *pm5.2* and *pm6.1* were used to identify candidate gene(s) associated with PM resistance. Genes showing polymorphism(s) between the PM-R and PM-S sequences were identified using the "find variation" function of Geneious Pro 9.1.8 (Biomatters, Auckland, New Zealand). All genic sequences showing polymorphism between the PM-R and PM-S sequences were re-aligned to the sequences of additional resistance (Gy14 and PI 288995) and susceptible lines (WMEJ, PI 173892, and PI 267942) to confirm conservation of the polymorphism, and those genes showing conserved polymorphism among these lines were selected. Candidate PM resistance genes were selected if one of the two following categories was met. First, the gene should show strong evidence of involvement in plant stress defense responses based on putative function (as determined by performing a sequence similarity search of the NCBI database). Second, the polymorphic sequence should induce a nonsense mutation. Finally, the selected genes were subjected to RT-PCR to determine the differential expression between PM-R and PM-S (see *Podosphaera xanthii* infiltration, RNA isolation, and expression analysis).

In silico *3D modeling of CsGy5G015660*

SWISS-MODEL (Waterhouse et al. 2018; https://swissmodel.expasy.org/) and Modeller software (version 9.25; https://salilab.org/modeller/download_installation.html) were performed as following their protocols to predict protein structure of a full length coding sequence of *CsGy5G015660*.

Table 1 List of the genes used for RT-PCR analysis

QTL	Genes ID	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	Amplicon size (bp)
pm5.2	*CsGy5G013040*	GACTGGCTGCGACAAAAGTG	AGATACTTAGCCTGAGCGAGC	319
	CsGy5G013070	GGAAAGACTGGCTGCGACAA	AAGCTGTGGGACCCTGAATT	258
	CsGy5G013450	GGCGTATCCACGAGCCAAAA	GCCCAACCTATGCACACCTC	113
	CsGy5G013460	CCATCAGTTGTGTCGGTAGTTC	GCACCTTTATTAAGTAATGCCCG	118
	CsGy5G014280	GTTGGGCACTGAAAGCG	GATATGAGAGAGTTCACAAGATGT	306
	CsGy5G014630	GAAGGAAGGCGGTGATGG	GGGATGACCATGAGAGTCGA	728
	CsGy5G015650	ACTGGTTTTCGAGCGAGC	ATCCCTACCCAGCTCATACC	322
	CsGy5G015660	TGCTACAACATGTCGGGTCT	TCTCCACGACTTGCTTCATTG	296
	CsGy5G015780	TCACATCGGTCGTTCGGT	GGAGCAAGGAAAGGACTCG	702
	CsGy5G015880	CAACGACGGAATGCTTGC	GTAGACGACAACTGCCACA	445
	CsGy5G016430	GGAAGCTCAAGCTGCTAATATC	CATATGCCAGTGGCAGAGG	123
	CsGy5G016450	CTTCGAGTTCCGCTCGCAAT	GTTCTTGTGGCTTCACTGGCT	124
	CsGy5G016490	GATCTTTTCGGTTCGCTCGC	GATTGCAGACCACACACTCC	324
	CsGy5G016560	TCCCTCGTAGCCAGTCAGAA	CGTGGTTTCGTGTCCTCACT	335
	CsGy5G016620	AGGCCATCTGACAAACCCCT	ATGCCAGTTTCAACACGACCC	95
	CsGy5G017310	TACTTCCCGCTGTTGGACAC	GATTTGCCCTAAACTACAACCC	449
	CsGy5G018400	GACCCCTTCAACCACTCCAG	TGTGGCGTTCATTCCTGTCA	286
pm6.1	*CsGy6G012810*	CCGGAGCGGGTAGTTTATGC	GCAGCTTGGCCGTTCATTTG	117
	CsGy6G012820	GCTTCCAACAACGTTGCCATC	CACTCAAGCAAAACCGAGCG	146
	CsGy6G012960	CACCCATAAGACCATCCCGA	TCCGTTTTCCCACCACCA	322
	CsGy6G013010	CTCCCGGAACTATCCGACGA	GTTGGATCCGTGGAAAGCGT	147
	CsGy6G013020	CGGTTTCCCTGACTCTCCT	CACAAGACAGCCAAGGACA	223
	CsGy6G013210	CGCCGCCATTTCTGACCA	CATTTCCTTGGCTGCTCTGG	318
	CsGy6G013270	GGCCCTAGTAAACGTGGTGT	TGGCACGGCAGAGAATGTAA	255
	CsGy6G013300	CGATCGCGATTGATCCTGGG	GCAAAGAGGTTCGGCCATCA	129
	CsGy6G013310	CCCCTCAATCATTGCCCAT	GCGGCTTCTCATCCCAAC	173
	CsGy6G013350	TGCTGATTCTTCCAATTCCGGA	TGTTCGTTTGCTGTTGTTGCT	350
	CsGy6G013800	CCACCAGATGTGGATGTTGATGA	GACAAGTTGTGACAGCCCCTT	133
	Cs-Actin	CCTCATTGGAATGGAAGCTGC	GAAGCACTTCCTGTGGACGATG	350

Development and application of a CsGy5G015660-derived CAPS and CsaMLO8-based InDel marker

To further confirm the correlation between the *CsGy5G015660* allele and the PM resistance phenotype, we developed the CAPS marker (CAPS_CsGy5G015660) from *CsGy5G015660* using the forward primer (5′-GCA ACAAGTTCAATGGACCAC-3′) and reverse primer (5′-GAATCTCTCCAGTCAAATTGTTTCC-3′) using the *HinfI* restriction enzyme (Table S2). CAPS_CsGy5G015660 was used to screen 40 F_2 lines [15 resistant, 15 susceptible, and 10 intermediate (a disease rating of 3 or 4)], and 35 inbred lines provided by Dongoh Seed Co. (Ichun, South Korea) (Tables S1, S4; Fig. S3). PCR reaction mixtures (20 µL) were prepared using a Maxime PCR PreMix (Intron Bio, Seoul, Korea) and amplifications were performed in a Bio-Rad T100™ Thermocycler (BioRad, CA, USA). Samples were amplified using the follow reaction program: 94 °C for 5 min, followed by 35 cycles of 94 °C for 20 s, 60 °C for 10 s, and 72 °C for 20 s, with a final cycle at 72 °C for 5 min. *HinfI* was added to PCR amplicons and incubated at 37 °C for 1 h. We also applied CsaMLO8_InDel marker (forward: 5′-TATGGCTGCCTTTCATCTCCT-3′, reverse: 5′-ATATCT TCAACTCGCTGATGGAAACAA-3′) to screen 35 inbred lines (Table S2). Samples were amplified using 94 °C for 5 min, followed by 30 cycles of 94 °C for 20 s, 65 °C for 20 s, and 72 °C for 2 min, with a final cycle at 72 °C for 5 min. The final reaction products were visualized on 2% agarose gels prepared with 0.5 × TBE buffer and stained with RedSafe.

Evaluation of the natural variation of CsGy5G015660

The sequences of 115 cucumber core germplasms (Qi et al. 2013) were used to evaluate the natural variation of the candidate gene, *CsGy5G015660*. All reads of the re-sequenced

115 core cucumber accessions (SRA0506480) were downloaded from the NCBI short read archive and imported into Geneious Pro 9.1.8 (Biomatters, Auckland, New Zealand). Sequence alignments were performed using the "Map to reference" function with *CsGy5G015660* used as a reference sequence. On the basis of alignments between *CsGy5G015660* and the short-read sequences of the 115 cucumber accessions, we identified the natural variation in eight SNPs in *CsGy5G015660* alleles.

Results

Genetic inheritance of PM resistance

The PM-resistant parental line (PM-R) was found to asymptomatic and was accordingly rated as 0 (no symptoms). In contrast, the susceptible line (PM-S) showed maximal disease symptoms and rated as 6 (severe disease symptom). The F_1 progenies showed intermediate symptoms and were assigned scores of 3 or 4. The mean disease score distribution of the 132 individuals in the F_2 mapping population showed a continuous frequency distribution over the range of 0 to 6, with an average score of 3.2 (Fig. 1a, b). In the $F_{2:3}$ families, the frequency distribution of the mean disease score was skewed toward the two parents, PM-R and PM-S (Fig. 1c). This bimodal frequency distribution might be expected, given that the $F_{2:3}$ families were derived from the two selected extreme groups of PM resistance.

Whole-genome re-sequencing of cucumber

In this study, we performed whole-genome re-sequencing of the two parental lines (PM-R and PM-S) and two bulks (resistant and susceptible bulks) comprising the extreme phenotypes identified among F_2 individuals. We accordingly obtained a total of 0.98 GB of raw data comprising 237.5 and 202.4 million paired-end short reads (100 bp in length) from PM-R and PM-S, respectively. Subsequent to a quality check, 0.93 GB of data remained, which were used for further analysis. The sequences of both parental genomes were aligned to the Gy14 reference genome (version 2.0), resulting in 20× depth with 98.92% and 98.61% genome coverage, respectively. Resistant and susceptible bulks generated 294.1 and 248.8 million raw paired-end short reads, respectively. After quality control and equalizing, the sequences of these bulks covered 99.05% and 99% of the Gy14 genome, respectively, (Table 2). In total, we identified 224941 polymorphic SNPs between the PM-resistant and PM-susceptible bulks of the F_2 progeny. The whole-genome sequences obtained in this study have been deposited in the GenBank/NCBI database under the Sequence Read Archive Accessions Nos. SRR11782917 and SRR11782916 for PM-R and PM-S, respectively.

Molecular mapping of QTLs associated PM resistance in the F_2 population using QTL-seq

To identify the QTL region(s) conferring PM resistance, we calculated the SNP-index of individual SNPs using the parental PM-S line as a reference genome and compared these with the sequences of both resistant and susceptible bulks. An SNP-index value of 0 indicates that the entire short reads are derived exclusively from the PM-S genome, whereas a value of 1 indicates that the reads are derived entirely from the PM-R genome. The average SNP-index values on each chromosome were calculated based on a 2-Mb genomic interval with a 10-kb increment and plotted for PM-resistant and PM-susceptible bulks against all chromosomes (Figs. 2, S1). To ensure efficient detection of the differences in SNP-indices between the two bulks, we measured the Δ (SNP-index) by integrating the SNP-index

Table 2 Statistics of QTL-seq sequencing

Sample	No. of raw reads	No. of filtered reads (%)[a]	No. of aligned reads (%)[b]	Genome coverage (%)[c]
PM-R	237,575,528	224,236,490 (94.39)	150,443,813 (87.87)	98.92
PM-S	202,477,822	190,888,534 (94.28)	104,432,302 (61.75)	98.61
R-Bulk	294,145,720	284,372,244 (96.68)	182,473,465 (89.03)	99.05
S-Bulks	248,858,030	232,971,186 (93.62)	162,018,937 (89.35)	99

[a]Percentage represents the ratio of number of filtered reads from number of raw reads

[b]Percentage represents the ratio of number of aligned reads to the cucumber Gy14 sequence from number of raw reads

[c]Gy14 sequence (genome version 2.0) was used as a cucumber reference genome sequence

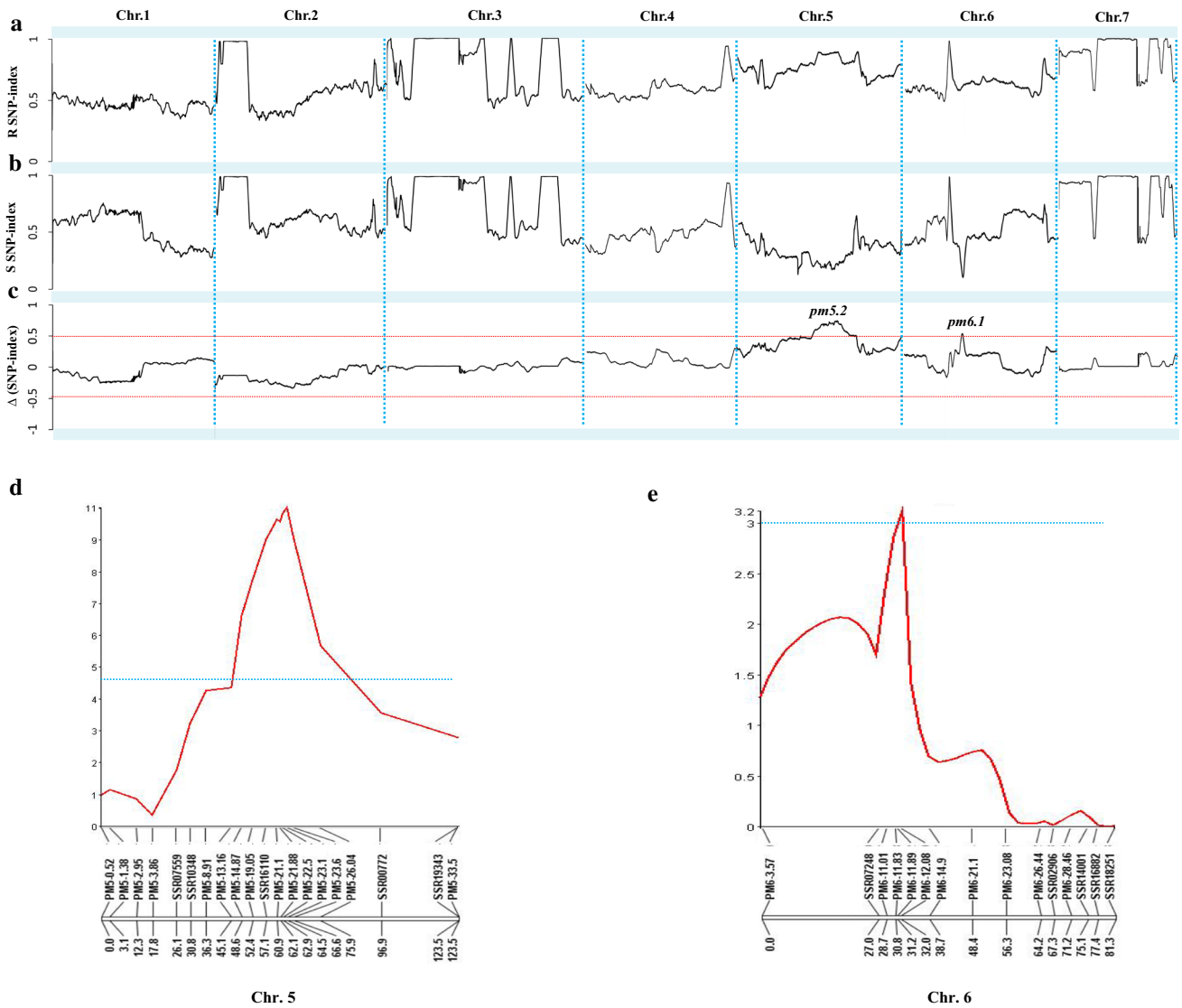

Fig. 2 Single-nucleotide polymorphism (SNP)-index graphs of resistant (**a**) and susceptible (**b**) bulks, and a Δ (SNP-index) graph (**c**) based on QTL-seq analysis of an F_2 population. The *X*-axis represents the position on seven chromosomes, and the *Y*-axis represents the SNP-index. The SNP-index was calculated based on 2-Mb intervals with a 10-kb sliding window. The Δ (SNP-index) graph (**c**) was plotted with a statistical confidence interval under the null hypothesis of no QTLs ($P < 0.05$). Two candidate QTLs, *pm5.2* and *pm6.1*, were defined using the criteria of SNP-index near 1 and 0 in resistant (**a**) and susceptible (**b**) bulks, respectively. Log of odds (LOD) score plots of powdery mildew resistance QTLs obtained by classical QTL analysis of chromosome 5 (**d**) and chromosome 6 (**e**) of an F_2 population. The significant threshold ($P < 0.01$) is indicated by the dotted horizontal line. The *X*-axis represents the molecular marker-linked position on chromosomes 5 and 6, and the *Y*-axis represents the LOD score

information of the PM-resistant and PM-susceptible bulks and plotted a statistical confidence interval according to the genomic locations (Mb) of the reference genome. Significant genomic positions were identified at a statistical significance of $P < 0.05$. By examining the Δ (SNP-index) plots, we identified two significant ($P < 0.05$) genomic regions harboring QTLs for PM resistance on chromosomes 5 and 6 in accordance with the criteria of SNP-index estimation defined in QTL-seq analysis (Fig. 2c). The genomic region from 16.35 to 24.99 Mb on chromosome 5 was characterized by an average SNP-index higher than 0.9 (highest value = 1.0) in the PM-resistant bulk and lower than 0.16 in the PM-susceptible bulk (lowest value = 0). Further analysis of this QTL indicated that most individuals in the PM-resistant bulk showed the PM-R allele, whereas most individuals in the PM-susceptible bulk had the PM-S allele. These observations accordingly indicate that PM resistance could be determined primarily by a factor(s) in this genomic region. Moreover, this genomic region had an average Δ (SNP-index) value of 0.73, which is significantly different from 0 at the

95% confidence interval (Fig. S2a). These results thus provide convincing evidence for the presence of a major QTL controlling PM resistance in the 16.35 to 24.99 Mb region on chromosome 5 of cucumber (Table 3; Figs. 2, S2a), which we accordingly designated *pm5.2*. Similarly, a second region extending from 11.01 to 12.42 Mb on chromosome 6 had an average SNP-index higher than 0.61, with a highest value of 0.73 in the resistant bulk, and with values lower than 0.24 (lowest values = 0.1) in the susceptible bulk. This region had an average Δ (SNP-index) value of 0.5, which is significantly different from 0 at the 95% confidence interval (Table 3; Fig. S2b). These results would therefore appear to indicate the presence of a second QTL conferring PM resistance within the region between 11.01 and 12.42 Mb on chromosome 6 of cucumber, which we designated *pm6.1* (Table 3; Figs. 2, S2b).

Validation of QTL-seq-derived QTLs based on classical QTL analysis

We performed classical QTL analysis to verify the accuracy of two QTLs identified by QTL-seq. A major genomic region from marker PM5-13.16 (45.1 cM) to SSR00772 (96.9 cM), designated as *pm5.2*, was physically located in the region between 13.16 and 27.82 Mb on chromosome 5 (Fig. 2d). The LOD value for this region was 11, with the highest peak value at marker locus PM5-23.1 (Fig. 2d). This region showed a positive additive effect, which could explain 30% of the observed phenotypic variation (Table 3). In addition, we mapped one minor QTL in the interval between PM6-11.83 (30.8 cM) and PM6_14.9 (38.7 cM) on chromosome 6, with a peak LOD value of 3.2 at marker locus PM6-12.08, which we designated *pm6.1* (Fig. 2e). This 7.9 cM target QTL region was found to show a positive additive effect associated with a 3.07 Mb region [PM6-11.83 (11.83 Mb) to PM6_14.9 (14.9 Mb)] on chromosome 6 and could explain 11% of the phenotypic variance (Table 3). This QTL interval corresponded to the genomic region of *pm6.1* identified by QTL-seq, which covered the region between 11.01 and 12.42 Mb. These results are thus broadly consistent with those obtained using QTL-seq analysis, although indicate a wider genomic region covered by the QTL.

Identification of candidate genes for PM-resistance QTLs

To identify potential candidate genes associated with PM resistance, we used two consecutive approaches. Initially, we identified all the genes located in the two detected QTLs (*pm5.2* and *pm6.1*) that showed polymorphism(s) between the genic sequences of PM-R and PM-S, and subsequently examined the expression levels of the selected genes to identify those showing differential expression levels between the PM-R and PM-S lines. On the basis annotation to the Gy14 reference genome (version 2.0), w *pm5.2* region of chromosome 5 contains 552 putative genes. Among these, 48 genes were identified as showing sequence polymorphism between two parental lines, 37 of which were nonsynonymous and 11 nonsense mutations (Table S3). Seventeen putative genes, seven of which are known to be involved in disease defense reaction(s) and the remaining 10 of which show nonsense mutations, were selected and transcript expression levels were examined based on RT-PCR analysis. Finally, we identified the gene *CsGy5G015660*, which encodes a putative LRR receptor-like serine/threonine-protein kinase RPK2, the transcripts of which showed distinct accumulation in the PM-R line at 1, 2, 5, and 7 dpi (Fig. 3).

The *pm6.1* region of chromosome 6 was found to contain 122 putative genes, among which, 32 showed sequence polymorphisms between the PM-R and PM-S lines. Non-synonymous SNPs were identified in 24 putative genes (Table S3). In contrast to the genes detected in the *pm5.2* region on chromosome 5, none of the analyzed genes in chromosome 6 showed significantly different transcriptional levels between the PM-R and PM-S lines.

Analysis of strong candidate genes controlling PM resistance

To confirm the involvement of *CsGy5G015660* in PM resistance, we developed the CAPS marker CAPS_CsGy5G015660 and used this to screen 40 F_2 individuals, among which there were 15 showing PM resistance, 15 showing PM susceptibility, and 10 showing intermediate resistance. Genotyping analysis using CAPS_CsGy5G015660 revealed that the 25 F_2 individuals with

Table 3 QTLs associated with powdery mildew resistance identified based on QTL-seq and classical QTL analysis

QTL-seq				Classical QTL			
QTL	Physical position (Mb)	Average Δ SNP-index	Source of allele	Physical Position (Mb)	Associated marker	LOD score	Phenotypic variation explain PM resistance (%)
pm5.2	16.35–24.99	0.73	PM-R	13.16–27.82	PM5-23.1	11	30
pm6.1	11.01–12.42	0.5	PM-R	11.83–14.9	PM6-12.08	3.2	11

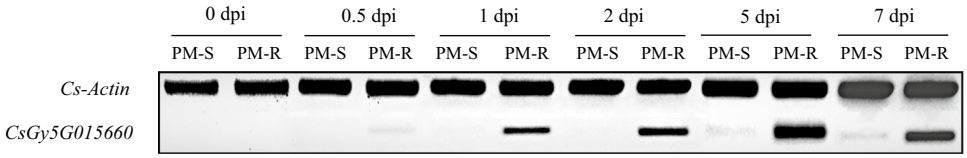

Fig. 3 Expression patterns of candidate powdery mildew (PM) resistance gene in cucumber. Leaves from the five-leaf-stage of PM-resistant and PM-susceptible lines were used for RT-PCR. The *Cs-ACTIN* gene was used as a positive reference control. dpi represents days post-inoculation of *Podosphaera xanthii*

intermediate or susceptible phenotypes showed either specific susceptibility (224 bp) or heterozygous bands (224 bp and 324 bp) (Fig. S3), which is consistent with the fact that PM resistance is associated the expression of a homozygous recessive resistance gene(s). However, among 15 individuals showing PM resistance, three lines showed a heterozygous genotype characterized by both resistance and susceptible bands (Fig. S3). The CAPS_CsGy5G015660-based genotyping analysis indicated that the CAPS_CsGy5G015660 marker showed a strong correlation with the PM resistance phenotype, with 92.5% consistency for selection of the PM-resistant line. Given that use of the CAPS_CsGy5G015660 marker showed a 7.5% inconsistency between phenotype and genotype, we identified four additional genes (*CsGy5G014630*, *CsGy5G015650*, *CsGy5G015780*, and *CsGy5G015880*) that showed polymorphism(s) between PM-R and PM-S and were located at distances up to 1628 and 280 kb either side of the *CsGy5G015660* gene. However, the expression patterns of these genes showed no difference between PM-R and PM-S in response to inoculation with *P. xanthii*. Collectively, these results provide compelling evidence in support of our contention that *CsGy5G015660* is a strong candidate gene responsible for PM resistance.

Furthermore, we also used the CAPS_CsGy5G015660 marker to screen 35 inbred lines, among which there were 31 and four lines with PM-resistant and -susceptible phenotypes, respectively. We detected no inconsistency between genotype and phenotype among the four lines that showed a susceptible phenotype, as they all showed heterozygous bands (Table S4). However, among the 31 PM-resistant lines, we detected four lines (12.9%) showing a heterozygous banding pattern (Table S4), thereby indicating that the CAPS_CsGy5G015660 marker could be used to screen for inbred PM resistance inbred with 87.1% accuracy. Furthermore, most of PM-resistant inbred lines identified using the CAPS_CsGy5G015660 marker were also confirmed using a *CsaMLO8*-based InDel marker (Tables S2, S4). Given that these two genes are located 8.8 Mb apart, *CsGy5G015660* and *CsaMLO8* could be independently introgressed from PM resistance sources (Fig. 4). Although we found that four PM-resistant inbred lines (lines #28, 33, 34, 35 in Table S4) could not be identified as such using the CAPS_CsGy5G015660 marker, three accessions (lines #9, 21, 26) could be exclusively genotyped using this marker (Table S4).

3D modeling of CsGy5G015660 and natural occurrence of the CsGy5G015660 allele in cucumber germplasm

Having identified the *CsGy5G015660* gene as a prominent factor association with PM resistance, we applied 3D modeling using a full length coding sequence of *CsGy5G015660* to identify the relationship between SNPs and protein structure of PM-S and PM-R. From this analysis, two additional α-helices and β-sheets were observed at a specific location of PM-S originated CsGy5G015660 protein (Fig. 5). The five additional α-helices were observed at a specific location of PM-R originated CsGy5G015660 protein (Fig. 5). These additional formations of α-helices and β-sheets induced overall structural difference between PM-R and PM-S, especially on inside concave surface structure (Fig. 5). Furthermore, we subsequently attempted to determine the frequency of SNPs of *CsGy5G015660* in diverse cucumber germplasms. We accordingly identified eight SNP loci, 1456, 1534, 1594, 1769, 1770, 1779, 2076, and 2220 in the coding sequence of *CsGy5G015660* showing differences between PM-R and PM-S (Fig. 6). The four SNPs were found to cause a nonsynonymous mutation of the CsGy5G015660 protein (Fig. 6). For this purpose, we used the genome sequences of 115 cucumber core accessions (Qi et al. 2013) for in silico analysis using BLASTN searches to detect the presence of eight SNPs (Table S5). *CsGy5G015660*-based in silico genotyping revealed that 48 (41.7%) and 67 (58.3%) accessions were PM-R and PM-S (homozygous and heterozygous alleles), respectively (Tables S5, S6).

Discussion

Genetic inheritance of PM resistance

Powdery mildew is one of the most severe and widespread diseases affecting cucurbit vegetables. To date, numerous studies related to genetic inheritance, QTL analysis of PM

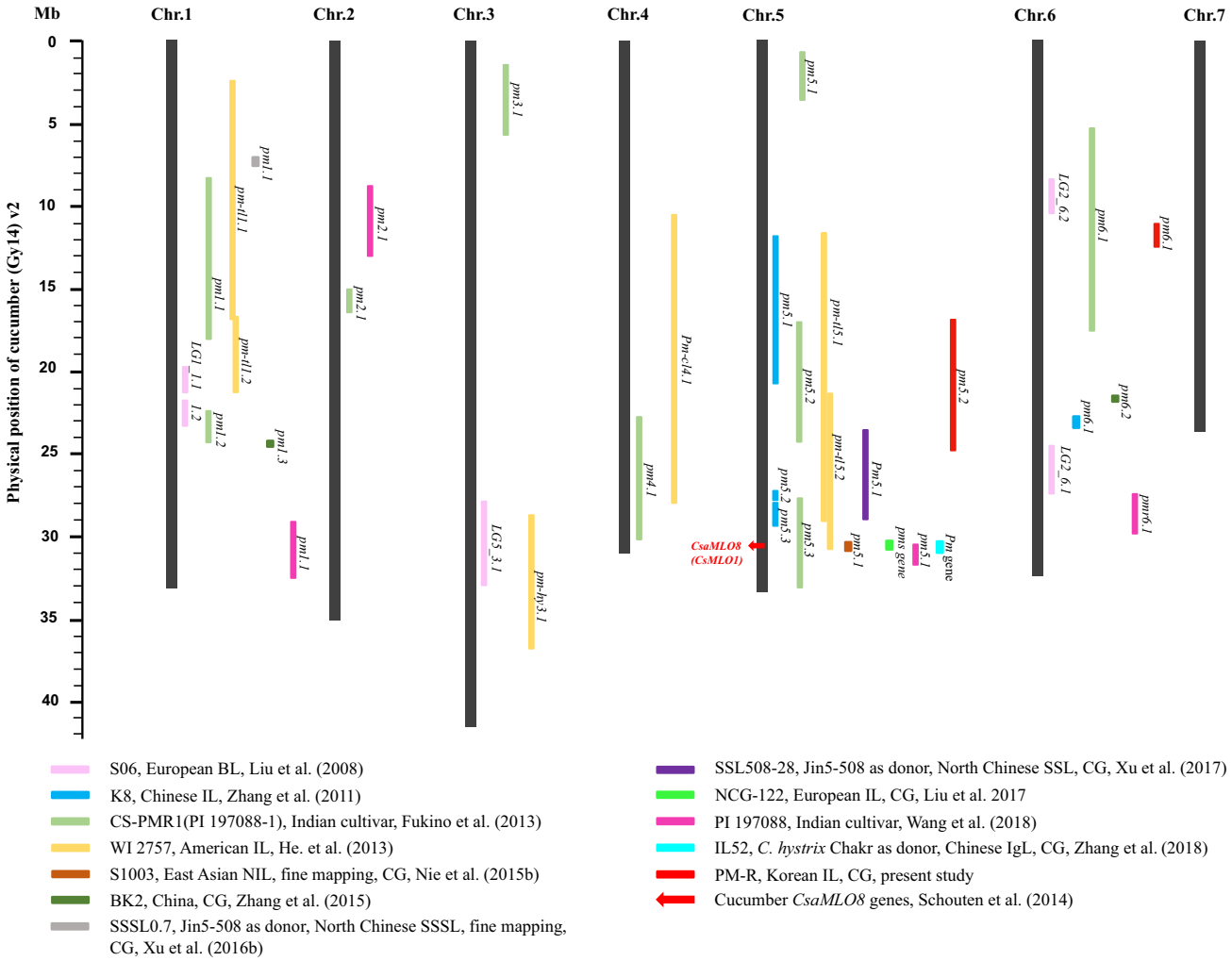

Fig. 4 Physical locations of previously identified powdery mildew (PM) resistance QTLs and the QTLs detected in the present study. The seven cucumber chromosomes are represented by black bars, and the physical positions of QTLs correspond to the scale on the left. The genomic regions related to PM resistance are presented on the right of each chromosome. *BL*, breeding line, *IL* inbred line, *NIL* near-isogenic line, *SSSL* single-segment substitution line, *SSL* segment substitution line, *IgL* introgression line, *CG* candidate gene

resistance, and selection of PM-resistant germplasm have been reported (Sakata et al. 2006; Liu et al. 2008; Zhang et al. 2011; Fukino et al. 2013; Nie et al. 2015a, b; Xu et al. 2016b; Wang et al. 2018), some of which have shown that the PM resistance of cucumber is controlled by a single recessive gene (Nie et al. 2015a; Liu et al. 2017; Zhang et al. 2018). In contrast, however, most of the relevant studies have indicated that PM resistance is a multi-gene quantitative trait (Sakata et al. 2006; Liu et al. 2008; Zhang et al. 2011; Fukino et al. 2013; Nie et al. 2015b; Xu et al. 2016b; Wang et al. 2018). The findings of the present tend to support the latter assumption. In general, quantitative resistance under polygenic control is more durable than that conferred by a single dominant gene (Kelly and Vallejo 2006). However, the quantitative inheritance of resistance based on disease severity is often characterized by low heritability and high environmental influence (Olczak-Woltman et al. 2009). In this regard, the development of resistant cultivars using the current cutting-edge molecular techniques and marker-assisted selection has been a more efficient strategy than phenotype-based PM disease screening, which can be inaccurate and influenced by environmental conditions.

Identification of QTLs associated with PM resistance and comparison with previously identified QTLs and candidate genes

In the present study, we used QTL-seq and classical QTL approaches to analyze an F_2 population of 132 individuals derived from a cross between the lines PM-R and PM-S, and the continuous frequency distribution of disease scores obtained for this population provided clear evidence for the

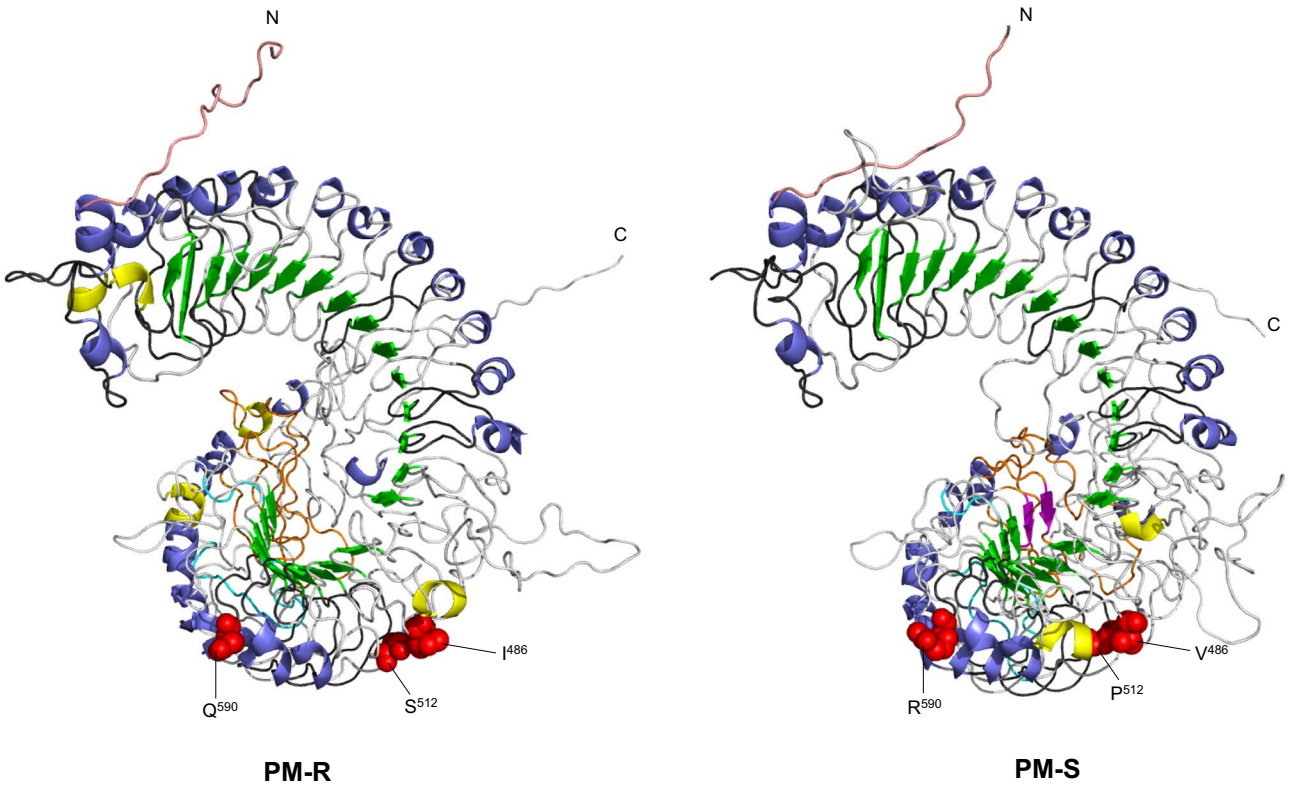

Fig. 5 In silico 3D modeling of PM-R (left) and PM-S (right) using a full length coding sequence of *CsGy5G015660*. The α-helices are shown as blue and yellow (yellow indicates the α-helices unique to PM-R or PM-S only); β-sheets are in green and pink (pink indicates the additional β-sheets on PM-S than PM-R). Each different domain was marked with a different color: signal peptide domain (salmon), LRR domain (black), transmembrane domain (cyan), and protein kinase domain (orange). Three amino acids with missense mutations were shown as red spheres. Positions of the N and C termini are shown in capital letters (color figure online)

polygenic control of PM resistance (Fig. 1b). Unlike classical QTL analysis, QTL-seq utilizes the integrated advantages of BSA and high-throughput genome sequencing. It is a powerful tool that in a single step can be used detect the target regions of interest based on the analyses of F_2 populations (Takagi et al. 2013; Clevenger et al. 2018). In the present study, QTL-seq analysis identified two genomic regions, *pm5.2* and *pm6.1*, conferring PM resistance, which were located in 16.35–24.99 Mb and 11.01–12.42 Mb genomic regions on chromosomes 5 and 6, respectively, (Figs. 2, S2). These QTLs had positive Δ (SNP-index) values of 0.73 and 0.5, respectively, indicating that the resistant parental line PM-R harbors alleles for PM resistance at the *pm5.2* and *pm6.1* loci. Similarly, classical QTL analysis enabled us to identify two genomic regions between 13.16 and 27.82 Mb and 11.83 and 14.9 Mb on chromosomes 5 and 6, with LOD values of 11 and 3.2, respectively (Fig. 2d, e). Although the genomic regions for PM resistance detected using classical QTL analysis were broader than those detected using the QTL-seq approach, it also enabled us to determine LOD values explaining the effect of each QTL for phenotypic variation. Therefore, the combination of QTL-seq and classical QTL analyses can represent a powerful tool for the identification of PM resistance over a short time period using an F_2 mapping population. Furthermore, the utilization of bulks of individuals with both extreme phenotypes means that identification of the genomic loci associated with PM resistance can be performed with a reasonably reliable degree of accuracy.

Given that the co-localization of QTLs derived from different germplasms may indicate that they belong to the same locus or are closely linked (Wang et al. 2020), we compared our PM resistance loci with those identified in previous studies (Fig. 4). We accordingly found that the genomic interval of the major-effect (30% R^2) QTL *pm5.2* corresponds exactly with the *pm5.2* (16.96–24.18 Mb) locus identified in the Indian temperature-independent PM-resistant line CS-PMR1 (PI197088-1) (Fukino et al. 2013). Moreover, the *pm-tl5.1* (11.53–20.09 Mb) locus identified in the USDA inbred line WI 2757 (He et al. 2013) completely encompassed the *pm5.2* locus identified in the present study. Furthermore, two *pm5.1* QTLs spanning 11.80–20.84 Mb and 23.56–28.68 Mb, identified in the Chinese PM-resistant inbred line K8 and the SSL508-28 substitution line (Zhang

Fig. 6 Schematic structure of *CsGy5G015660*. **a** Exon–intron structure of *CsGy5G015660* and the position of SNPs found in *CsGy5G015660*. Black and grey boxes indicated exons and intron, respectively. **b** Amino acid sequences of translated proteins of *CsGy5G015660* of PM-R and PM-S. Red and yellow dots showed the non-synonymous and synonymous substitution, respectively. The bars under the amino acids sequences represented the following domain: signal peptide domain (salmon), leucine–rich domain (LRR) domain (black), transmembrane domain (cyan), and protein kinase domain (orange) (color figure online)

et al. 2011; Xu et al. 2017), were found to partially overlap our *pm5.2* locus. These results tend to indicated that the same or a closely linked locus is possibly derived from diverse PM-resistant germplasms, including PM-R. Furthermore, it could be proposed that appropriate introgression of chromosome 5 harboring a locus conferring PM resistance is the most important approach for breeding PM-resistant cultivars. In contrast to *pm5.2* on chromosome 5, only a single QTL spanning the 5.31–17.89 Mb region on chromosome 6, identified by Fukino et al. (2013), shows co-location with our minor-effect (11% R^2) QTL *pm6.1*, with which it partially overlapped. This finding may thus indicate that *pm6.1* originates from a specific PM-resistant germplasm that lacks closely linked loci.

To date, several candidate PM resistance genes have been proposed based on QTL analysis using diverse PM-resistant germplasms (Nie et al. 2015b; Zhang et al. 2015, 2018; Xu et al. 2017; Liu et al. 2017). Most of the proposed candidate genes are located within the QTLs detected on chromosome 5 and these QTLs have been extensively dissected. On the basis of these efforts, the genes *Csa5M623470* (9930) [*CsGy5G026660* (Gy14)] encoding *CsaMLO8*, *Csa5M579560* (9930) [*CsGy5G019870* (Gy14)] encoding an LRR-receptor-like protein kinase, *Csa5M622830* (9930) [*CsGy5G026540* (Gy14)] encoding a GATA transcription factor, *Csa5M622800* (9930) [*CsGy5G026510* (Gy14)] encoding a glycosyl transferase, and *Csa5M623490* (9930) [*CsGy5G026680* (Gy14)] encoding a serine carboxypeptidase-like protein have been identified as candidate genes associated with PM resistance (Xu et al. 2016b; Liu et al. 2017; Zhang et al. 2018). Given that these genes map to positions within or close to (less than 3 Mb) the *pm5.2* region, we compared the sequence polymorphism(s) between these genes in the PM-R and PM-S lines. However, we found that none of the analyzed candidate genes showed sequence polymorphisms between these two lines. These findings thus indicate that although the PM resistance QTLs of PM-R are similar to those of other PM-resistant germplasms, the PM resistance gene(s) identified in PM-R could differ from the candidate genes identified by other research groups.

Identification of the candidate genes associated with PM resistance QTLs

Although considerable effort has been made to dissect the QTLs associated with PM resistance in cucumber, few candidate genes are currently available for use in the MAS of PM-resistant germplasm (Wang et al. 2020). To identify plausible candidate genes lying within the *pm5.2* region detected in the present study, we examined sequence polymorphisms and differential gene expression levels in both parental lines. Among the 552 annotated genes located in the *pm5.2* region, 10 nonsense mutant-type genes and/or seven stress resistance-related genes were selected for RT-PCR expression analysis at six different time points: 0, 0.5, 1, 2, 5, and 7 dpi (Tables 1, S3). Among these genes, we identified *CsGy5G015660* as showing specifically enhanced transcript levels in the PM-R line at 1, 2, 5, and 7 dpi (Fig. 3). Similarly, among the 122 putative genes located in the *pm6.1* region, we selected 11 genes based on their possible involvement in disease resistance reaction(s) using sequence similarity comparisons. However, we were unable to identify any candidate gene(s). Thus, although several genes, including *CsaMLO8* (*CsMLO1*) and *CsGy5G015660*, have been identified of chromosome 5, there have to date been no candidate genes detected on chromosome 6. Therefore, further studies on dissection of the *pm6.1* QTL, based on recombinant inbred line (RIL) or near isogenic line (NIL)-assisted fine mapping analyses, will be necessary to identify the PM resistance-associated factor lying within this region.

Sequence similarity analysis indicated that *CsGy5G015660* may encode the LRR receptor-like serine/threonine-protein kinase RRK2. It is well established that disease resistance genes typically have a conserved protein domain, such as the LRR sequence (Staskawicz et al. 1995; Berg 2019). Similarly, a putative LRR receptor-like kinase (LRR-RLK) has been identified as contributing to downy mildew disease resistance in *Arabidopsis* (Hok et al. 2011). Genes encoding LRR receptor-like serine/threonine-protein kinase-like proteins have also been demonstrated to be responsible for resistance to the scab disease of apple and downy mildew disease of pearl millet (Kulkarni et al. 2016; Padmarasu et al. 2018). From the respective sequence-based functional annotation, *CsGy5G015660* could serve as a PM resistance accompany with sequence polymorphism and differential expression levels between PM-R and PM-S.

We detected eight SNPs between the sequences of *CsGy5G015660* in the PM-R and PM-S lines, among which, only four are associated with missense mutations, whereas the remaining four SNPs result in silent mutations (Fig. 6). Of the four SNPs resulting in missense mutations, two are located in the LRR domain (Fig. 6b), and thus it is conceivable these sites could play an important role in conferring PM resistance. In order to identify this possibility, we applied in silico 3D modeling using a full length coding sequence. The result confirmed that there was a structural difference between PM-R and PM-S. The 3D modeling showed that new α-helices and β-sheets were formed uniquely on CsGy5G015660 protein of each parental line (Fig. 5). The LRR protein was usually composed by solenoid structure where concave side was defined by a parallel β-sheets (Bella et al. 2008). Furthermore, it has been considered that the concave curved surface of LRR domain usually contained ligand binding site, protein–protein interaction module or recognition site of elicitors from pathogens (Bella et al. 2008; Głowacki et al. 2011). The biggest structural difference between CsGy5G015660 protein of PM-R and PM-S were concave side structure. The protein of PM-S showed deformed curved concave surface by appearance of two additional β-sheets (Fig. 5). However, the protein of PM-R still maintained the curved concave surface. Overall results suggested that the SNPs associated with missense mutations affect the overall protein structure formation rather than change the protein structure in the region. The conformational change of the inside concave surface caused by SNPs, which probably control binding site for recognition of pathogen elicitors. This hypothesis should be verified in further studies.

In order to verify the correlation between the *CsGy5G015660* allele and the PM-resistant phenotype, we developed a CAPS marker, CAPS-CsGy5G015660, and used this to screen 40 F_2 individuals (Fig. S3). The genotyping result suggested that resistance of *CsGy5G015660* is not complete dominant or recessive, because both bands of PM-R and PM-S were observed in the intermediate lines. Since the frequency distribution of the disease score in F_2 population was skewed toward PM-S lines, we considered that *CsGy5G015660* followed incomplete recessive. Of course, the possibility of other gene(s) being involved could not be ruled out. The exception of three individuals in R-bulk also observed. The inconsistency shown by these latter three individuals could be attributed to the fact that either *CsGy5G015660* is not a PM resistance gene or that additional genes having minor effects are necessary for complete PM resistance. To assess these possibilities, we examined the polymorphic sequences in four additional genes (*CsGy5G014630, CsGy5G015650, CsGy5G015780*, and *CsGy5G015880*) flanking *CsGy5G015660*, all of which showed similar expression patterns between the PM-R and PM-S lines. On the basis of this information, *CsGy5G015660* appears to be a strong candidate gene responsible for PM resistance in the PM-R line. The possible contribution of additional genes that may play weaker roles in controlling PM resistance should, nevertheless, be examined in further studies.

Evaluation of cucumber germplasm from the perspectives of CsGy5G015660 and CsaMLO8

To date, *CsaMLO8* has been considered to be a gene associated with PM susceptibility in the hypocotyl of cucumber seedlings (Berg et al. 2015). Studies have indicated, however, that the loss of *CsaMLO8* function is not sufficient for complete resistance to PM in cucumber (Nie et al. 2015a, b). Nevertheless, at present, *CsaMLO8* is the only known key gene involved in the regulation of PM resistance. However, the resistance of PM-R used in the present study does not appear to be associated with a loss of *CsaMLO8* function, as we failed to detect any polymorphism in the gene sequence. We can thus reasonably assume that *CsGy5G015660* is the major source of PM resistance in the PM-R line. Therefore, we compared the natural variation of *CsGy5G015660* and *CsaMLO8* alleles (Table S5). *CsGy5G015660*-based genotyping revealed that 48 (41.7%) and 67 (58.3%) accessions were PM-R and PM-S (homozygous and heterozygous alleles), respectively, whereas *CsaMLO8*-based genotyping indicated that 21 (18.3%) and 94 (81.7%) accessions were PM-R and PM-S, respectively (Tables S5, S6). Although 115 cucumber core germplasm showed inconsistent genotypes between *CsGy5G015660* and *CsaMLO8* alleles, 35 inbred lines showed highly consistent genotype between both alleles (Tables S4, S5). Given that these two genes are located 8.8 Mb apart (Fig. 4), *CsGy5G015660* and *CsaMLO8* could be independently introgressed into the inbred lines through continuous crossing and selection practices by breeders. These issues should be further examined in future studies.

Screening Korean inbred cucumber lines using the CAPS_CsGy5G015660 marker

In order to establish the applicability of CAPS_CsGy5G015660 in cucumber breeding programs, we used this marker to screen 35 inbred lines, among which 31 and four lines showed PM-resistant and PM-susceptible phenotypes, respectively. Using the marker, the four PM susceptible inbred lines were clearly genotyped as PM susceptible lines, whereas among the 31 PM-resistant lines, four (12.9%) showed a heterozygous banding pattern (Table S4), which could be attributable to the fact these inbred lines originated from different sources of PM-R. Overall, however, the percentage inconstancy between phenotype and CAPS_CsGy5G015660-based genotype was less than 13% in the analyzed inbred lines (Table S4). These results thus indicate that CAPS_CsGy5G015660-based genotyping could serve as an efficient screening tool for selection of PM resistance in the cucumber breeding programs of Korean seed companies.

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Author contributions statement SL designed the research. KTW, CYZ, and MBA developed the plant materials and performed field work and genetic analysis. CYZ, MBA, and SB conducted the sequence data analysis. CYZ and SL wrote the manuscript. CYZ and MBA conducted the statistical analysis and RNA expression-level analysis. All authors reviewed and approved publication of this study.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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