Identification of novel loci and candidate genes for resistance to cucumber powdery mildew using GWAS

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Research article

Keywords:

Posted Date: June 10th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-33121/v1>

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Abstract

Background

Powdery mildew (PM) is one of the most serious diseases in cucumber and causes huge yield loss. Multiple quantitative trait loci (QTLs) for PM resistance have been detected in previous studies, however, none of these studies used the approach of genome-wide association analysis (GWAS). In this study, a cucumber core germplasm (CG) consisting of 109 resequenced lines was evaluated for PM resistance in three seasons.

Results

twelve loci (*pmG1.1*, *pmG2.1*, *pmG2.2*, *pmG3.1*, *pmG4.1*, *pmG4.2*, *pmG5.1*, *pmG5.2*, *pmG5.3*, *pmG5.4*, *pmG6.1* and *pmG6.2*) associated with PM resistance were detected on all chromosomes except for Chr.7 using GWAS. Among these loci, *pmG2.1*, *pmG4.1* and *pmG6.1* were the novel ones that have not been reported in previous studies. The loci of *pmG2.1*, *pmG5.2*, *pmG5.3* showed stronger signal in three seasons, for which three candidate genes *Csa2G022790*, *Csa5G484620* and *Csa5G604330* were respectively predicted using pairwise LD correlation and qRT-PCR analysis. Further, *Csa4G022270* in *pmG4.1*, *Csa6G022350* and *Csa6G022370* in *pmG6.1* might be the candidate genes based on the annotation of homologous genes in *Arabidopsis*.

Conclusion

Twelve Loci related to powdery mildew resistance in cucumber were detected by genome-wide association analysis, three of which namely *pmG2.1*, *pmG4.1* and *pmG6.1* are novel. Three candidate genes within the genomic region of locus *pmG2.1*, *pmG5.2* and *pmG5.3* were identified, respectively. These results provide targets for gene cloning and genetic breeding of PM resistance in cucumber.

Introduction

Cucumber (*Cucumis sativus* L.) is an important vegetable crop with a long history of cultivation. Powdery mildew (PM) is one of the most important diseases in cucumber and occurs extensively in various countries all over the world [1–3]. PM infection in cucumber at flowering stage could reduce the yield by 20% ~ 40%. Development of resistant cultivars is the most economic and effective method to control PM in cucumber.

Many studies showed that cucumber resistance to PM was determined by quantitative trait loci (QTLs) [3–5]. The mapped QTLs (Fig. 1) are scattered over all the seven cucumber chromosomes. For some chromosomes, more than one QTLs are clustered. For example, three QTLs (*pm5.1*, *pm5.2*, *pm5.3*) are located on chromosome 5 that are reported in different studies [4, 6–11].

Most of the QTLs showed recessive inheritance, indicating that the resistance is governed by loss-of-function mutant alleles of certain susceptible genes [12, 13]. Indeed, a mutant allele of the cucumber susceptibility gene *CsaMLO8* (named as *CsMLO1* in [14] explains the contribution to PM resistance by the QTL *pm5.1* [15]. Except for the *pm5.1*, causal genes of other mapped QTLs for PM resistance in cucumber have not been discovered yet. Thus, further researches are needed to fine-map and clone the QTLs, which may be combined with an approach to search for mutations in susceptibility genes co-localized with mapped QTLs (Schouten et al. 2014).

Genome-wide association analysis (GWAS) is an effective and feasible way for the analysis of disease resistance traits [16]. With the development of sequencing technology and the reduction of sequencing costs, GWAS has been applied in many species, such as corn [17], rice [18–20], maize [21], sorghum [22], and foxtail millet [23]. In cucumber, GWAS has been used to map multiple genes. For example, the bitterness gene *Bi* were detected via GWAS in 115 diverse cucumber lines [24]. Downy mildew (DM) resistance was analyzed in 395 accessions selected from 1234 USDA cucumber lines [25]. Using GWAS in combination with QTL mapping, a candidate gene related to callus regeneration was identified (Wang et al. 2018c). Bo et al. (2019) identified a novel locus *fsd6.1* combined with *fsd6.2* (*Csgl3*) which regulate fruit spine density by GWAS.

In this study, 109 core germplasm (CG) lines [11, 27] were used for a GWAS on PM resistance. In total, 12 QTLs were detected, of which three are novel and nine were reported in previous studies. Further, potential candidate genes for certain QTLs were studied by evaluating their expression in contrast lines with high resistance or susceptibility.

Results

Genetic diversity of PM resistance in the CG population

The 109 CG lines were evaluated for PM resistance three times, in spring 2013 (pm_2013S), spring 2014 (pm_2014S) and fall 2014 (pm_2014A). For each line, the DI of each experiment was calculated and shown in Supplementary Table 1. The mean DI of the three experiments varies among the CG lines between 25.9 and 29.4 (Fig. 2a). The DI correlation among the three experiments was significant with a $R^2 = 0.7 \sim 0.81$ (Fig. 2b). Based on the SNP in the three loci and the DIs of three seasons, we detected the genotypic effect at each of the two seasons (Supplementary Fig. 1) [28]. Results showed that the three loci were independent in the three seasons, which indicated that these three loci work independently for powdery mildew.

The mean DI per line of the CG population was used for phylogenetic analysis to study the PM resistance diversity, resulting in three clusters based on the complete method (1.5) of SAS (Supplementary Fig. 2). The first cluster contains 34 lines with a DI < 20. Among these lines, 23 lines showed a DI < 10 and classified as highly resistant (HR) lines. Of these HR lines, nine (CG7, CG9, CG32, CG35, CG44, CG63, CG70, CG82 and CG101) showed a consistent DI score in all three experiments. The second cluster

contains 33 lines with a DI > 35 and regraded as highly susceptible (HS) lines. Five HS lines, CG3, CG23, CG31, CG55, CG209 showed a comparable DI among the three tests. Interestingly, seven tested Japanese type (CG3, CG4, CG28, CG29, CG31, CG103 and CG104) showed high sensitivity. The third cluster includes 30 sensitive lines with a DI between 20 and 35.

Genome-associated analysis of powdery mildew resistance

In order to identify genetic loci that are associated with PM resistance in the CG population, DIs of each experiments was used for GWAS. Total twelve loci (*pmG1.1*, *pmG2.1*, *pmG2.2*, *pmG3.1*, *pmG4.1*, *pmG4.2*, *pmG5.1*, *pmG5.2*, *pmG5.3*, *pmG5.4*, *pmG6.1* and *pmG6.2*) were detected, which are scattered on six chromosomes (Fig. 3). Three loci, *pmG2.1*, *pmG5.2* and *pmG5.3* were detected in all the three experiments with a higher p-value than others, indicating that these loci were stable and strongly related to PM resistance. Four loci, *pmG1.1*, *pmG4.1*, *pmG4.2* and *pmG5.4*, were detected in two of the three experiments. Five loci, *pmG2.2*, *pmG3.1*, *pmG5.1*, *pmG6.1* and *pmG6.2*, were detected in one of three experiments. Among the 12 loci, 9 loci were located in chromosomal regions where QTLs for PM resistance were reported in previous studies [6, 10, 11, 29]. While, the other three (*pmG2.1*, *pmG4.1* and *pmG6.1*) have not been reported yet (Fig. 1) and thus representing novel cucumber loci for PM resistance.

Candidate genes for the PM resistance

Aiming at identifying potential candidate genes of the stable loci (*pmG2.1*, *pmG5.2* and *pmG5.3*) and novel loci (*pmG4.1* and *pmG6.1*), chromosomal region of 50 kb around the peak SNPs were further analyzed.

Candidate gene analysis for novel loci (*pmG4.1* and *pmG6.1*)

Physical distance of the peak SNP in *pmG4.1* was at Chr.4:2,436,922 bp. Based on the cucurbit genomics (<http://cucurbitgenomics.org/>), 14 genes were predicted in the *pmG4.1* region (Supplement table S2). In this region, *Csa4G022270* encodes NPR1-like protein that regulates salicylic acid (SA)-mediated plant defense [30]. Physical distance of the peak SNP in *pmG6.1* was at Chr.6:2,291,66 bp. Ten genes were predicted in the *pmG6.1* candidate region (Supplement table S3). *Csa6G022350* encodes splicing factor U2AF subunit and its homologous gene in *Arabidopsis* is involved in pathogen response via the salicylic acid pathway [31]. *Csa6G022370* encoded MAC/Perforin domain containing protein and its homologous gene in *Arabidopsis* is related to response during geminivirus infection [31]. These two genes might be the candidate genes of *pmG6.1*.

Candidate gene analysis for *pmG2.1*

For the *pmG2.1* locus, SNPs of the chromosome region Chr. 2: 2,710-2,949 kb were analyzed by pairwise LD correlations with a focus on candidate genes in the interval from 2.715 to 2.768 Mb (Fig. 4a). Based on the Cucumber Genome Browser (<http://www.icugi.org/cgi-bin/ICuGI/index.cgi>), 11 candidate genes were located in this interval. Three genes, *Csa2G022770*, *Csa2G022780* and *Csa2G022790* were selected, since they all encode a disease resistance-like protein although the DNA sequence similarity among them

is very lower than 25%. The relative expression of the three genes were analyzed by qRT-PCR (Fig. 4d-f) with gene specific primers (Supplement table S4) in the selected HR and HS lines. Results showed that *Csa2G022790* was significantly up-regulated in three of the four HR lines at 12 h after PM inoculation. In one of the three HR lines, CG44, the induction was also seen at 8 d after PM inoculation. A single nucleotide polymorphisms (SNP) (G to T) in the promoter of *Csa2G022790* was located on the TCA-element (salicylic acid responsiveness) with the G presents in HR lines (Fig. 4c). Salicylic acid is a key signaling component involved in the activation of certain plant defense responses [32]. In the HS lines the expression of *Csa2G022790* was down-regulated in the CG3 line and no difference was found in the other lines (Fig. 4g). These results indicated that *Csa2G022790* might be the candidate gene of the *pmG2.1* locus.

Candidate gene analysis for pmG5.2

For the *pmG5.2* locus, SNPs in the chromosomal region of Chr.5: 16,650 – 17,150 kb were analyzed (Fig. 5a). We focused on the interval from 16,878,456 bp to 16,879,990 bp based on pairwise LD correlations ($r^2 \geq 0.6$). *Csa5G484620* was identified in this region, encoding an NBS resistance protein. Two SNPs (SNP2431819 and SNP2431821) were found in this gene (Fig. 5c). Twenty nine out of 30 HR lines were G/G and the other one is A/C, whereas 9 of 29 S lines were A/C and the others were G/G. Based on the qRT-PCR, the *Csa5G484620* was significantly up-regulated in CG44 (HR) at the 12 h and down-regulated at 24 h after PM inoculation (Fig. 5d). Then, four HR lines and four HS lines were applied to qRT-PCR (Fig. 5e). In the HS lines, the expression of *Csa5G484620* was down-regulated in CG23 while up-regulated in CG55, and no difference in the other two lines. Whereas the expression of all the HR lines were significantly up-regulated at 12 h after PM inoculation. These results indicated that *Csa5G484620* might be the candidate gene of the *pmG5.2* locus.

Candidate gene analysis for pmG5.3

For the *pmG5.3* locus, SNPs in the chromosomal region of Chr.5: 22,230 – 22,610 kb was analyzed with the focus on the interval from 22,506 kb to 22,518 kb (~ 12 kb) using pairwise LD correlations ($r^2 \geq 0.6$) (Fig. 6a). Two annotated genes were identified; *Csa5G604330* encoding alpha-amylase and *Csa5G604340* encoding Pectate lyase. The latter is homologous to the Arabidopsis *PMR6* gene [13]. SNP_2526314 was on the CDS of *Csa5G604340*. For this site, 29 of 30 HR lines was A, the other HR lines was G, whereas that 7 of 29 HS lines was G, the other HS lines was A (Fig. 6b). Then, four HR lines and four HS lines were applied to analyze the expression of *Csa5G604340* using qRT-PCR. Two of the HS lines (CG23 and CG31) and three of the HR lines showed up-regulated expression at 12 h after PM inoculation (Fig. 6c). We focus on all the 18 European type lines, all the G genotype in this site was HS lines, and 55.6% of A genotype were HR lines, which showed that the gene *Csa5G604340* could be related to the PM resistance of European type (Fig. 6d). *Csa5G604340* might be the candidate gene of the *pmG5.3* locus.

Discussion

The inheritance of PM resistance is complicated, most studies showed that the PM resistance was a quantitative trait that controlled by multiple genes [4–9, 11, 33–35]. In this study, we applied GWAS on a CG population, which contains 97 lines representing a corn collection of worldwide cucumber germplasm [27] and evaluated three times for PM resistance in different years and seasons. The frequency distributions of DI indicated that PM resistance of cucumber were controlled by multiple loci (Fig. 2), which is consistent with previous studies [7, 14].

Many QTLs for PM resistances were identified and mapped on all seven chromosomes in different studies [6–10, 29, 34]. In the current study, twelve loci associated with PM were detected by GWAS, of which nine loci were reported in previous studies (Fig. 1) and three loci (*pmG2.1*, *pmG4.1* and *pmG6.1*) are novel. The *pm-QTL* (LGIII, 20C) on Chr.7, *pm-QTL* (LGI, 26C) on Chr.6 [5] and *pm1.1* on Chr.1 [6] was not detected by our study, which may be due to the different environments. Our results show that GWAS may be a complementary approach to other QTL studies for PM resistance in cucumber.

Previous studies showed that most of the QTLs for PM resistance in cucumber are recessively inherited [6, 7, 34], indicating that the resistance is governed by impaired susceptibility (S) genes [12, 13] mapped cucumber genes that are homolog to know plant S genes for PM and downy mildew (DM) resistance. Some of the QTLs identified in our study co-localize with some of the cucumber S gene homologs. For example, The MLO-like gene *CsaMLO8* [13] located nearby *pmG5.4* [15]. A DMR and PMR candidate gene *Csa5M622830* (Chr.5: 24735193... 24738505) was also nearby the *pmG5.4* [34]. The candidate gene *Csa5G604340* for *pmG5.3* is a homology of *AtPMR6* [13].

Additional to check the overlap between S genes and QTLs for mildew resistance, we focused also on resistance (*R*) genes in this study. NBS and leucine-rich repeat (LRR) domains were important features for *R* gene family [36–39]. In our study, three genes were predicted for three major loci, *pmG2.1*, *pmG5.2* and *pmG5.3*, respectively. In *pmG2.1*, a gene encoding disease resistance-like protein (*Csa2G022790*) was identified. The expression of *Csa2G022790* was significantly up-regulated in the HR lines and the SNP located on the promoter of the gene is shared by 28 of 29 HS lines. In *pmG5.2*, the expression of *Csa5G484620* was significantly up-regulated in the HR lines. Almost all HR lines have the same SNP in this gene. These indicated that the gene may be a resistance gene. For *pmG5.2*, the candidate gene *Csa5G604340* is a homolog of the *AtMLO6* gene, thus a S-gene [13]. The identified SNP in this gene was shared by all the HS of European type.

PM resistance is a complex trait and it often involves multiple genes and multiple metabolic pathways [40, 41]. Few genes, which are significantly effect on the traits, will be mapped by one populations. Because different disease resistant materials have different disease resistant genes, it is impossible to clone all the PM genes from a group or a specific material. However, GWAS can effectively mine genes related to PM in core germplasm due to most of the genetic variation are existed in the core germplasm of cucumber. It can detect disease resistance genes in all genetic resources at the same time and excavate disease resistance genes in the whole genome. GWAS provides a very effective mean for comprehensive research on the mechanism of powdery mildew resistance.

Conclusions

Twelve Loci related to powdery mildew resistance in cucumber were detected by genome-wide association analysis, three of which namely pmG2.1, pmG4.1 and pmG6.1 are novel. Three candidate genes within the genomic region of locus pmG2.1, pmG5.2 and pmG5.3 were identified, respectively.

Materials And Methods

Plant materials

A cucumber CG population consisting of 109 lines was provided by the cucumber research group in the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, China. This CG population was selected from more than 3,000 germplasms worldwide and resequenced [27]. Sequencing data can be found on the Cucumber Genome website (<http://www.icugi.org/cgi-bin/ICuGI/index.cgi>).

Disease tests and disease index calculation

The phenotyping of PM resistance was carried out in the greenhouse of Nankou farm (40°13'N, 116°09'E) and Shunyi farm (40°15'N, 116°83'E) in Beijing from 2013 to 2014. Disease tests with PM were performed three times, in Spring 2013 (pm_2013S), Spring 2014 (pm_2014S) and Fall 2014 (pm_2014A). In each test, three replicates were applied and six plants per line were used for each replicate.

Naturally infection of PM occurred at adult plant stage. PM appeared about four weeks after sowing. From five weeks after sowing, PM symptoms was scored once a week for three times by ranking the disease of each plant with 0, 1, 3, 5, 7 and 9, where 0: no symptom; 1: $\leq 1/3$ th of all leaves with powdery mildew spots; 3: $1/3 - 2/3$ of all leaves with powdery mildew spots; 5: $\geq 2/3$ of all leaves with powdery mildew spots; 7: PM spots covers the whole leaf; 9: $\geq 2/3$ of the browning leaves [4]. For each line, a disease index (DI) was calculated. $DI = 100 \times \sum (\text{Number of plants with disease rating} \times \text{Disease rating}) / (\text{Total number of plants} \times \text{Highest disease rating})$ [29].

Genetic diversity of PM resistance in the CG population

Using the DI of each line per experiment, a mean DI per line was calculated over the three experiments and used as input to study the genetic diversity of the CG population. A phylogenetic tree was constructed with SAS 9.0 [42].

GWAS

FastLMM was used for association tests of resistance with an estimated relatedness matrix as covariate. GWAS was conducted and the genome-wide lowest P value was recorded. The 5% lowest tail was taken from the 200 recorded minimal P values as the threshold for genome-wide significance. The Manhattan map for GWAS was generated using the R package CMplot (<https://github.com/YinLiLin/R-CMplot>). The

SNP data used for the association analysis were downloaded from the cucumber genome website: <http://www.icugi.org/cgi-bin/ICuGI/index.cgi>[27].

Linkage-disequilibrium (LD) analysis

The software Plink [43] was used to calculate the LD coefficient (R^2) between pairwise high-quality SNPs; the parameters were set as: '-r2 -ld-window 999999 -ld-window-kb 1000 -ld-window-r2 0', and the results were used to estimate LD decay.

Analysis of novel loci for the PM resistance

The mapped QTLs of this study were compared with published studies to identify the novel ones, for which candidate region was analyzed within 50 kb around peak SNPs. The three strongest candidate region was analyzed by the position ($-\log_{10}$ (p-value) = 5 as the critical). The candidate gene was annotated. The physical distance was based on the cucurbit genomics (<http://cucurbitgenomics.org/>).

RNA extraction and qRT-PCR

Four highly sensitive (HS) lines (CG3, CG23, CG31 and CG55) and highly resistant (HR) lines (CG7, CG9, CG35 and CG44) were used for gene expression study. Plants were inoculated with PM inoculum (1×10^6 spores/ml) when the seedlings were at two-mature-leaf stage. Leaves (First true leaf per plant) were sampled at four time points (12 hours, 24 hours, 3 days and 8 days post inoculation) from three plants per line and per time point. Total RNA was extracted according to the instructions of the manufacturer TIANGEN kit (Invitrogen, Carlsbad, CA, USA). RNA purity was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA), 1% formaldehyde gel electrophoresis, and a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

For real-time RT-PCR (qRT-PCR) analysis, primers were designed using DNAMAN according to the gene CDS sequences (Supplementary Table S1). First-strand cDNA was synthesized using the PrimeScript™ RT reagent Kit (TAKARA BIO, Inc., Shiga, Japan). qRT-PCR was performed with the SYBR Premix Ex Taq™ Kit (Takara) with the following cycling parameters: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 55 °C for 30 s, with a final cycle of 95 °C for 15 s, 55 °C for 60 s and 95 °C for 15 s. Relative transcription levels were analyzed using the $2^{-\Delta\Delta CT}$ method [44]. qRT-PCR was performed in a BIO-RAD CFX96 system (Bio-Rad, USA), and the cucumber actin1 gene (ID) was employed as the internal control [45]. Three technical replicates were performed for each gene and relative transcription levels were analyzed using the $2^{-\Delta\Delta CT}$ method [44].

Supplemental Material

Supplementary Materials: Figure S1: Genotypic effects of the *dmG1.2*, *dmG2.1*, *dmG5.2* and *dmG7.1* in the dm_2013S and dm_2014A; Figure S2: The phylogenetic tree of phenotype of CG; Table S1: Phenotype of core germplasm. Table S2: genes were predicted in the *pmG4.1* region. Table S3: genes were predicted in the *pmG6.1* region. Table S4: qRT-PCR primers sequences in this study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the National Natural Science Foundation of China (31672172); the earmarked fund for Modern Agro-industry Technology Research System (CARS-23), The Agricultural Science and Technology Innovation Program of the Chinese Academy of Agricultural Sciences (CAAS-ASTIP-IVFCAAS) and the Key Laboratory of Biology and Genetic Improvement of Horticultural Crops, Ministry of Agriculture and Rural Affairs, P. R. China.

Authors' contributions

XL wrote and revised the manuscript. HL, PL and HM investigated disease index data. YB, XG and SZ conceived of the study and critically reviewed the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors thank the Key Laboratory of Biology and Genetic Improvement of Horticultural Crops, Ministry of Agriculture, China.

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Figures

Figure 1

Locations of QTLs/genes for PMR in cucumber reported in previous studies and GWAS loci in this study.

Figure 2

Box plots and heatmap depicting the phenotypic distribution of DI of PM resistance in 3 environments.

Figure 3

GWAS Mahattan plots of PM resistance in three seasons. a: pm_2013s; b:pm_2014s; c:pm_2014a.

Figure 4

Identification of the causal gene for the peak pmG2.1. a: Local Manhattan plot (top) and LD heatmap (bottom) surrounding the peak pmG2.1; b: Dashed lines indicate the candidate region (~ 45 kb) for the peak. Eleven genes were predicted in the pmG2.1 candidate region. c: SNP variation of candidate gene in the core germplasm; d-f: Expression pattern analysis of the three predicted genes among HR and HS lines. **Significant difference ($P < 0.01$); g: Expression of Csa2G022790 among the four HR and HS lines, respectively.

Figure 5

Identification of the causal gene for the peak pmG5.2. a: Local Manhattan plot (top) and LD heatmap (bottom) surrounding the peak pmG5.2; b: Local LD heatmap on the 16,857kb to 16880kb. c: SNP variation of candidate gene in the core germplasm; d: Expression pattern analysis of the four predicted genes among HR and HS lines; e: Expression of *Csa2G022790* among the four HR and HS lines, respectively.

Figure 6

Identification of the causal gene for the peak pmG5.3. a: Local Manhattan plot (top) and LD heatmap (bottom) surrounding the peak pmG5.3; b: Dashed lines indicate the candidate region (~ 18 kb) for the peak. Two genes were predicted in the pmG2.1 candidate region. SNP variation of candidate gene in the core germplasm; c: Expression of Csa2G022790 among the four HR and HS lines, respectively. **Significant difference ($P < 0.01$); d: Genotype variation in the European type.

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