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

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Abstract

Background

Downy mildew (DM) is one of the most serious diseases in cucumber and brings the loss of yield and profit. Multiple QTLs for DM resistance have been detected, however, no loci related to resistance was reported using genome-wide association analysis (GWAS). In this study, the core germplasm (CG) of cucumber lines that had been constructed and resequenced were used to identify DM resistance Loci using GWAS technology.

Results

Thirteen loci (dmG1.1, dmG1.2, dmG2.1, dmG2.2, dmG3.1, dmG4.1, dmG4.2, dmG5.1, dmG5.2, dmG6.1, dmG6.2, dmG7.1 and dmG7.2) associated with DM resistance were detected on all the seven chromosomes. Among these loci, dmG2.1 and dmG7.1 were novel loci compared with previous studies. Based on the annotation of homologous genes in Arabidopsis and pairwise LD correlations, Csa1G575030 could be the most likely candidate gene of dmG1.2; Csa2G059820 and Csa2G060360 could be the candidate gene of dmG2.1. A WRKY transcription factor Csa5G606470 could be the candidate gene of dmG5.2. Csa7G004020 could be the candidate gene of dmG7.1.

Conclusions

These results identify five candidate genes for four loci related to DM resistance in cucumber which provide theoretical basis for gene cloning and genetic breeding of DM resistance in cucumber.

Introduction

Cucumber (Cucumis sativus L.) is an economically important vegetable crop grown all over the world. Downy mildew (DM) is one of the most serious diseases and causes significantly production lost, it has been found all over the world (Perez-Garcia et al. 2009; Thomas C E, 1986). The symptoms of DM mainly appeared on the upper of leaf surfaces. Although there are many studies on DM that have been reported, its mechanism was not clear and there were few genes been identified.

Downy mildew (DM) is caused by the obligate biotrophic oomycete Pseudoperonospora cubensis [(Berkeley & M.A. Curtis) Rostoyzev]. DM was occurred in many species, such as pearl millet [1], Arabidopsis thaliana [2–4], Triticum aestivum [5, 6], Helianthus annuus L.[7, 8] and Cucumis melo[9]. Downy mildew was also devastating in cucumber. Many researches on downy mildew in cucumber have been reported [10–12].

Zhang et al. (2013) constructed the F₂ and F₂:3 population based on the cross of inbred line K8 and K18 and identified 5 QTLs (dm1.1, dm5.1, dm5.2, dm5.3 and dm6.1) associated with DM on the Chr1, Chr5 and Chr6, respectively. And the QTLs in Chr5 could also be detected by other studies (Bai et al. 2008). By using F₂ population constructed by the cross between resistance line IL52 and susceptible line ‘Changcunmici’, Peng
et al. (2013) detected two QTLs ($DM_{5.1}$ and $DM_{5.2}$). Yoshioka et al. (2014) found two QTLs on Chr5 by using RILs population derived from CS-PMR1 and the old Japanese cultivar Santou. Four QTLs ($dm2.1$, $dm4.1$, $dm5.1$, and $dm6.1$) were detected by the cross between DM-resistant inbred line WI7120 and susceptible '9930', and $dm4.1$ and $dm5.1$ were major effect QTLs [13]. Five QTLs ($dm2.2$, $dm4.1$, $dm5.1$, $dm5.2$, and $dm6.1$) were found using NGS-assisted BSA, and $dm2.2$ and $dm5.2$ were major effect loci for DM resistance [14]. Wang et al. (2018) reported that $dm5.1$, $dm5.2$, and $dm5.3$ were major-effect QTLs contributing to DM resistance. Wang et al. (2019) identified a DM resistance gene STAYGREEN, which also located on Chr5 [12]. Therefore, we proposed that the sites located on Chr5 was the most likely loci on DM resistance. Thirteen MLO-like genes and three homolog DMR genes were identified in cucumber [15].

Genome-wide association analysis (GWAS) is an effective and feasible method of analyzing 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, multiple genes were identified by GWAS. The bitterness gene $Bi$ was detected by the genome-wide association of 115 diverse cucumber lines [24]. Wang et al. (2018) found a candidate gene related to callus regeneration by GWAS combined with QTL mapping [25]. Bo et al. (2019) identified a novel locus $fsd6.1$ combined with $fsd6.2$ ($Csgl3$) which regulates fruit spine density by GWAS [26]. GWAS has been widely used for gene mapping of multiple species and complex traits.

In this study, 97 core germplasms (CG) lines [27] were used to perform genome-wide association analysis on downy mildew resistance and multiple loci were detected. These loci provided strong support for the comprehensive utilization of cucumber resistance to downy mildew genetic resources.

**Materials And Methods**

**Plant materials**

97 CG lines of cucumber were 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 was on the Cucumber Genome website (http://www.icugi.org/cgi-bin/ICuGI/index.cgi).

**Investigation of disease index of the core germplasm resistance**

All materials were grown in the greenhouse of Nankou farm (40°13′N, 116°09′E) and Shunyi farm (40°15′N, 116°83′E) in Beijing in 2014. Disease tests for DM resistance were performed twice, in Spring 2014 (dm_2014S) and Autumn 2014 (dm_2014A), respectively. In each test three replicates were applied and 6 plants were used per line. Naturally DM occurred at adult stage. Downy mildew symptom appeared at about five weeks after sowing. The disease was identified three times at about six weeks after sowing, and once a week.
Disease rating scale for each seedling was as follows: 0: no symptom; 1: ≤1/10 of all leaves with downy mildew spots; 3: 1/10 ~ 1/4 of all leaves with downy mildew spots; 5: 1/4 ~ 1/2 of all leaves with downy mildew spots; 7: 1/2 ~ 3/4 of all leaves with downy mildew spots, or the whole leaf dead [28]. DI = 100 × Σ (Number of plants with disease rating × Disease rating)/(Total number of plants × Highest disease rating) [28].

All statistics for significant differences among different materials were performed using two-way analysis of variance or two-tailed, two-sample Student’s t-test.

**Genetic diversity of DM resistance in germplasm**

The phylogenetic tree was constructed based on the genotype and phenotype of germplasm using SAS 9.0 [29]. According to the mean of DI, three groups were clustered using the complete. The distance of the complete was 1.5.

**Genomewide association analyses of DM resistance**

FastLMM was used for genome wide association analyses, with an estimated relatedness matrix as covariate, and the genome-wide minimal $P$ value was recorded. The 5% lowest minimal $P$ values were as the threshold for genome-wide significance. The Manhattan map for GWAS was generated using R package CMplot [30]. SNP data used for the association analysis were from the cucumber genome website (http://www.icugi.org/cgi-bin/ICuGI/index.cgi) [27].

**Linkage-disequilibrium analysis**

The software Plink [31] 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 and stronger signal loci for the DM resistance**

The novel loci were compared with previous studies. The 50 kb around peak SNPs were considered as candidate region. The candidate regions were predicted using the LDblock and the candidate genes were identified by the annotation of homologous gene in *Arabidopsis* and the SNP variation. The candidate gene was analyzed by the gene annotation. The physical distance was based on the cucurbit genomics (http://cucurbitgenomics.org/). The effect of the QTLs was performed using the R/qtl package with the multiple-QTL model (MQM) [32, 33].

**Results**

**Genetic diversity of DM resistance in CG**

The DM resistance of the CG materials were phenotyped in the spring of 2014 (dm_2014S) and the autumn of 2014 (dm_2014A), respectively. The mean DI of dm_2014A was higher than that of dm_2014S, which indicated that DM was more severe in the autumn of 2014. The coefficient of variation (CV) was 27.4% and
59.3%, respectively. The DI in the two years were similarly (Fig. 1b), so the phenotype of DM resistance was effective and accurate for the GWAS analysis. The frequency distribution of DI from different environments were illustrated in Fig. 1a. Distribution of DI in two experiments showed a bimodal model, which suggested that DM resistance in cucumber was controlled by multiple QTLs.

According to the mean of DI in CG materials, there were four categories clustered based on the complete method (1.5) of SAS (Supplementary Fig. 1). Highly resistance (HR) (DI < 10) contained 6 lines (“CG64”, “CG70”, “CG72”, “CG75”, “CG81” and “CG86”). Resistance (R) (10 < DI < 27) contained 34 lines. Sensitive (S) (27 < DI < 39) contained 34 lines. And highly sensitive (HS) (DI > 39) contained 23 lines (Supplementary Table S1). South China Banna type was distributed in the resistance and highly resistance category. Japanese type was mainly sensitive to DM. Due to HR lines distributed in the India and Xishuangbanna, we proposed that the resistance gene to DM might be derived from India or Xishuangbanna (Fig. 2c).

**Genome-associated analysis of DM resistance**

Thirteen loci (dmG1.1, dmG1.2, dmG2.1, dmG2.2, dmG3.1, dmG4.1, dmG4.2, dmG5.1, dmG5.2, dmG6.1, dmG6.2, dmG7.1 and dmG7.2) for DM resistance were detected, distributed across 7 chromosomes (Fig. 2). The detailed SNPs were listed in Table 1. Four loci (dmG2.1, dmG6.1, dmG6.2 and dmG7.2) were detected in dm_2014A. Five loci (dmG1.1, dmG2.2, dmG4.1, dmG4.2 and dmG5.1) were detected in dm_2014S. Three loci (dmG1.2, dmG5.2 and dmG7.1) were repeatedly detected in two years, and could be considered as stable and major loci related to DM. dmG2.1 and dmG7.1 were novel loci based on previous study (Discussion). These novel and major loci (dmG2.1, dmG1.2, dmG5.2 and dmG7.1) were used for further candidate genes analysis.
| Locus   | SNP name  | Chromosome | SNP Position   | Flanking markers          | Physical location (Mb) | Reference          |
|---------|-----------|------------|----------------|----------------------------|------------------------|--------------------|
| dmG-1.1 | SNP187059 | 1          | 10,520,443     | UW084539                   | 8.72                   | Zhang et al. 2018  |
|         |           |            |                | SSR16055                   | 13.30                  |                    |
| dmG-1.2 | SNP398090 | 1          | 21,761,223     | Marker1_15701257           | 15.70                  | Wang et al. 2018   |
|         |           |            |                | Marker1_26471365           | 26.47                  |                    |
| dmG-2.1 | SNP639496 | 2          | 4,618,724      | Novel                      |                        |                    |
| dmG-2.2 | SNP730945 | 2          | 8,449,502      | SSR13532                   | 5.64                   | Win et al. 2017    |
|         |           |            |                | SSR13105                   | 10.28                  |                    |
| dmG-3.1 | SNP1139014| 3          | 8,524,603      | SSR16264                   | 9.15                   | Win et al. 2017    |
|         |           |            |                | SSR13312                   | 10.25                  |                    |
| dmG-4.1 | SNP1793602| 4          | 5,302,408      | SSR00012                   | 4.11                   | Cavagnaro et al. 2011 |
|         |           |            |                | SSR13159                   | 20.10                  |                    |
| dmG-4.2 | SNP193892 | 4          | 13,242,382     | SSR00012                   | 4.11                   | Cavagnaro et al. 2011 |
|         |           |            |                | SSR13159                   | 20.10                  |                    |
| dmG-5.1 | SNP2452535| 5          | 18,119,570     | Marker5_16446482           | 16.44                  | Wang et al. 2018   |
|         |           |            |                | Marker5_18140438           | 18.14                  |                    |
| dmG-5.2 | SNP2531703| 5          | 22,899,752     | SSR01498                   | 22.66                  | Zhang et al. 2018  |
|         |           |            |                | InDel82                    | 25.75                  |                    |
| dmG-6.1 | SNP13977656| 6         | 13,977,656     |                           | 5.54                   | Win et al. 2016    |
|         |           |            |                |                            | 17.28                  |                    |
| dmG-6.2 | SNP3104337| 6          | 25,297,012     | SSR01148                   | 25.95                  | Wang et al. 2016   |
|         |           |            |                | SSR05946                   | 28.09                  |                    |
| dmG-7.1 | SNP3165616| 7          | 84,900         | Novel                      |                        |                    |
| dmG-7.2 | SNP3517753| 7          | 18,251,423     | SSR33278                   | 17.52                  | Yoshioka et al. 2014 |
|         |           |            |                | SSR477                     | 19.19                  |                    |
Candidate gene analysis of novel locus dmG2.1

In order to identify potential candidate genes in novel and stable major loci, the 50 kb region around the peak SNPs were used for further analysis (Fig. 3a). Based on the cucurbit genome (http://cucurbitgenomics.org/), six genes were presented in the dmG2.1 region. In this region (Fig. 3b), Csa2G059820 encodes Pentatricopeptide repeat-containing protein; Csa2G059830 encodes homogentisate phytyltransferase; Csa2G059840 encodes Lysine–tRNA ligase; Csa2G060350 is a transcription factor/ transcription regulator; Csa2G060360 is an unknown protein; Csa2G060370 encodes Fe/S biogenesis protein. Expression of six genes were analyzed based on the RPKM in the RNA-transcriptome of PRJNA285071 (http://cucurbitgenomics.org/rnaseq/home). The expression of Csa2G059820, Csa2G060360 and Csa2G060370 were up-regulated in the resistant line PI 197088 (Fig. 3c). The homolog gene of Csa2G059820 (AT2G18940) and Csa2G060360 (AT5G57345) in Arabidopsis were differentially expressed in infected rosette leaves at 12 days post inoculation of Cabbage leaf curl virus [34]. These two genes could be the candidate gene of dmG2.1.

Candidate gene analysis of novel locus dmG7.1

Fifty kb around the peak SNPs were further used to analyze the candidate gene associated with the locus dmG7.1 (Fig. 3d). Based on the cucurbit genome (http://cucurbitgenomics.org/), 4 genes were predicted in the dmG7.1 region. All the four genes were the isoflavone reductase related protein (Fig. 3e). Phylogenetic tree was constructed based on the amino acids in the reference genome. Phylogenetic tree showed that Csa7G002050 was homologous with Csa7G004020 (Fig. 3f). The results showed that dmG7.1 could be related to the isoflavone reductase. The expression of Csa7G004020 was up-regulated at the 3-day and 6-day after DM infection in the resistant line PI 197088 based on the RPKM in the RNA-transcriptome of PRJNA285071 in http://cucurbitgenomics.org/rnaseq/home (Fig. 3g). Therefore, we proposed that Csa7G004020 could be the candidate gene of dmG7.1.

Candidate gene analysis for stronger loci dmG1.2

For the dmG1.2, the candidate region (Chr.1: 21,711 – 21,781 kb) were analyzed by pairwise LD correlations (Fig. 4a). We focused on the locus mapped from 21,756,442 bp to 21,774,330 bp by LD block. Based on the Cucumber Genome Browser (http://www.icugi.org/cgi-bin/ICuGI/index.cgi), two candidate genes were located in this region. The two genes Csa1G575020 and Csa1G575030 both encode kinase family protein. No SNPs located in the CDS of Csa1G575020. Seven SNPs variation which result in amino acid variation within the CDS region of Csa1G575030 (Fig. 4c). We proposed that Csa1G575030 was the candidate gene of dmG1.2.

Candidate gene analysis for stronger loci dmG5.2
For the *dmG5.2*, 50 kb around the peak SNPs region were further analyzed (Fig. 5a). The candidate region from 22,883 kb to 22,913 kb (~30 kb) was estimated using pairwise LD correlations ($r^2 \geq 0.6$) (Fig. 5b). According to the Cucumber Genome Browser (http://cucurbitgenomics.org/organism/2), five annotated genes were identified in this candidate region (Fig. 5b). *Csa5G606470* is a WRKY transcription factor 2–4; *Csa5G606480* is a eukaryotic translation initiation factor 5A; *Csa5G606490* is a cell division topological specificity factor; *Csa5G606500* encodes a putative uncharacterized protein RAF9-1; *Csa5G606510* is an unknown protein. Based on the homologous gene in *Arabidopsis*, the gene *AT1G13960* (Homologous of *Csa5G606470*) in response to salicylic acid in leaf proteome profile of *Arabidopsis thaliana* [35].

SNP_2531403 was on the CDS of *Csa5G606470*. On this site, 29 of 30 HR lines was G (Pro), the other one was A (Ser), whereas that 7 of 29 HS lines was G (Pro), the others were A (Pro) (Fig. 5c). *Csa5G606470* could be the candidate gene of *dmG5.2*.

**Discussion**

Inheritance of DM resistance in cucumber was complex. Angelov showed that the resistance of DM in cucumber was controlled by a recessive gene [36] and this gene was linked with dull green fruit skin color related gene D [37]. Most studies have reported that DM was controlled by multiple genes in cucumber, such as a dominant susceptible gene and a recessive gene [38], a pair of dominant and recessive interacting genes [39], three recessive genes [9]. In our study, the results showed that DM resistance was controlled by multiple genes. The different results could be due to different materials and different environments. The core germplasm we used possesses the DM resistance with most variation.

In recent years, GWAS is widely used to detect genes in many species. Our study firstly used the GWAS to detect loci and genes for DM resistance in cucumber. Several loci distributed on all 7 chromosomes have been identified by different populations. Based on previous studies, two loci were mapped on chr1, chr2, chr3, and chr6, respectively, one loci on chr4 and chr7, three loci (*dm5.1, dm5.2* and *dm5.3*) on chr5 [10, 13, 14, 28, 40–45]. In our study, a total of thirteen loci (*dmG1.1, dmG1.2, dmG2.1, dmG2.2, dmG3.1, dmG4.1, dmG4.2, dmG5.1, dmG5.2, dmG6.1, dmG6.2, dmG7.1 and dmG7.2*) distributed on 7 chromosomes were detected by CG lines. Compared with the previous QTLs, the loci on the Chr1, Chr3, Chr4, Chr5 and Chr6 had been previously reported. Two loci on the Chr2, Chr7 was new reported, respectively. (Table 1). Based on the loci listed in Table 1, eleven of thirteen loci were also identified in QTL mapping. The candidate gene, *Csa5G606470*, mapped in *dmG5.2*, had a SNP in the CDS. And the SNP variation result in amino acid changes. The candidate gene was the same as *dm5.2* based on BSA-seq analysis in Zhang et al’s study [41]. The variation was effect on the 3’ UTR, which could be due to different materials. Three loci could be identified in two years, which showed that the results in our study were consistent. Ten of thirteen loci could be identified in one year, which could be due to different environments. In our study, thirteen loci were consistent with previous studies, which showed that GWAS could detect more variations [46]. So, GWAS was efficient to detect DM resistance related QTLs.

DM resistance is a complex trait that involves in multiple genes and metabolic pathways. Some of the major loci were interacted with each other. Three major QTLs in Chr5 were negative additive effects [42]. Wang et al. (2016) detected the effect of four QTLs (*dm2.1, dm4.1, dm5.1* and *dm6.1*). *dm6.1* could be interacted
with \textit{dm2.1}, \textit{dm4.1} and \textit{dm5.1}. And \textit{dm2.1} could be affected by \textit{dm5.1}. In our study, the peak SNPs in \textit{dmG1.2}, \textit{dmG2.1}, \textit{dmG5.2} and \textit{dmG7.1} were applied to detect the effect code of each other. \textit{dmG1.2} interacted with \textit{dmG7.1} in the two experiments. \textit{dmG5.2} had effect on \textit{dmG1.2} and \textit{dmG7.1} in one season. \textit{dmG1.2} was independent of \textit{dmG2.1} and \textit{dmG5.2}, and \textit{dmG2.1} was independent with \textit{dmG7.2}.

(\textbf{Supplementary Fig. 2}) These results showed that the DM resistance is a complex trait and with multiple gene interactions, which was consistent with previous studies [42, 47]. Therefore, GWAS is an effective strategy to identify complex traits.

\section*{Conclusions}

Thirteen loci associated with DM resistance were detected in cucumber. Two novel loci were identified compared with previous studies. Based on the annotation of homologous genes in \textit{Arabidopsis} and pairwise LD correlations, five candidate genes for four loci related to DM resistance were identified in cucumber. This study provides theoretical basis for gene cloning and genetic breeding of DM resistance in cucumber.

\section*{Supplementary Materials}

Figure S1: The phylogenetic tree of phenotype of CG; Figure S2: Genotypic effects of the \textit{dmG1.2}, \textit{dmG2.1}, \textit{dmG5.2} and \textit{dmG7.1} in the dm_2014S and dm_2014A; Table S1: Phenotype of core germplasm.

\section*{Declarations}

\textbf{Ethics approval and consent to participate}

Not applicable.

\textbf{Consent for publication}

Not applicable.

\textbf{Availability of data and materials}

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

\textbf{Competing interests}

The authors declare that they have no competing interests.

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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.

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Figures
Figure 1

Box plots and heatmap depicting the phenotypic distribution of DI of DM resistance in two environments.
Figure 2

GWAS Mahattan plots of DM resistance in two experiments, and the distribution of 109 cucumber CG lines in different continents based on DI of DM resistance. a: dm_2014A; b: dm_2014S; c: distribution of 109 cucumber CG lines. The red spot represents HR; Blue HS; Yellow represents R; Green represents S.
Figure 3

Identification of the causal gene for the peak dmG2.1 and dmG7.1. a: Local Manhattan plot surrounding the peak dmG2.1; b: Six genes were predicted in 50 kb around the peak dmG2.1; c: RPKM of these six genes based on the transcriptome of PRJNA285071; d: Local Manhattan plot surrounding the peak dmG7.1; e: Four genes were predicted in 50 kb around the peak dmG2.1; f: Phylogenetic analysis of four genes in dmG7.1 candidate region; g: RPKM of four genes in dmG7.1 candidate region based on the transcriptome of PRJNA285071.
Identification of the causal gene for the peak dmG1.2. a: Local Manhattan plot (top) and LD heatmap (bottom) surrounding the peak dmG5.2; b: SNP variation of candidate gene in the core germplasm.
Figure 5

Identification of the causal gene for the peak dmG5.2. a: Local Manhattan plot (top) and LD heatmap (bottom) surrounding the peak dmG5.2; b: Dashed lines indicate the candidate region (~ 30 kb) for the peak. Five genes were predicted in the dmG2.1 candidate region. c: SNPs variation of candidate gene in the core germplasm.

Supplementary Files

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