Identification and Molecular Mapping of a Gummy Stem Blight Resistance Gene in Wild Watermelon (*Citrullus amarus*) Germplasm PI 189225

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Abstract

Gummy stem blight (GSB), caused by *Stagonosporopsis cucurbitacearum* (syn. *Didymella bryoniae*), is a destructive foliar disease of watermelon in areas with hot and humid climates. The wild watermelon germplasm PI 189225 is a known source of resistance to GSB. The identification and use of molecular markers linked to resistance genes in the wild-type germplasm will speed up the introgression of GSB resistance into new watermelon varieties. An F₂ segregating population was obtained from a cross between the resistant wild watermelon genotype PI 189225 and the susceptible genotype K3. The F₂-derived F₃ families were inoculated with a single isolate of *S. cucurbitacearum* (JS002) from Jiangsu Academy of Agricultural Sciences. The results of the genetic analysis demonstrated that GSB resistance in PI 189225 was controlled by a major quantitative trait locus (QTL), temporarily designated *Qgsb8.1*. Based on the results of bulk segregant analysis and sequencing, one associated region spanning 5.7 Mb (10,358,659 to 16,101,517) on chromosome 8 was identified as responsible for the resistance to GSB using the Δ (single-nucleotide polymorphism [SNP]-index) method. The

result of a QTL linkage analysis with Kompetitive allele-specific PCR (KASP) SNP markers further mapped the GSB resistance locus between the SNP markers KASP_JS9383 and KASP_JS9168 in a region of 571.27 kb on chromosome 8. According to the watermelon gene annotation database, the region contains approximately 19 annotated genes and, of these 19 genes, 2 are disease resistance gene analogs: *Clao01017* (coiled-coil nucleotide-binding site leucine-rich repeat resistance protein) and *Clao01019* (pathogenesis related). Reverse-transcription quantitative PCR demonstrated that the expression of the two genes changed following *S. cucurbitacearum* infection, suggesting that they play important roles in GSB resistance in watermelon. This result will facilitate fine mapping and cloning of the *Qgsb8.1* locus, and the linked markers will further provide a useful tool for marker-assisted selection of this locus in watermelon breeding programs.

Keywords: gummy stem blight, molecular marker, resistance gene, wild watermelon germplasm

Watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) is a globally important vegetable fruit crop. The total yield in 2017 was over 100 million tons, accounting for 10.8% of total vegetable production (FAO Statistics 2017; <http://www.fao.org/faostat/zh/#data/QC>).

Gummy stem blight (GSB), caused by *Stagonosporopsis cucurbitacearum* (Fr.) Aveskamp, Gruyter & Verkley (syn. *Didymella bryoniae*), is a major fungal disease of watermelon and other cucurbits in areas with hot and humid climates (Keinath et al. 1995; Stewart et al. 2015; van Steekelenburg 1981, 1983, 1984, 1985a,b). The disease can cause crown blight, extensive defoliation, fruit rot, and loss of fruit during storage and transportation (Norton 1979; Sowell and Pointer 1962). Yield losses attributed to GSB in watermelon production can reach as high as 100% in watermelon-growing regions with high disease pressure (St. Amand and Wehner 1995a; Power et al. 1992; Sitterly and Keinath 1996). In recent years, many types of fungicides have been applied to control GSB disease; however, only partial control of the disease can be achieved with fungicides. The use of fungicides also causes serious environmental problems and may

induce fungicide resistance. Utilization of host resistance is considered to be the most important and preferred method to control the disease.

Owing to the narrow genetic diversity in cultivated watermelon (Levi et al. 2001a,b; Yang et al. 2016), wild watermelon germplasms with high levels of resistance to diseases and other stresses provide important sources for watermelon improvement. The wild watermelon germplasm PI 189225 has been reported to be resistant to GSB (Gusmini et al. 2005, 2017), powdery mildew (Tetteh et al. 2010, 2013), and anthracnose (Boyhan et al. 1994b). To effectively utilize the comparably high level of resistance against GSB in PI 189225, it is necessary to determine the mode of inheritance and identify the molecular markers linked to GSB resistance.

With the development of next-generation sequencing (NGS) technologies and the availability of reference genome sequences of watermelon, bulked segregant analysis (BSA) in combination with whole-genome resequencing is regarded as an efficient and cost-effective approach to rapidly locate genomic regions containing genes or quantitative trait loci (QTL) affecting traits of interest (Liu et al. 2012; Mokry et al. 2011; Takagi et al. 2013). This approach has been successfully applied in several plant species, including *Oryza sativa* (Abe et al. 2012; Takagi et al. 2013), *Zea mays* (Haase et al. 2015; Liu et al. 2012; Silva et al. 2012; Xu et al. 2014), *Hordeum vulgare* (Mascher et al. 2014), *Cucumis sativus* (Lu et al. 2014), *Brassica rapa* (Wang et al. 2016), *B. juncea* (Zhao et al. 2017), *B. napus* (Yao et al. 2017), *Allium cepa* (Kim et al. 2015), and *C. melo* (Zhang et al. 2016).

In the present study, we employed BSA in combination with NGS (BSA-seq) and the Kompetitive allele-specific PCR (KASP) single-nucleotide polymorphism (SNP) genotyping method for identification and mapping of the GSB resistance loci in the wild watermelon germplasm PI 189225.

Materials and Methods

Plant materials and DNA extraction. The plant population consisted of 264 F₂ individuals and 211 F_{2,3} families derived from a

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cross with the cultivated watermelon breeding line K3 (*Citrullus lanatus*) as the female parent and the wild watermelon accession PI 189225 (*C. amarus*) as the male parent. The F₂ population was first seeded in trays with 50 compartments (5.0 cm in length by 5.0 cm in width by 4.5 cm in depth) filled with a standard potting medium (Pindstrup peat substrate; Pindstrup Horticulture Ltd., Ryomgård, Denmark) and grown in a growth room at optimal growth temperatures of 25 to 30°C for watermelon. Seedlings at the two-leaf stage were then transplanted to plastic greenhouse at the Luhe experimental base of the Jiangsu Academy of Agricultural Sciences (JAAS) (118°621'N, 32°488'E), Nanjing, China, and compost fertilizer (consisting of 17% N, 17% P₂O₅, and 17% K₂O; Jiangsu Xintiandi Amino Acid Fertilizers Ltd., Jiangsu, China) and organic fertilizer (consisting of poultry manure; Jiangsu Xintiandi Amino Acid Fertilizers Ltd.) were applied as base fertilizers before transplanting. The application rates were 50 and 1500 kg ha⁻¹ for compost fertilizer and organic fertilizer, respectively. The plants were grown in two-row plots of 50 m, with 0.5 m between adjacent rows. The distances between plots and adjacent plants were 0.5 and 0.6 m, respectively.

The F₂ plants were self-pollinated to produce the F_{2,3} generation. Each plant was tagged individually, and the plant identification was used in the plastic greenhouse to associate the F₂ genotype with the corresponding phenotypes of the derived F_{2,3} families. At 65 days after transplanting, flowers were checked frequently in the afternoon to determine the status of the flowers and both the female and the male flower buds were tagged, showing the date at the latest 1 day before anthesis, and also bagged by paper caps to prevent contamination. Self-pollination was carried out by hand between 9 and 11 A.M. of the next day using fresh pollen after the flowers opened. After pollination, paper caps were not removed until the fruit setting was observed. Standard cultivation management was followed throughout the growing season. At 40 days after pollination, watermelon seed were manually harvested.

Fungal isolate and conidial production. One virulent monoclinal isolate of *S. cucurbitacearum* (isolate JS002; JAAS), collected from Ningbo (eastern China), was used as the pathogen material; this isolate was identified and chosen based on its capacity for abundant sporulation and its aggressiveness (Li et al. 2015). The conidial production was conducted following the procedure of Li et al. (2015). Briefly, small (5 mm in diameter) surface peels of cucumber fruit were first cut off with an approximate thickness of 1.5 mm. Agar discs (5 mm in diameter) cut from the margins of actively growing *S. cucurbitacearum* colonies on potato dextrose agar were then subcultured on the cucumber fruit and incubated at 25°C with a 12-h photoperiod and 90% relative humidity (RH) for approximately 6 days. Cucumber tissues with black fruiting bodies were macerated, and the suspension was filtered through two layers of sterile cheesecloth and diluted in distilled water. The spore concentration was determined by counting in a hemocytometer under an Olympus BX41 microscope (Olympus, Tokyo, Japan) and adjusted to 1 × 10⁶ conidia/ml for inoculation of plants.

GSB testing and statistical analysis. GSB testing was conducted in a greenhouse at JAAS during the spring and autumn seasons in 2016. Twenty-four plants from each of the parents, the F₁ generation, and the F_{2,3} families were tested in the greenhouse. When the seedlings had grown to the four-leaf stage, they were inoculated by spraying a suspension of *S. cucurbitacearum* spores (1 × 10⁶ conidia/ml), to which 0.05% (vol/vol) Tween-20 had been added. This was followed by incubation at 25°C in a plastic mist tunnel at 90 to 100% RH for 24 h in the dark to promote spore germination and infection, and the plants were then kept in a greenhouse under natural light. For each F_{2,3} family, each individual seedling was scored 10 days after inoculation based on a disease rating scale ranging from 0 to 9, where 0 = absence of GSB spots, 1 = ≤5% GSB spots, 3 = >5% but ≤25% GSB spots, 5 = >25% but ≤50% GSB spots, 7 = >50% but ≤75% GSB spots, and 9 = >75% GSB spots or whole-leaf death. The disease index (DI) was calculated from the disease rating scale using the following formula: $DI = 100 \times \sum [(\text{number of plants with disease rating} \times \text{disease rating}) / (\text{total number of plants} \times \text{highest disease rating possible})]$. The disease rating scale was estimated by the percentage of symptomatic disease spot over the true leaves of each plant. Each

of the two experiments was arranged in a completely randomized design with two replications, each consisting of 211 F_{2,3} families.

Analysis of variance (ANOVA) was performed for DI to determine whether there were statistical differences among the F_{2,3} families using the AOV function in QTL IciMapping (version 4.1) software with the default parameters. The data were also presented with means, ranges, and least significant difference. A Spearman correlation analyses was carried out between the DI from the two seasons using the statistical package of SPSS 19.0 (SPSS Inc., Chicago, IL, U.S.A.).

BSA and DNA extraction. Based on the GSB reactions of the F_{2,3} families, the corresponding F₂ individual plants were classified into three categories according to the DI: resistant (all plants resistant), intermediate (most plants susceptible and some resistant), and susceptible (all plants susceptible). The resistant and susceptible bulks were constructed by combining equal amounts of DNA from 12 homozygous resistant F₂ plants and 12 homozygous susceptible F₂ plants, respectively. Genomic DNA was extracted from freeze-dried leaf tissue collected from F₂ seedlings using a cetyltrimethylammonium bromide method, as modified by Ren et al. (2015). DNA quality and concentration were determined by agarose gel analysis and spectrophotometry (NanoDrop ND-1000; Thermo Scientific, Wilmington, DE, U.S.A.), and the concentration was adjusted to be in the range of 50 to 100 ng μl⁻¹.

Construction of sequencing libraries and Illumina sequencing. Approximately 2 μg of DNA from the two bulks and the two parental lines was used to construct paired-end sequencing libraries according to the manufacturer's instructions (Illumina TruSeq library construction). The constructed libraries were subjected to whole-genome resequencing with Illumina HiSeq 2500. Raw sequencing reads from the Illumina HiSeq 2500 system (Illumina) were subjected to quality control using FASTQC (Brown et al. 2017). Low-quality paired-end reads (Q ≤ 5) with more than 50% bases in any single-end reads or the reads containing adapter (reads containing more than five adapter-polluted bases) or poly-N sequences (the number of N bases accounting for >5% of total bases) were filtered. After removing adapters and low-quality reads, the clean reads were aligned against the reference genome of watermelon (97103 v1) by using Burrows-Wheeler Aligner software (Li and Durbin 2009). The Genome Analysis Toolkit was used to call SNPs and small indels across the parental lines and bulks with the standard filter method (McKenna et al. 2010).

SNP-index association analysis. SNP-index association analysis was used to determine genotype frequency differences between the two bulks (Abe et al. 2012) by calculating the Δ(SNP-index). In this study, M stands for the female parent (susceptible, K3), P stands for the male parent (resistant, PI 189225), aa denotes the genotype from the R pool, and ab denotes the S pool. Δ(SNP-index) values were calculated as follows: SNP-index(ab) = Mab/(Pab+Mab), SNP-index(aa) = Maa/(Paa+Maa), and Δ(SNP-index) = SNP-index(aa) – SNP-index(ab), where Mab indicates the depth of the ab population derived from M and Pab indicates the depth of the ab population derived from P. Maa indicates the depth of the aa population derived from M, and Paa indicates the depth of the aa population derived from P. A sliding window analysis was applied, averaging the ΔSNP-index values within a 1-Mb-sized window and 10-kb step increment using an in-house-developed Python script (available on request) based on the program developed by Takagi et al. (2013). The average was plotted for all chromosomes and regions in which the average Δ(SNP-index) of a locus was more close to the target characteristic than that of surrounding regions, and windows that showed an average P value < 0.01 were considered candidate genomic regions harboring a locus associated with resistance to GSB.

Development and analysis of KASP markers. To develop markers, biallelic SNPs were randomly selected in the mapped regions according to a high-resolution genetic map constructed previously (Ren et al. 2015) and the watermelon reference genome sequence (Guo et al. 2013). The selected SNPs were first tested for their ability to differentiate the polymorphism by genotyping the two parents and a few F₂ individuals with KASP genotyping assays at China Golden Marker Biotech Company (Beijing, China), and the primers that detected clear polymorphism were utilized to genotype the entire mapping population.

One common reverse primer and two allele-specific forward primers based on the flanking sequences around the variant position (SNP) were designed using Primer3 (Rozen and Skaletsky 2000). PCR was performed in 96-well PCR plates in a 5- μ l solution comprising 2.5 μ l (50 ng) of template DNA, 2.5 μ l of KASP 2 $\times$ master mix (LGC, Hoddesdon, U.K.), and 0.07 μ l of primer mix (12 μ l of each allele-specific forward primer [100 μ M], 30 μ l of reverse primer [100 μ M], and 46 μ l of TRIS [10 mM, pH 8.3]; LGC, Hoddesdon, U.K.). Amplification was performed in a water bath-based thermal cycler instrument (Hydrocycler; LGC, Middlesex, UK) programmed at 94°C for 15 min, 10 touchdown cycles of 94°C for 20 s and 61 to 55°C for 60 s (dropping 0.6°C per cycle), and 26 cycles of 94°C for 20 s and 55°C for 60 s. Genotyping data obtained based on the fluorescence detected from the KASP assay were viewed and analyzed through KlusterCaller software (LGC Genomics, Beverly, MA, U.S.A.). Complete details on the principles and procedures of the KASP assays are available at <https://biosearch-cdn.azureedge.net/assetsv6/KASP-genotyping-chemistry-User-guide.pdf>.

Fig. 1. Gummy stem blight (GSB) reactions on the leaves of resistant parent PI189225, F_1 and susceptible parent K3, and representative disease rating scales corresponding to 0 = absence of gummy stem blight (GSB) spots, 1 = $\leq$ 5% GSB spots, 3 = $>$ 5% but $\leq$ 25% GSB spots, 5 = $>$ 25% but $\leq$ 50 GSB spots, 7 = $>$ 50% but $\leq$ 75% GSB spots, and 9 = $>$ 75% GSB spots.

Fig. 2. Frequency distribution of disease index among the $F_{2:3}$ families derived from the cross K3 $\times$ PI 189225 in response to gummy stem blight inoculation in controlled greenhouse conditions in the 2016 spring and autumn seasons.

Linkage map construction and QTL analysis. Linkage map construction and QTL analysis were performed using QTL IciMapping V3.2 (Wang 2009) (http://www.isbreeding.net/default.aspx?aspxerrorpath=/download_software_ICIM.aspx). The linkage map was obtained using the MAP function in software QTL IciMapping 3.2 with a minimum logarithm of odds (LOD) value of 3.0. Recombination values used to map distances in centimorgans were calculated based on the Kosambi mapping function (Kosambi 1943).

The mean DI of GSB of each $F_{2:3}$ family calculated from 24 plants per family was used as the phenotypic data for the QTL mapping analysis. QTL analysis was conducted using the BIP functionality (QTL mapping in biparental populations) in QTL IciMapping. The inclusive composite interval mapping of additive (ICIM-ADD) QTL method with mapping parameters of a walking step of 1.0 centimorgan (cM), and a probability of the stepwise regression of 0.001 was used for significance of QTL identification. Significant LOD threshold value was calculated using 1,000 permutations, with a type I error of 0.05. The confidence interval of the QTL was determined by 1-LOD intervals surrounding the QTL peak. Missing phenotypes were deleted using the 'Deletion' command. The additive effect and phenotypic variance explained of the QTL were also estimated using QTL IciMapping V3.2.

Identification of potential candidate genes. The physical location of the region between the SNP marker KASP_JS9383 and the SNP marker KASP_JS9168 was identified in the watermelon genome sequences and, according to syntenic chromosome relationships among melon, cucumber, and bottle gourd, sequences of the region between the two markers were further used to search the corresponding chromosomes in the reference genome sequence of melon, cucumber, and bottle gourd at the Cucurbit Genomics Database (<http://cucurbitgenomics.org/>).

RNA isolation and reverse-transcription quantitative PCR analysis. Total RNA was extracted from the leaf tissues of the two parents following the protocol of Tiangen RNAprep Pure FFPE kit (Tiangen Biotech Co., Ltd., Beijing, China) and then converted to cDNA using the PrimeScript RT reagent kit (both from Tiangen Biotech Co., Ltd.). The quantity of the RNA was assessed spectroscopically and the quality was assessed by agarose gel electrophoresis. The qPCR reactions were performed using SYBR Premix ExTaq kit (TaKaRa, Biotechnology Co., Ltd., Dalian, China) in a total volume of 20 μ l containing 10.0 μ l 2X SYBR Premix Ex Taq II (TaKaRa, Biotechnology Co., Ltd.), 2 μ l cDNA, 1 μ l each primer (10 μ M) and 6.0 μ l ddH₂O. The PCR was performed using a Light-Cycler 480 II (Roche Diagnostics, Rotkreuz, Switzerland) and the thermocycling conditions were as follows: denaturation at 95°C for 30 s followed by 40 cycles of 95°C for 10 s and 60°C for 30 s, and one cycle of 95°C for 15 s, 60°C for 60 s and 95°C for 15 s for dissociation. Gene-specific primers were designed using NCBI primer BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and the primers were synthesized by TSINGKE Biological Technology Co., Ltd. (Nanjing, China). The primer sequences of the genes are listed in Supplementary Table S1. Three biological replications were used for each parent and each biological replication with three technical replicates. Relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001).

Results

Genetic analysis of GSB resistance in PI 189225. In the greenhouse test under controlled conditions, the PI 189225 seedlings were resistant and exhibited brown necrotic lesions at the sites of infection throughout the leaf at 5 days after inoculation. As the disease developed, the necrotic lesions became darker and were limited to the sites of infection, indicating a resistant response, and the plants were scored with DI. In contrast, the susceptible reaction of the susceptible parent K3 involved the development of large areas of necrosis or extensive chlorosis that became larger and coalesced as the disease progressed, and such plants were scored with DI ranging from 7 to 9. All of the F_1 plants displayed the susceptible reaction, which was similar to that of K3 (Fig. 1). In the $F_{2:3}$ population, the DI data showed a largely normal distribution and continuous variation, with one peak (Fig. 2). ANOVA revealed differences ($P < 0.01$) among the $F_{2:3}$

families for the DI obtained in both the 2016 spring season and 2016 autumn season, which ranged from 13.5 to 77.1 and 14.3 to 76.2, respectively (Table 1). The correlation analysis showed that the mean DI of GSB from the two seasons were significantly correlated ($r = 0.92$, $P < 0.01$), suggesting the stability of the two measurements of GSB resistance.

Resequencing data analysis and identification of candidate intervals. In total, 63.39 Gb of clean data of high quality ($Q30 \geq 95.86\%$) were obtained by filtering and trimming the raw data generated through parental genome resequencing and BSA. The GC content ranged from 35.07 to 36.68% (Table 2). The clean data from the four samples were aligned to the watermelon reference genome sequence, and the average sequence depths were 51.47-fold in

susceptible parent (SP), 35.49-fold in resistant parent (RP), 30.25-fold in susceptible bulk (SB), and 48.58-fold in resistance bulk (RB) (Table 2). The number of mapped reads was 136,043,523 (99.4%) in SP; 89,955,069 (98.9%) in RP; 78,109,328 (98.77%) in SB; and 128,471,984 (98.58%) in RB (Table 2), with an average of 432,579,904 (98.94%), indicating a high level of mapping to the reference genome.

In total, 3,500,079, 3,526,565, 199,641, and 3,562,294 SNPs were obtained for RP, RB, SP, and SB, respectively (Table 2). With the criteria for selecting the SNPs that are homozygous for each parent and show polymorphism between the parents, in total, 195,939 high-quality SNPs that are polymorphic between the resistant pool and the susceptible pool were used for subsequent SNP-index and $\Delta(\text{SNP-index})$ calculation. The identification of resistance-related candidate regions was accomplished by screening genotypic frequency differences between the resistant pool and the susceptible pool. One region spanning 5.7 Mb (10,358,659 to 16,101,517) on chromosome 8 with average $\Delta(\text{SNP-index})$ values above the confidence value (99% [red line]) (Fig. 3) were defined as primary regions associated with GSB resistance.

Selection and validation of a SNP subset in a KASP genotyping assay. To further narrow the associated regions, in total, 52 KASP markers were designed from the corresponding sequence of the region harboring SNPs surrounding the resistance loci and

Table 1. Means, ranges, least significant differences (LSD), and P values of disease index of the parents and $F_{2:3}$ population in the spring and autumn seasons of 2016

Environment	Parents and F_1			$F_{2:3}$ population			
	PI 189225	K3	F_1	Mean	Range	P value	LSD
Spring	14.5	67.4	66.1	44.5 ± 16.3	13.5–77.1	<0.01	6.46
Autumn	15.8	63.7	62.2	43.2 ± 16.2	14.3–76.1	<0.01	7.32

Table 2. Summary of sequencing data and the data aligned to the watermelon reference genome for the parental lines and the resistant and susceptible pools

Samples ^a	Clean reads ^b	Clean data ^c	GC content (%)	Q30 (%)	Avg depth ^d	Reads ^e	Mapped reads (%) ^f	SNPs ^g
RP	43,505,510	13,051,653,000	36.68	97.49	35.49	90,953,525	98.90	3,500,079
RB	63,114,485	18,934,345,500	35.49	95.86	48.58	130,321,985	98.58	3,526,565
SP	66,326,158	11,503,943,100	35.07	96.04	51.47	136,871,441	99.40	199,641
SB	38,346,477	11,503,943,100	35.07	90.49	30.25	79,082,342	98.77	3,562,294

^a SP = susceptible parent, RP = resistant parent, SB = susceptible bulk, and RB = resistance bulk.

^b Total number of pair-end reads in the clean data.

^c Total number of bases in the clean data.

^d Average read depth of each sample.

^e Number of reads aligned to the watermelon reference genome. Both ends are counted separately.

^f Percentage of reads aligned to the watermelon reference genome.

^g Single-nucleotide polymorphisms.

Fig. 3. Graph of $\Delta(\text{single-nucleotide polymorphism [SNP-index]})$ from the analysis of bulked segregant analysis in combination with next-generation sequencing marker-trait association to identify regions of the watermelon genome associated with resistance to gummy stem blight. The x-axis represents chromosomal position and the y-axis represents $\Delta(\text{SNP-index})$ values. Black lines are average $\Delta(\text{SNP-index})$ values determined by sliding window analysis. The dashed line indicates the association threshold value (0.84) at the significance level of $P < 0.01$, which was calculated by Loess regression.

tested for polymorphisms with the two parental lines and part of the population. Using KASP assays, genotyping results were obtained with 12 SNPs (Table 3), revealing the biallelic codominant polymorphism expected between the resistant and susceptible parents and in the F₂ population. The remaining 40 SNPs failed due to nonreadable signals or failed probe design. The QTL analysis showed that one major QTL, designated as *Qgsb8.1*, was detected in both seasons with LOD peak values of 13.6 and 16.4 in the 2016 spring and autumn seasons, respectively, and a 1-LOD support interval ranging from 10.3 to 12.2 cM on chromosome 8. It explained 32.42% (2016 spring season) and 31.54% (2016 autumn season) of the phenotypic variance with additive effects of -10.7 (2016 spring season) and -11.6 (2016 autumn season), respectively. The negative value of additive effects also confirmed the donor of PI 189225 for the GSB resistance allele. The QTL was located between SNP marker KASP_JS9383 and SNP marker KASP_JS9168, spanning an interval of approximately 1.9 cM, and the region was estimated to be of 571.27 kb (11,424,655 to 11,995,922 bp) on chromosome 8 of *C. lanatus* (Fig. 4).

Annotation and orthologous analysis. According to the watermelon gene annotation database (<http://cucurbitgenomics.org/organism/1>), the region contains approximately 19 annotated genes, and out of the 19 genes, two showed disease resistance gene analogs, *Cla001017* (coiled-coil nucleotide-binding site leucine-rich repeat

[CC-NBS-LRR] resistance protein) and *Cla001019* (pathogenesis-related) (Table 4). The functionality of the other genes in the region includes 1 encoding a RING finger protein, 1 encoding a receptor protein kinase-like protein, 1 encoding a pentatricopeptide repeat protein, 1 encoding Ras small GTPase proteins, 2 encoding a DNA mismatch repair protein, and 11 encoding proteins of unknown function (Table 4). The orthologous genes were also searched in melon, cucumber, and bottle gourd. The 19 annotated genes are orthologous to 8 annotated melon genes, 6 annotated cucumber genes, and 10 annotated bottle gourd genes (Table 2). The two disease-related genes *Cla001017* and *Cla001019* are orthologous to *Lsi08G002220* and *Lsi08G002200*, respectively, in bottle gourd.

Expression profiles of the candidate genes. We further conducted a reverse-transcription quantitative PCR (RT-qPCR) assay to determine the expression patterns of the two candidate genes in the susceptible parent K3 and resistant parent PI 189225 after inoculation with *S. cucurbitacearum*. The RT-qPCR results demonstrated that the expression of *Cla001017* in the K3 was higher than in the PI 189225 before inoculation, and the gene was highly upregulated following inoculation in the K3 (Fig. 5). In contrast, the expression of *Cla001017* in PI 189225 was first highly induced at 6 h postinoculation (hpi) and then decreased at 12 and 24 hpi. The relative expression of *Cla001019* in the resistant parent PI 189225 was higher than that in the susceptible parent K3 and we found that, after inoculation, *Cla001019* showed

Fig. 4. Genetic map and physical map showing the location of the *Qgsb8.1* locus on watermelon chromosome 8 and the candidate genes located in a 571.27-kb region flanked by two closely linked markers. **A**, Genetic map of watermelon chromosome 8; marker names are indicated on the left side of the map. Map distances (in centimorgans) are shown on the right side. Map position likelihood confidence intervals are shown as a black box with 1-LOD confidence intervals. **B**, Physical map of candidate genes in the region flanked by the two linked markers; the names of the candidate genes are indicated to the right of watermelon chromosome 8, and the physical locations are shown on the left side of chromosome 8. KASP = Kompetitive allele-specific PCR.

much higher relative expression levels at 6 hpi in the resistant parent PI 189225 than in the susceptible parent K3, and that the change trends in relative expression levels were similar to the gene *Clao01017* from 6 to 24 hpi in both of the parents (Fig. 5). The above results indicated that the two candidate genes should play important roles in GSB resistance in watermelon.

Discussion

GSB is one of the most serious foliar diseases of watermelon and can significantly affect yield and fruit quality (Crinò et al. 2007; Keinath 2000; Keinath et al. 2003; Vawdrey 1994). To date, relatively few studies have been performed on the genetics of GSB resistance in watermelon. Among these few studies related to the inheritance of resistance to GSB, watermelon-related studies were first carried out by Norton (1979), who reported that resistance to GSB in watermelon was controlled by one recessive gene (*db/db*). St. Amand and Wehner (2001) reported that the major effect conferred by *db/db* is complemented by the expression of modifier genes, and these genetic factors are weaker than environmental factors. The most recent studies of the genetics of GSB resistance in watermelon were described by Gusmini et al. (2017), who studied the inheritance of resistance to GSB using three PI accessions of watermelon; they reported that the genetic basis of resistance in PI 189225 previously reported by Norton (1979) was more complex than a single recessive gene. They also suggested that the GSB resistance in PI 482283 and PI 526233 may be under the control of a more complex genetic system.

In our previous study, we applied multiple inoculation strategies for GSB resistance on stems and leaves with five different isolates

of *S. cucurbitacearum* collected from different places in east China (Li et al. 2015). Different reactions in the stem and leaves of PI 189225 suggest that different genetic factors may influence the host's response to GSB infections in the leaves and stems (data not shown); this possibility was also raised in the studies conducted by Zhang et al. (2017) and Liu et al. (2017), who found that the GSB resistance in the stem and seedlings of cucumber accession PI 183967 was controlled by different QTL on different chromosomes. Although the parental lines employed in our study differed in their resistance to GSB and wide segregation was observed among the $F_{2:3}$ families, the corresponding F_2 families did not strictly fit the ratio expected under the hypothesis of a single recessive resistance gene.

Another possible reason for the inconsistency with a single resistance gene is that the population in the present study was derived from an interspecific cross, which can result in segregation distortion in some specific regions associated with gamete selection. Segregation distortion is a common phenomenon that can be influenced by factors affecting gametophyte selection (Lytte 1991; Xu et al. 1997). In the present study, compared with other factors such as marker types, environmental effects, and genetic population types, the interspecific cross was the most likely reason and, in our previous study, a segregation distortion region (SDR) was found on the short arm of chromosome 8 with another F_2 population derived from the same parents (Ren et al. 2015). However, whether this deviation from the expected ratio for a single recessive gene is due to segregation distortion or incomplete resistance remains to be examined.

S. cucurbitacearum can infect plants at any stage of development and affects all parts of the host, including the leaves, stems, and fruit.

Table 3. List of Kompetitive allele-specific PCR (KASP) markers used in this study

Marker name	Fluorescent primer	Sequence (5'-3')	Location ^a	Allele	LOD ^b
KASP_JS9388	Allele-specific 1 (FAM)	GCAGTGCCACACAGAAATTGATTG	13,959,138	C	1.40
	Allele-specific 2 (HEX)	TTGGCAGTGCCACACAGAAATTGATTA	...	T	...
	Common	GGTTCGTGATGCATGTTTGCCACCAT	...	...	...
KASP_JS9387	Allele-specific 1 (FAM)	AAGAAAAAGTTTGTTAAGTCTTGAGACGA	13,604,914	A	1.40
	Allele-specific 2 (HEX)	AAGAAAAAGTTTGTTAAGTCTTGAGACGT	...	T	...
	Common	CTCTCAAACAAAAACCCACGAACAATCAA	...	...	...
KASP_JS9385	Allele-specific 1 (FAM)	TCAGTAGGTAGATTCTCATCAATTAGG	12,684,112	G	1.40
	Allele-specific 2 (HEX)	CTCAGTAGGTAGATTCTCATCAATTAGA	...	A	...
	Common	CTGCTGATTCTATTCTTGCTCTATGTCTA	...	...	...
KASP_JS9383	Allele-specific 1 (FAM)	TCTGCTCTACCATAATGTAAGCCC	11,424,655	C	1.19
	Allele-specific 2 (HEX)	TATCTGCTCTACCATAATGTAAGCCT	...	T	...
	Common	TCTCTTCTTAACCTGCCATCTCCCA	...	...	...
KASP_JS9382	Allele-specific 1 (FAM)	CTTTAAAAAGTTGTAGCATGAGTCATTCA	11,344,469	A	1.10
	Allele-specific 2 (HEX)	TTTAAAAAGTTGTAGCATGAGTCATTCTG	...	G	...
	Common	GATTTGTGTATGGATTGTGCCCTTAAATT	...	...	...
KASP_JS9381	Allele-specific 1 (FAM)	CTCGCTCTCGCTTCCTTTCCTCA	11,151,442	T	1.22
	Allele-specific 2 (HEX)	TCGCTCTCGCTTCCTTTCCTCG	...	C	...
	Common	GTGAGAGAGAGCGAGAGAGATAGAT	...	...	...
KASP_JS9379	Allele-specific 1 (FAM)	CTCCGACTTCTAGAGAAGAAGG	10,659,204	G	1.22
	Allele-specific 2 (HEX)	CCTCCGACTTCTAGAGAAGAAGA	...	A	...
	Common	GAGAGCCAAAAGTGAGCTTAAATCAATGAT	...	...	...
KASP_JS9376	Allele-specific 1 (FAM)	TGCCAGAAATTAAGGATGCCTATGA	9,024,470	T	1.65
	Allele-specific 2 (HEX)	CCCAGAAATTAAGGATGCCTATGC	...	G	...
	Common	TCTTAGCAATCTTATTCCTATGCTTTTAAT	...	...	...
KASP_JS9373	Allele-specific 1 (FAM)	GTGACATTAGGAAAAAGTAAATGGAAAAAGA	7,892,440	T	1.05
	Allele-specific 2 (HEX)	TGACATTAGGAAAAAGTAAATGGAAAAAGG	...	C	...
	Common	CGGCGCTACAAATCATATGCAAGTA	...	...	...
KASP_JS9168	Allele-specific 1 (FAM)	TGAACCAATATTTAGCCTATCAGACC	11,995,922	C	1.19
	Allele-specific 2 (HEX)	ATTTGAACCAATATTTAGCCTATCAGACT	...	T	...
	Common	CACAACCATAGGTGGTCTTGGACAT	...	...	...
KASP_JS9166	Allele-specific 1 (FAM)	GTTTGAACGCATGATGGTATTGTATAGT	9,506,943	T	1.57
	Allele-specific 2 (HEX)	TTTGAACGCATGATGGTATTGTATAGC	...	C	...
	Common	GAACATTCTCAAAACCTTATGCTTATCTTT	...	...	...
KASP_JS9165	Allele-specific 1 (FAM)	AAATGTTATATGTGCCAAAATTGGAGTTC	8,429,182	C	1.01
	Allele-specific 2 (HEX)	AAAAAATGTTATATGTGCCAAAATTGGAGTTT	...	T	...
	Common	CAAGTTCCTAGTCAACTAGTCACATACAA	...	...	...

^a Location on chromosome 8.

^b Logarithm of odds.

Resistance to GSB disease in cucurbits is typically evaluated using spray inoculation with a conidial suspension at the four- to six-leaf stage of seedlings (Boyhan et al. 1994a; St. Amand and Wehner 1995a,b; Van Der Meer et al. 1978; van Steekelenburg 1981; Wehner and St. Amand 1993; Zhang et al. 1995, 1997). In the host and pathogen system, apart from the resistance in the host, other important factors favorable for GSB infection include temperature, humidity (particularly the presence of discontinuous water droplets on the foliage), and the length of incubation time (Army and Rowe 1991; St. Amand and Wehner 1995b; Wehner and St. Amand 1993; Zhang et al. 1997). In our preliminary experiment, the resistant parent and the susceptible parent were tested for differences in pathogenicity and aggressiveness in controlled conditions (unpublished data) with six isolates that were selected as the most aggressive based on our previous work (Li et al. 2015), and isolate JS002, which was also able to sporulate abundantly, was chosen for disease phenotyping. To simplify the possible complex disease resistance in PI 189225, we used the optimized inoculation method and only tested the GSB disease on leaves. In the present study, we also applied the foliar-spray inoculation method, and we kept the inoculated seedlings at 100% RH for approximately 24 h, which resulted in a significant

difference in resistance between the resistant parent PI 189225 and the susceptible parent K3. However, when the inoculated plants were kept for 48 h, both the resistant parent PI 189225 and the susceptible parent K3 were susceptible, with little difference in resistance (unpublished data). For most foliar fungal diseases, the presence of tiny, discontinuous water droplets on the foliage is important in bringing the germinating spores close to the leaf surface for initial infection, and continuous 100% RH is required for subsequent lesion expansion (Bushnell 1972). Army and Rowe (1991) also reported that 1 h of free water on leaves was sufficient for infection and that lesion expansion required continued leaf wetness in the infection of cucumber by *S. cucurbitacearum*. Thus, the inoculation method used in the present study was proper for phenotyping the responses to GSB disease in the segregating population. The possible disease resistance in stems could also be a valuable aspect of the disease resistance in PI 189225, and identification and molecular mapping of the resistance in stems could be tested later for comparison between these two kinds of resistance in the same accession.

In the present study, we mapped the resistance locus within the interval of 571.27 kb flanked by the SNP markers KASP_JS9383 and KASP_JS9168 on the short arm of chromosome 8 by using BSA-seq

Table 4. Positions and annotations of candidate genes in the genomic region of gummy stem blight resistance quantitative trait locus *Qgsb8.1* and the corresponding orthologous genes in melon, cucumber, and bottle gourd^a

Gene names	Start position	End position	Annotation	Melon (DHL92)	Cucumber (PI 183967)	Bottle gourd (USVL1VR-Ls)
<i>Cla003808</i>	11,475,588	11,476,601	Unknown protein	<i>MELO3C010590</i>	<i>CSPI06G02740</i>	<i>Lsi08G002710</i>
<i>Cla003809</i>	11,480,534	11,480,695	Unknown protein	—	—	—
<i>Cla003810</i>	11,517,939	11,518,190	Unknown protein	—	—	—
<i>Cla003811</i>	11,529,072	11,529,810	Unknown protein	—	—	—
<i>Cla003812</i>	11,547,432	11,547,788	Unknown protein	—	—	—
<i>Cla003813</i>	11,564,048	11,564,476	Unknown protein	—	—	—
<i>Cla001011</i>	11,644,962	11,648,496	RING finger protein	<i>MELO3C025008</i>	<i>CSPI06G02530</i>	<i>Lsi08G002360</i>
<i>Cla001012</i>	11,652,980	11,656,861	DNA mismatch repair protein	<i>MELO3C025009</i>	<i>CSPI06G02520</i>	<i>Lsi08G002350</i>
<i>Cla001013</i>	11,664,076	11,664,884	DNA mismatch repair protein	<i>MELO3C025010</i>	—	—
<i>Cla001014</i>	11,677,709	11,679,703	Receptor protein kinase-like	<i>MELO3C025012</i>	<i>CSPI06G02510</i>	<i>Lsi08G002340</i>
<i>Cla001015</i>	11,713,055	11,713,339	Unknown protein	—	—	—
<i>Cla001016</i>	11,747,241	11,749,340	Pentatricopeptide repeat protein	—	—	<i>Lsi08G002230</i>
<i>Cla001017</i>	11,759,681	11,762,959	CC-NBS-LRR resistance protein ^b	—	—	<i>Lsi08G002220</i>
<i>Cla001018</i>	11,804,110	11,807,365	Unknown protein	<i>MELO3C025014</i>	<i>CSPI06G02500</i>	<i>Lsi08G002210</i>
<i>Cla001019</i>	11,825,798	11,827,654	Thaumatococcus-like protein and pathogenesis-related protein	—	—	<i>Lsi08G002200</i>
<i>Cla001020</i>	11,853,693	11,857,508	Ras-related protein	<i>MELO3C025015</i> or <i>MELO3C011704</i>	<i>CSPI06G02490</i>	<i>Lsi08G002190</i> or <i>Lsi08G002830</i>
<i>Cla002100</i>	11,937,120	11,937,422	Unknown protein	—	—	—
<i>Cla002101</i>	11,943,731	11,944,738	Unknown protein	—	—	—
<i>Cla002102</i>	11,971,970	11,972,143	Unknown protein	—	—	—

^a Symbol: — indicates no hit.

^b CC-NBS-LRR = coiled-coil nucleotide-binding site leucine-rich repeat.

Fig. 5. Expression profiles of the two candidate genes (*Cla001017* and *Cla001019*) in susceptible parent K3 and resistant parent PI 189225 after inoculation of *Stagonosporopsis cucurbitacearum* via reverse-transcription quantitative PCR.

and KASP marker analysis. The physical location of the region based on the watermelon genome sequence (Guo et al. 2013) indicated that this region contained only one resistance-related gene, *Cla001017*, annotated as encoding the CC-NBS-LRR resistance protein containing the InterPro domain IPR002182 NB-ARC, suggesting that the resistance locus was close to the *Cla001017* locus or that it was the *Cla001017* gene. Another possible reason for only one resistance-related gene in the region is that *C. lanatus* and *C. amarus* are different species and only the *C. lanatus* genome was used for this genetic analysis. The gene conferring GSB resistance may not be listed in the candidate genes because it may not exist in *C. lanatus*. Moreover, the presence of an SDR also influences the estimation of recombination frequency and, thus, may decrease the accuracy of gene or QTL mapping. An area containing 3 or more skewed markers was defined as an SDR. According to the results of genomic variance analysis of SDRs (Ren et al. 2015), the QTL for GSB resistance identified in the present study were located in the middle of SDR8.1 on chromosome 8. All 45 bin markers in SDR8.1, including the bin marker SNP_chr08_bin10 between the SNP markers KASP_JS9383 and KASP_JS9168, were skewed in favor of susceptible parent K3, indicating that this QTL region in SDR8.1 is a hot spot for genetic variation between *C. lanatus* and *C. amarus*. Therefore, care should be taken in identification and mapping of genes or QTL from crosses between feral and cultivated types of crops.

There is collinearity among watermelon chromosome 8, melon chromosome 3, and cucumber chromosomes 2 and 6 (Huang et al. 2009; Li et al. 2011). Based on the collinearity relationship, one QTL (*gsb-s2.1*) on chromosome 2 and 3 QTL (*gsb-s6.1*, *gsb-s6.2*, and *gsb-s6.3*) on chromosome 6 for resistance to GSB in cucumber stems have been detected (Zhang et al. 2017), and one QTL (*gsb6.1*) on chromosome 6 for resistance to GSB in cucumber seedlings has been detected (Liu et al. 2017). Lou et al. (2013) also reported that the one QTL for resistance to GSB in cucumber was identified on chromosome 6. According to the reference genome sequence 97103, disease-resistance genes are also enriched in the distal part of the short arm of chromosome 8, which is near the interval of the resistance gene; this result suggested that the resistance gene may be associated with the QTL for GSB detected in melon and cucumber or with other resistance gene clusters. However, the actual relationship between these loci requires additional fine mapping.

In summary, this is the first report on the genetics and mapping of resistance to GSB in watermelon, and the resistance gene and linked molecular markers mapped in this study are important for watermelon breeding programs and will contribute to marker-assisted selection procedures in watermelon resistance breeding. Additionally, the markers can be employed in further fine mapping and positional cloning to gain a more complete understanding of resistance to GSB in PI 189225.

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