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Development of a Muskmelon Cultivar with Improved Resistance to Gummy Stem Blight and Desired Agronomic Traits Using Gene Pyramiding

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Abstract

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Gummy stem blight (GSB), caused by *Didymella bryoniae* (Auersw.) Rehm., is a severe disease affecting Cucurbitaceae crops including melons. The resistance of current melon varieties that carry a single *Gsb* resistance gene is insufficient to protect against the abundant variation of the *D. bryoniae* isolates. Pyramiding multiple *Gsb* resistance genes into melon cultivars is an effective way to develop a broad resistance spectrum and to increase the duration of GSB resistance. In this study, two resistance genes (*Gsb-4* and *Gsb-6*) from two resistant accessions, PI482398 and PI420145, were pyramided into one variety using marker-assisted selection (MAS). The donor parent 4598 that contained *Gsb-4* and *Gsb-6* was hybridized with muskmelon Baipicui to produce BC_1F_1 and BC_2F_1. Phenotyping and MAS enabled identification and pyramiding of two *Gsb* genes in individuals of F_1, BC_1F_1 and BC_2F_1. Field cultivation showed that individuals carrying both *Gsb-4* and *Gsb-6* had improved resistance to GSB and improved fruit quality. The results indicated that MAS-based pyramiding is an effective strategy for breeding melon cultivars with increased resistance to GSB.

Keywords: *Cucumis melo*; *Didymella bryoniae*; disease resistance breeding; fruit quality; marker-assisted selection

Gummy stem blight (GSB) is a disease caused by *Didymella bryoniae* (Auersw.) Rehm., which causes severe yield losses in melons (*Cucumis melo* L.), watermelons (*Citrullus vulgaris* Schrad.), cucumbers (*Cucumis sativus* L.) and other Cucurbitaceae crops (Keinath *et al*. 1995). GSB was found out in the early 1980s in Europe (Lebeda 1985; Frantz & Jahn 2004), in the United States (Amand & Wehner 1991; Gusmini *et al*. 2003, 2005), China, Japan and other tropical and subtropical countries (Wako *et al*. 2002; Tsutsumi & Silva 2004). Breeding cultivars with major resistance genes is the most effective and economical strategy to control GSB disease.

Qualitative gene-for-gene resistance against some pathogen races, as conferred by major genes, is the easiest resistance that can be incorporated into breeding programs. However, because of rapid changes in pathogen virulence or population biotype, the qualitative resistance is not durable (Suh *et al*. 2013). One strategy for breeding cultivars with durable and broad-spectrum resistance is pyramiding resistance loci. Owing to the dominance and epistasis effects

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of resistance genes, it is difficult to pyramid genes using conventional breeding methods. Molecular markers can be successfully used to identify and pyramid desirable and multiple alleles for biotic and abiotic stress resistance in a collection of diverse genotypes (Jena & Mackill 2008).

PI482398 and PI420145 are the widely accepted GSB resistance resources in the world (Zhang *et al*. 1997; Wolukau 2007). Independent genes with dominant resistance alleles have also been identified in PI482398 (*Gsb-4*) and PI420145 (*Gsb-6*) (Zhang *et al*. 1997; Frantz & Jahn 2004). Our previous studies have reported some markers linked to the *Gsb* resistance genes. E-TG/M-CTC_{200}, E-AT/M-CTG_{90}, E-TC/M-CAG_{60}, and E-TG/M-CTA_{70} amplified fragment length polymorphism (AFLP) markers have been identified in positions of 2.0, 6.0, 5.4, and 6.0 cM, respectively, from the *Gsb-6* locus (Wolukau 2007). The CMTA170a simple sequence repeat (SSR) marker is linked to *Gsb-4* at 5.14 cM (Wang *et al*. 2012). This study sought to successfully transfer and pyramid two *Gsb* resistance genes from PIs into an elite muskmelon cultivar using MAS. Evaluation of agronomic traits was performed in developing populations and selected resistant plants.

MATERIAL AND METHODS

Plant materials. Baipicui, a Honey Dew muskmelon cultivar from the Xinjiang Province in western China, is highly susceptible to GSB. Cultivar 4598, which contains *Gsb-6* and *Gsb-4*, was used as the donor parent for transferring GSB resistance genes into Baipicui. Baipicui was crossed with 4598 to obtain F_1. Then, F_1 was backcrossed with the recurrent parent Baipicui to produce BC_1F_1. Phenotype identification and MAS were used to select BC_1F_1 individuals with high resistance and two resistance genes. The selected plants were backcrossed to obtain BC_2F_1 and self-pollinated to produce BC_2F_2. Parental plants and progeny were grown in the greenhouse at the Jiangpu Experiment Farm of Nanjing Agricultural University (NJAU) in Nanjing, China, during the spring and autumn from 2012 to 2014.

Inoculation and progeny seedling selection. The A1 isolate of *D. bryoniae* collected from the field of Horticulture College of NJAU was used for all resistance tests. It was maintained on the potato carrot agar medium (PCA) at 4°C and multiplied on the potato dextrose agar medium (PDA) at 25°C in the dark for 7 days, then subjected to intermittent UV irradiation (12 h UV/12 h dark) for 4 days at 25°C. Then, the spore suspension was adjusted to 5×10^5 spores per ml using a haemacytometer. Seedlings at the stages of three to five true leaves were inoculated by spraying the fresh made inoculum with a hand sprayer. Resistance evaluation was conducted according to Zhang *et al.* (1997).

Amplification of AFLP-specific fragments. DNA was isolated from lyophilized leaves by a modified CTAB extraction method (Rogers & Bandich 1994). AFLP reactions were performed as described by Vos *et al.* (1995) with some modifications. PCR-selective amplification of primers E-TG/M-CTC_{200}, E-AT/M-CTG_{90}, E-TG/M-CTA_{70} and E-TC/M-CAG_{60} (Table 1) was performed in a 20 µl reaction mixture containing 5 µl diluted production from the preamplification reaction, 10 ng *Eco*RI primer, 10 ng *Mse*I primer, 2.0 µl 2 mM dNTP, 2.0 µl 10× PCR reaction buffer, and 1U Taq DNA polymerase. PCR conditions were denaturation for 3 min at 94°C, followed by 30 s at 94°C, 30 s at 65°C, 60 s at 72°C for 11 cycles. The annealing temperature was then lowered by 0.7°C per cycle during the 11 cycles; then 30 s at 94°C, 30 s at 56°C, 60 s at 72°C for 23 cycles; and a final extension at 72°C for 2 min. The PCR products were separated on a 6% non-denaturing polyacrylamide gel, stained by a simple silver staining method described by Ashutosh *et al.* (2007).

Conversion of specific AFLP fragments to SCARs. The AFLP fragments were converted to sequence characterized amplified regions (SCAR) according to the method of Mienie *et al*. (2002) with modifications. The re-amplified fragments were cloned into the pGEM-T easy vector (Promega, Madison, USA) and transformed into competent *Escherichia coli* JM 109 cells according to the manufacturer's recommendations. Then, the cloned fragments were sequenced by the Biotech Company, Jinsirui, Nanjing, China. The primers for the cloned fragments were designed by Primer 6.0 software. The setting parameter of the Tm value was 50–60°C. The primer lengths were set to 18–22 bp.

Marker-assisted selection. Primers $SGSB_{1800}$ and CMTA170a were synthesized by the Biotech Company Jinsirui (Table 1). The PCR of $SGSB_{1800}$ was carried out in a 20 µl reaction mixture containing 10.8 µl sterilized ddH_2O, 2.0 µl 10× PCR buffer, 1.5 µl $MgCl_2$ (25 mM), 2.0 µl dNTP (2.5 mM), 1.0 µl each primer (20 ng/µl), 0.2 µl Taq polymerase (5 U/µl), and 1.5 µl template DNA (20 ng/µl). PCR conditions were denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 80 s, and a final extension at 72°C for 7 min. The PCR products were separated on

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Table 1. Primer sequences used to pyramid the GSB resistance genes in muskmelon

Name	Forward primer (5'→3')	Reverse primer (5'→3')
E-TC/M-CAG$_{60}$	GACTGCGTACCAATTCTC	GATGAGTCCTGAGTAACAG
E-TG/M-CTA$_{70}$	GACTGCGTACCAATTCTG	GATGAGTCCT,GAGTAACTA
E-AT/M-CTG$_{90}$	GACTGCGTACCAATTCAT	GATGAGTCCTGAGTAACTG
E-TG/M-CTC$_{200}$	GACTGCGTACCAATTCTG	GATGAGTCCTGAGTAACTC
CMTA170a	TTAAATCCCAAAGACATGGCG	TTTCGATTGGCAGGAAGCAGA
SGSB$_{1800}$	GTTGCGTTCTCTGCTTGGA	AGGTAATTGAGGTGTCGTCTTA

1% agarose gel in 1× TAE, and visualized by staining with ethidium bromide. The PCR reaction mixture of CMTA170a contained 11.3 µl sterilized ddH_2O, 2.0 µl 10× PCR buffer, 1.5 µl $MgCl_2$ (25 mM), 2.0 µl dNTP (2.5 mM), 1.0 µl of each primer (20 ng/µl), 0.2 µl Taq polymerase (5 U/µl), and 1.0 µl template DNA (20 ng/µl) in a volume of 20 µl. PCR conditions were the same as those for SGSB$_{1800}$ except for the annealing temperature (58°C). The PCR products were separated on a 6% non-denaturing polyacrylamide gel in 1× TBE for 90 min after silver staining.

Evaluation of agronomic traits. BC_2F_1 was planted in a greenhouse to investigate the GSB resistance and commercial quality. Baipicui, PI420145, PI1482398 and 4598 were used as controls. The plants were grown in single rows (1.2 × 0.4 m) at a plant density of 0.6~0.7 plants/m^2. Hanging vine and single vine pruning were used to cultivate the melons. The fruit setting was on the 10th to 15th node per plant. Nine mature fruits (45 days after pollination) were used to evaluate fruit traits. Fruit pulp firmness (kg/m^2) was determined with a penetrometer (GY-1 Texture Analyzer, Zhenjiang Top Instrument Company, Hangzhou, China) fitted with a 3.5-mm diameter round head probe on three discs of the skin surface from the fruit equatorial area according to the manufacturer's description. The mean fruit weights, flesh thickness, soluble solids content (SSC), and vitamin C (Vc) content were determined for nine fruits. A calliper was used to measure the flesh thickness of each fruit. Total SSC in juice was determined with a refractometer as °Brix according to the instructions. The Vc content (mg/100g) was measured by a spectrophotometer method (Zhang & Fan 2007). All data were evaluated using the SPSS 19.0 Statistics software.

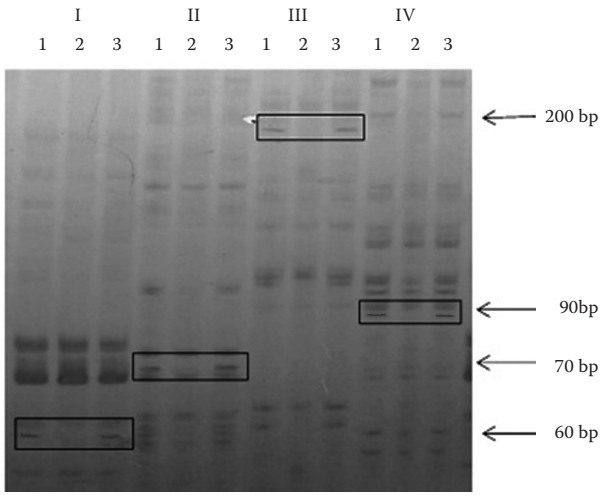

Figure 1. The amplification of E-TC/M-CAG$_{60}$ (I), E-TG/M-CTA$_{70}$ (II), E-TG/M-CTC$_{200}$ (III), E-AT/M-CTG$_{90}$ (IV) in PI420145 (1), Baipicui (2) and 4598 (3)

RESULTS

Development of a SCAR marker linked to *Gsb-6*. Polymorphic DNA fragments of 200, 90, 70 and 60 bp were separately amplified by four AFLP markers E-TG/M-CTC$_{200}$, E-AT/M-CTG$_{90}$, E-TG/M-CTA$_{70}$ and E-TC/M-CAG$_{60}$ in PI420145 and 4598 but not in Baipicui (Figure 1). Four cloned and sequenced polymorphic fragments were subjected to BLAST analysis. However, only the E-TG/M-CTC$_{200}$ clone with a 134-bp long fragment (GenBank accession No. KX867995) showed high homology with the melon genome sequence (CM3.5_scaffold00075). On the basis of sequence information of the E-TG/M-CTC$_{200}$ clone, a pair of SCAR primers named SGSB$_{1800}$ was

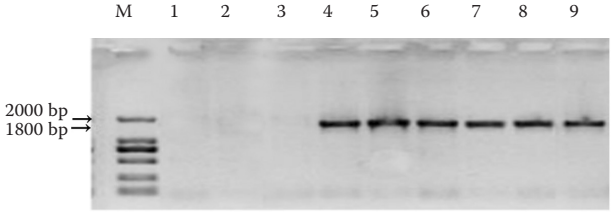

Figure 2. The amplification of SGSB$_{1800}$ in Baipicui (1, 2, 3), PI420145 (4, 5, 6) and 4598 (7, 8, 9)

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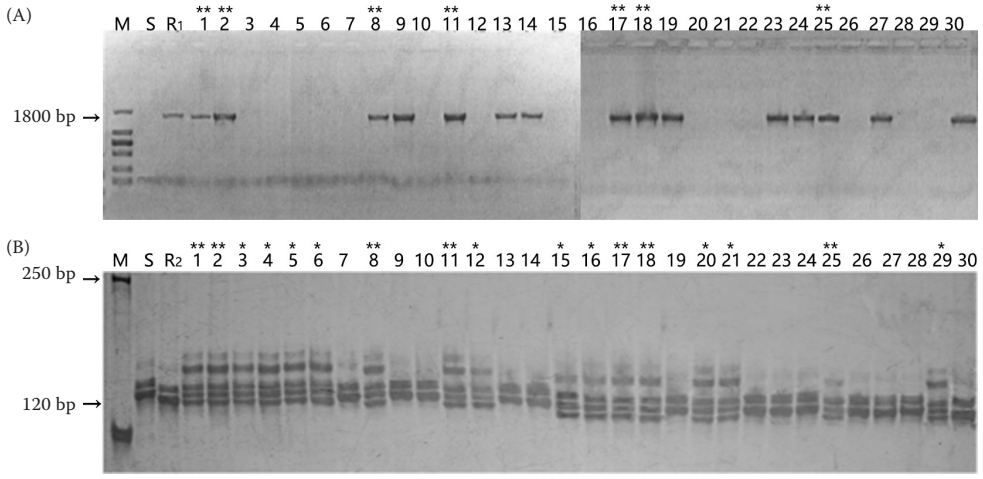

Figure 3. The amplification of $SGSB_{1800}$ (A) and CMTA170a (B) in BC_2F_1 individuals of muskmelon; **individuals with both bands amplified by $SGSB_{1800}$ and CMTA170a; *individuals with only one band amplified by CMTA170a; M – Marker; S – Baipicui; R_1 – PI420145; R_2 – PI482398; 1–30 – BC_2F_1

designed by the Primer 6.0 software (Table 1). One 1800-bp polymorphic DNA fragment was amplified by primers $SGSB_{1800}$ in PI420145 and 4598 (Figure 2). Therefore, $SGSB_{1800}$ was able to identify *Gsb-6* instead of E-TG/M-CTC_{200} in pyramiding breeding.

Resistance genes pyramided in BC_2F_1. $SGSB_{1800}$ and CMTA170a were used to screen for *Gsb-6* and *Gsb-4*. The SSR marker CMTA170a linked to *Gsb-4* amplified a 120-bp long fragment in PI482398 and 4598. Therefore, one amplified fragment indicated one *Gsb* resistance gene, and two amplified fragments indicated two *Gsb* resistance genes; thus this technique was useful in pyramiding (Figure 3). Moreover, phenotypic evaluation of resistance was conducted at each backcross to ensure the MAS accuracy. Finally, only 11 BC_2F_1 individuals were identified as having two pyramided target genes (Figure 4).

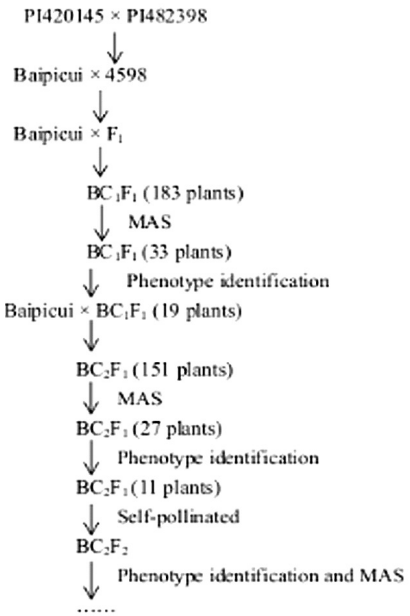

Figure 4. The breeding diagram of pyramiding two *Gsb* resistance genes into muskmelon

Desired agronomic traits improved in BC_2F_1. Nineteen selected BC_1F_1 plants showed segregation in morphological traits including leaf shape, plant type and fruit quality. The plants resembling the recurrent parent Baipicui were preserved for the next backcross. The 11 selected BC_2F_1 plants were more similar to Baipicui than BC_1F_1 despite the segregation of fruit peel types and colour. All BC_2F_1 plants

Figure 5. The phenotype of Baipicui × (PI420145 × PI482398) BC_2F_1 (left) and Baipicui (right) at the late stage in a greenhouse

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Table 2. Resistance and fruit quality statistics of Baipicui, 4598 and selected BC_2F_1

Material	Fruit shape	Fruit firmness (kg/cm^2)	Flesh colour	Flesh thickness (cm)	Flesh texture	Soluble solid content (%)	Vc (mg/100g)	Fruit weight (kg)	Resistance to GSB
Baipicui	olive	15.0 ± 0.181^a	orange	3.0 ± 0.450^a	crispe	8.3 ± 0.418^a	10.319 ± 0.391^b	1.5 ± 0.133^a	high susceptible
4598	cylindrical	10.7 ± 0.227^b	white	1.1 ± 0.271^b	soft	5.6 ± 0.474^c	13.936 ± 0.363^a	0.3 ± 0.067^c	high resistance
Selected BC_2F_1	olive	14.4 ± 0.117^a	orange	2.8 ± 0.298^a	crispe	7.6 ± 0.583^b	12.399 ± 0.797^a	1.3 ± 0.149^b	high resistance

Vc – vitamin C; GSB – gummy stem blight; data in the same column followed by different lowercase letters are significantly different at $P = 0.05$

carrying *Gsb-4* and *Gsb-6* displayed high GSB resistance compared with that of the susceptible cultivar Baipicui (Figure 5). In addition, we also examined several fruit traits among the BC_2F_1, 4598 and Baipicui (Table 2). The results showed that the fruit shape, flesh colour and flesh texture of BC_2F_1 individuals and Baipicui were the same (Table 2). The appearance of BC_2F_1 fruits was similar to that of Baipicui fruits (Figure 6). The average fruit firmness and flesh thickness of BC_2F_1 were not significantly different from the recurrent parent but were significantly different from 4598. There were significant differences in SSC and fruit weight between the three sources. The average SSC of BC_2F_1 individuals was about 0.7% lower than that of Baipicui, but was about 2.0% higher than that of 4598. The average fruit weight of selected BC_2F_1 individuals was about 0.2 kg lower than that of Baipicui, but was about 1.0 kg higher than that of 4598. The mean Vc content of BC_2F_1 line was not significantly different from that of 4598, but was significantly different from that of Baipicui. The average Vc content of BC_2F_1 was about 2.08 mg per 100 g higher than that of Baipicui.

Figure 6. The fruit of Baipicui × (PI420145 × PI482398) BC_2F_1 (left) and Baipicui (right)

DISCUSSION

D. bryoniae is well known for its capacity to quickly overcome resistance in melon cultivars. During the long-term cultivation of melon cultivars containing a single resistance gene, the resistance to gummy stem blight gradually becomes insufficient, and the resistance is not durable in melon plants (Sowell 1981; Sitterly & Keinath 1996). Moreover, resistance from six resources (PI420145, PI140471, PI157082, PI511890, PI482398 and PI482399) shows a decreasing trend in the progeny when they are separately introgressed into susceptible melon cultivars compared with the resistant donor parents (Zhang *et al.* 1997). Broad and durable resistance to a wide spectrum of pathogen races/strains is desirable but it is often affected by multiple factors such as pathogen populations with various mutation rates, an environment favourable for the pathogen, the extent and duration of a particular resistance gene existing in cultivars, and the crop reproduction and mating systems that affect the resistance gene flow, among other factors (McDonald & Linde 2002; Kang *et al.* 2005; Hanson *et al.* 2016). Consequently, a gene pyramiding strategy can effectively improve the chances of conferring durable resistance to plant diseases (Vidavski *et al.* 2008; Nowicki *et al.* 2012). In a previous study, PI420145 and PI482398 have been found to exhibit the same level of resistance to GSB. However, because of the variation of *D. bryoniae* isolates, most of the resistant lines are only

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moderately resistant (Zuniga *et al*. 1999). Therefore, we attempted to pyramid two *Gsb* resistance genes into a single genotype and then we transferred the combined resistance into the muskmelon cultivar Baipicui using marker-assisted backcrossing.

Gene pyramiding is difficult to achieve through conventional breeding approaches, owing to the low accuracy in the identification of desirable genotypes, and therefore it can be laborious and time consuming (Ye & Smith 2010). MAS offers possibilities to improve the selection strategies in pyramiding breeding in a shorter period of time. This study demonstrated that MAS can deliver targeted genotypes in a small population. Owing to the high cost, complicated operation and strict technical requirements of AFLP, the AFLP marker E-TG/M-CTC$_{200}$ was converted to SCAR$_{1800}$ before pyramiding. Because only two markers associated with *Gsb-4* and *Gsb-6* were selected in the previous study, the validation of MAS lines was performed by phenotype evaluation. Seedling inoculation was used to confirm the accuracy of selecting genes from pyramided plants. In this study, only 11 out of the 151 BC_2F_1 plants were identified to contain the targeted genes, possibly because two markers CMTA170a and SGSB$_{1800}$ were not closely linked to *Gsb-4* and *Gsb-6*, respectively. Thus, the complete co-segregation of the two genes could not occur in the progeny, and the MAS showed low efficiency. Nevertheless, this is the first report of pyramiding with two *Gsb* resistance genes in a melon line. This study provides insights into pyramiding breeding of melons to improve GSB resistance. The next research steps will be: (1) to identify more markers that are closely linked to the resistance genes; (2) to use 11 BC_2F_1 backcrossing or self-pollinating plants to obtain a variety with high resistance and good prospect for commodification.

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