

Full Length Research Paper

# A RAMP marker linked to the tobacco black shank resistant gene

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**Bulk segregant analysis (BSA) and randomly amplified microsatellite polymorphism (RAMP) were employed to analyze F<sub>2</sub> individuals of the Yunyan 317×Hubei 517 to screen and characterize molecular markers linked to black shank resistant gene. A total of 800 arbitrary decamer oligonucleotide primer-pairs were used for RAMP analysis. Primer pair GT (CA)<sub>4</sub>/S89, producing one RAMP marker GT (CA)<sub>4</sub>/S89<sub>550</sub>, was tightly linked to the black shank resistant gene. Results of Southern blot suggest that the fragment GT (CA)<sub>4</sub>/S89<sub>550</sub> was existed in Yunyan 317 and resistant plants, and absent in Hubei 517. Linkage analysis was carried out using marker GT (CA)<sub>4</sub>/S89<sub>550</sub> on 752 black shank high-resistant individuals of F<sub>2</sub> progenies from crossing between Yunyan 317 and Hubei 517. Our results indicated that the genetic distances between GT (CA)<sub>4</sub>/S89<sub>550</sub> and black shank resistant gene was 1.4cM.**

**Key words:** Tobacco, black shank resistant gene, RAMP, molecular marker.

## INTRODUCTION

Black shank [caused by *Phytophthora parasitica* (Dast.) var. *nicotianae* (B. de Haan) Tucker] is among the most destructive and widespread of all tobacco diseases in the world scope (Li et al., 2006). This disease is most prevalent in poorly-drained areas where tobacco was planted the previous year. For coping with crop diseases, breeding new cultivar of disease-resistant is one of the best ways. Hence, it is significant to find the markers linked to black shank resistant gene on the DNA level. Random amplified microsatellite polymorphism (RAMP) technology has a lot of merits such as easy to operate, cheap in cost, speedy and accurate (Cheng et al., 2001). And bulked segregator analysis (BSA) was one of the most useful methods in finding linkage marker of gene with molecular marker technology (Michelmore et al., 1991; Zhu et al., 2004; Zhang et al., 2008). In this paper, the results of combination with RAMP and BSA to identify

linkage molecular maker of black shank resistant gene of tobacco were presented.

## MATERIALS AND METHODS

### Plant materials

Parent tobacco plants, Hubei 517 is highly susceptible to *P. parasitica*, while Yunyan 317 is highly resistance (Rei et al., 1997). Hubei 517, Yunyan 317 and a total of 1012 F<sub>2</sub> plants were used in the experiments.

### Inoculation and classification

Evaluation of F<sub>2</sub> plants for their responses to *P. parasitica* var. *nicotianae* was performed using the small-plant technique (Litton et al., 1970a). Pathogen culture and inoculum preparation were performed as described as Litton et al. (1970a). Twenty-six days after seeding, the roots of the plants were inoculated by submerging the tubes in a suspension of active zoospores of *P. parasitica* var. *nicotianae* in a one-third Hoagland's solution (Litton et al., 1970a). The rating was made 10 to 14 d after inoculation with an isolate of *P. parasitica* var. *nicotianae* race 1, when the

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susceptible control plants (Hubei 517) were dead or almost dead. The root systems were washed free of vermiculite and each individual root was examined. Plants that were alive and free of obvious symptoms in their roots were classified as resistant, and those that had symptoms as susceptible. The susceptible plants include those that were either dead or alive but having obvious root system symptoms, with single or multiple roots being necrotic.

#### DNA extraction

Ten susceptible and 752 resistant  $F_2$  plants were used for DNA extraction. DNA was extracted from shoots by the CTAB method (Zhang et al., 2006). Samples were ground to powder in liquid nitrogen, using a mortar and pestle. The powder was transferred to a 25 ml sterile tube with 10 ml of CTAB buffer. The extraction buffer consisted of 2% (w/v) CTAB (cetyltrimethyl ammonium bromide, Sigma), 1.5 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 9.5, and 0.2% (v/v)  $\beta$ -mercaptoethanol. After incubating the homogenate at 65°C for 1 h an equal volume of chloroform was added and centrifuged at 10,000 rpm for 20 min. DNA was precipitated with 1/10 volume (ml) of 3 M sodium acetate and an equal volume of isopropanol followed by centrifugation at 10,000 rpm for 10 min. The DNA pellet was washed with 70% ethanol, air-dried, and resuspended in TE-buffers (10 mM Tris pH 8.0 and 0.1 mM EDTA). DNA quantity was estimated spectrophotometrically by measuring absorbance at 260 nm. DNA samples were diluted in sterile deionized water and maintained at -20°C.

#### Gene pool construction and RAMP analysis

The BSA technique was used to screen markers co-segregated with black shank resistant gene as described by Michelmore et al. (1991). Equal amounts of DNA from 10 resistant and 10 susceptible  $F_2$  plants were mixed to form a resistant pool and a susceptible pool, respectively. Amplifications were carried out in 25  $\mu$ L of reaction mixture containing 2.5  $\mu$ L 10  $\times$  PCR buffer, 0.2 mmol/L dNTPs, 2 mmol/L of  $MgCl_2$ , 1 U of Taq DNA polymerase (Shanghai Sangon Biological Engineering Technology and Services Co.), 20 ng of each primer pairs (Shanghai Sangon Biological Engineering Technology and Services Co.) and 20 ng of template DNA. The reaction mixture was incubated at 94°C for 120 s, followed by 40 cycles of 94°C for 45 s, 37°C for 45 s, and 72°C for 60 s. The PCR was terminated following incubation at 72°C for 6 min. The amplifications added with 2  $\mu$ L loading buffer (95% v/v formamide and 0.08% w/v bromophenol blue in 20 mM EDTA, pH 8.0) were electrophoresed through 1.0% in an agarose gel containing 0.5  $\mu$ g/mL ethidium bromide, and were visualized and photographed on an UV transilluminator.

#### Southern blot analysis

The polymorphic fragment GT (CA)<sub>4</sub>/S89<sub>550</sub> were <sup>32</sup>P-labeled as described previously (Zhang et al., 2007), and used as probes in Southern blot analysis. DNA transfer and hybridization procedure was performed as Sambrook et al (1989). The DNA was digested with *Bam*H I, electrophoresed through 0.8% agarose, and transferred to nylon membranes. A radioactive probe for hybridization was made from a 0.55-kb GT (CA)<sub>4</sub>/S89<sub>550</sub> fragment with <sup>32</sup>P using random-primed labeling (Feinberg and Vogelstein, 1983). The prehybridization blots were hybridized with GT (CA)<sub>4</sub>/S89<sub>550</sub> probe overnight at 65°C. They were then washed 3 times with sodium phosphate buffer (pH 7.2) and 1% SDS at 65°C for 5, 30, and 15 min and visualized by autoradiography.

#### Linkage analysis

Recombination rate (cM) = recombinator / (no. of individual of  $F_2$  generation  $\times$  2)  $\times$  100%, in which 2 stands for diploid genome (Zhang et al., 2008).

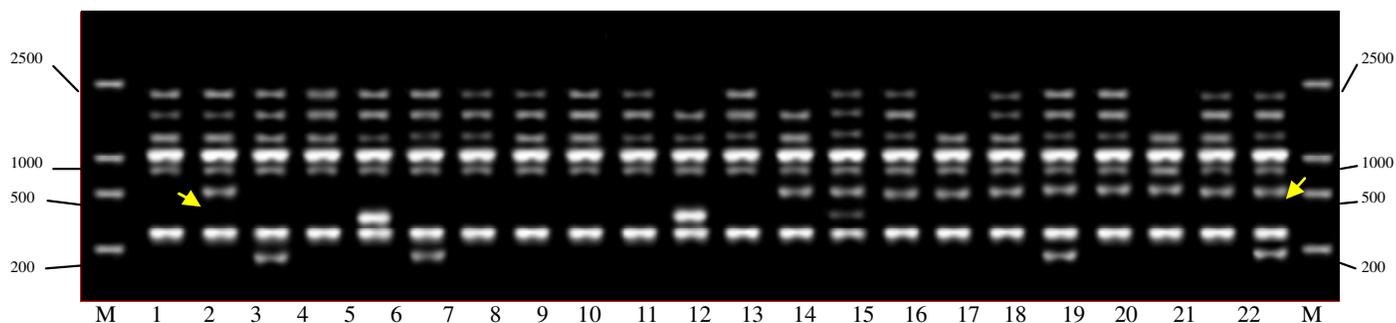
## RESULTS AND DISCUSSION

Resistance or susceptible reaction of each individual plant was significant, making the phenotypic identification easier. Among the 1012 inoculated  $F_2$  plants, 752 resistant and 260 susceptible plants were identified. Statistical analysis ( $X^2$ ) showed that distribution at 3:1 segregation ratio of the resistance trait was significant, indicating that the resistance of Yunyan 317 was controlled by one gene or by closely linked genes.

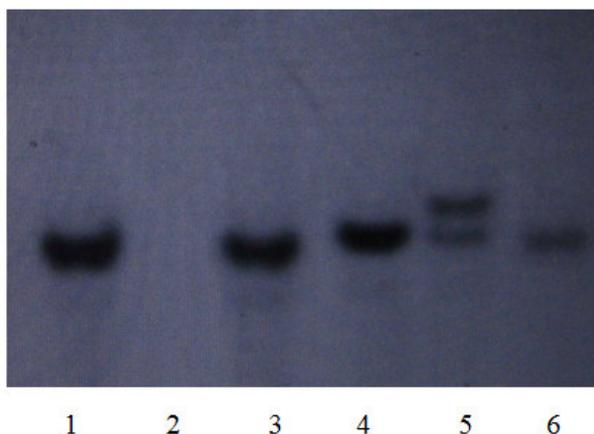
800 primer pairs were used in this study, 243 (30.4%) produced amplification products that were too faint to score or could not be consistently reproduced, and 519 (64.9%) produced monomorphic banding patterns. Thus only 38 (4.8%) out of 800 primer pairs produced polymorphic banding patterns. One to nine bands generated by a primer pair of variable lengths were detected. More than two thirds of the bands were larger than 600 but smaller than 3600 base pairs, and less than 8% larger than 3600 base pairs, that is, mostly of small and medium size. Then, these RAMP primer pairs were further used to test the 20 individuals of the resistant and susceptible pools. Results showed that only one out of 38 primer pairs, GT (CA)<sub>4</sub>/S89, reproducibly produced polymorphism GT (CA)<sub>4</sub>/S89<sub>550</sub> (about 550 bp) in plant Yunyan 317 and resistant plants, but no corresponding band was obtained in Hubei 517 and susceptible plants (Figure 1). The results primarily confirmed that black shank resistant gene was linked with the RAMP markers.

Yunyan 317, Hubei 517 and some resistant plants with polymorphic fragment GT (CA)<sub>4</sub>/S89<sub>550</sub> plants were selected for Southern blot hybridization. This experiment was conducted with a view to estimating the fragment GT (CA)<sub>4</sub>/S89<sub>550</sub> copy number in the putative resistant plants. Figure 2 showed the results of parents and resistant DNA hybridized with <sup>32</sup>P-dCTP labeled GT (CA)<sub>4</sub>/S89<sub>550</sub> probes respectively. The patterns of hybridization with GT (CA)<sub>4</sub>/S89<sub>550</sub> fragment probe suggested that the fragment GT (CA)<sub>4</sub>/S89<sub>550</sub> was existed in Yunyan 317 and resistant plants, and absent in Hubei 517. The amplified products of GT (CA)<sub>4</sub>/S89 showed that 21 plants have no GT (CA)<sub>4</sub>/S89<sub>550</sub> in 752  $F_2$  resistant plants. The linkage analysis indicated that the genetic distance between GT (CA)<sub>4</sub>/S89<sub>550</sub> and black shank resistant gene was 1.4 cM.

It was worth noting that only 10 susceptible  $F_2$  plants were used to screen and characterize molecular markers, in order to avoid the mistakes. The small-plant technique used in this study which was originally developed by Stokes and Litton (1966) and later modified by Litton et al. (1970a), had been used as a standard technique for



**Figure 1.** Amplification products of GT (CA) <sub>4</sub>/S89 in parent tobacco plants and F<sub>2</sub> individuals. Lane 1, Hubei 517; lane 2, Yunyan 317; lanes 3-12, different accession of susceptible F<sub>2</sub> individuals; lanes 13-22, different accession of resistant F<sub>2</sub> individuals; yellow arrows in the figure indicated the polymorphic band GT (CA) <sub>4</sub>/S89<sub>550</sub>.



**Figure 2.** Southern blot hybridization patterns of Yunyan 317, Hubei 517 and some resistant F<sub>2</sub> individuals. Lane 1, Hubei 517; lane 2, Yunyan 317; lanes 3-6, different accession of resistant F<sub>2</sub> individuals.

evaluating the response of tobacco to *P. parasitica* var. *nicotianae* (Litton et al., 1970b). This technique had the advantage that it allowed the screening of a large number of candidate plants in a short period of time (Litton et al., 1970b), but the disadvantage was that mistakes sometimes happened. Tobacco plants were typically subjected to *P. parasitica* var. *nicotianae* only when they became older, after being transplanted in the field. Plant materials that were identified to be resistant when they were only small seedlings using this technique were more than likely to be more resistant when they became older. The reason was that older plants were generally more resistant to plant pathogens than those that were younger. Hence, much more resistant F<sub>2</sub> plants were used to screen and characterize molecular markers in this study.

Molecular marking technologies which base on revealing polymorphism of the allele areas, have become

importantly technological methods in the crop heredity and breeding, which could be used to assist selecting the goal gene in the early stage of plant breeding. Make good use of them could improve the selecting efficiency and shorten the breeding period. In recent years a lot of important quality trait genes of main crops were marked such as the nuclear fertility restorer gene in rice and the common bunt resistance gene in wheat (Zhu et al., 2004, Demeke et al., 1996). Black shank is one of the most devastating diseases in tobacco (*Nicotiana tabacum* L.). *P. parasitica* var. *nicotianae* race 1 is the most damaging race of this fungus. In this paper, combined with BSA and RAMP, we found a marker GT (CA) <sub>4</sub>/S89<sub>550</sub>, closely linked to the black shank resistant gene against *P. parasitica* var. *nicotianae* race 1. The marker GT (CA) <sub>4</sub>/S89<sub>550</sub> got in this study could be used in molecular assisting breeding. Molecular-assisting breeding of black shank resistant tobacco plants could avoid most inoculation procedure of *P. parasitica* to cut down on workload, could identify the resistance of plants in the early stages of breeding, increasing selection veracity and efficiency, and could accelerate reasonable and rapid utilization of the resistant gene, shortening the breeding cycle.

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