

## Short Communication

# Screening and characterization a RAPD marker of tobacco brown-spot resistant gene

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**Bulk Segregant Analysis (BSA) and Randomly Amplified Polymorphic DNA (RAPD) methods were used to analyze F<sub>2</sub> individuals of 82-3041 × Yunyan 84 to screen and characterize the molecular marker linked to brown-spot resistant gene. A total of 800 arbitrary decamer oligonucleotide primers were used for RAPD analysis. Primer S361, producing one RAPD marker S361<sub>650</sub>, was tightly linked to the brown-spot resistant gene. Linkage analysis was carried out using marker S361<sub>650</sub> on 1042 individuals of F<sub>2</sub> progenies from the crossing between 82-3041 × Yunyan 84. The results demonstrated that the genetic distances between S361<sub>650</sub> and brown-spot resistant gene was 2.98 cM.**

**Key words:** Brown-spot, resistance gene, bulk segregant analysis, molecular marker, and linkage analysis.

## INTRODUCTION

Tobacco brown spot caused by *Alternaria alternata* is an important disease of tobacco plant worldwide (Adachi and Tsuge, 1994). The disease mainly occurs in the later stage of growth of the tobacco plant in the field, and can cut down the quality of tobacco (*Nicotiana tabacum* L.) leaves. The most economic and effective method to control the disease is the utilization of natural genetic resistance and breeding of resistant cultivars. The finding of linked marker of brown-spot resistant gene on the DNA level can be applied to assist selection, increase breeding efficiency and provide the base for gene cloning later. Until now there are no scientific reports focusing tobacco brown-spot resistant gene linked to a molecular marker. Methods for the localization of molecular markers, such as Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), RAPD and Sequence Characterized Amplified Regions (SCAR), have been used extensively to localization genes of interest and in molecular-assisted plant breeding (Zhu et al., 2004). RAPD markers have certain advantage over

other methods because they are easy to generate and do not require the use of isotopes (Zhang et al., 2005) and have been used successfully to generate markers for tobacco (Bai et al., 1995; Yi et al., 1998). In this paper, the preliminary results in the study of identification of RAPD marker tightly linked to brown-spot resistant gene is presented.

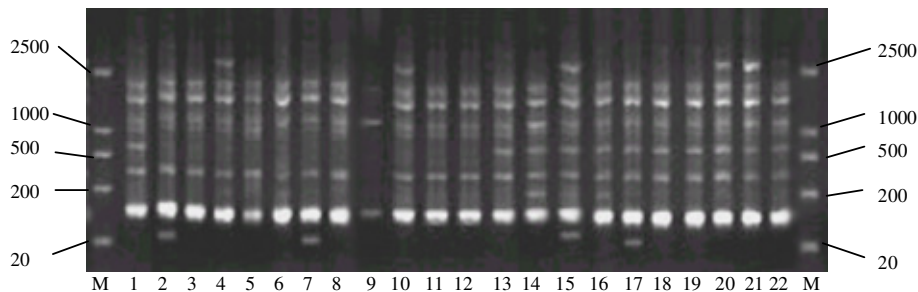
## MATERIALS AND METHODS

### Tobacco materials

The seeds of parent tobacco plants, 82-3041 and Yunyan 84 were kindly provided by South Center of China Tobacco Breeding Research (SCCTBR). 82-3041 is highly susceptible to *A. alternata*, while Yunyan 84 is highly resistance (Rei et al., 1997). F<sub>2</sub> progenies of 82-3041 and Yunyan 84 were used in the experiments.

The inoculation of tobacco leaves, belonging to F<sub>2</sub> progenies, was performed as described by Guo et al. (1997). The resistant grade was scored as described by Guo et al. (1997). Lesion area was measured 9 days after inoculation and a five-grade system of resistance was used to characterize the responses of plants in regard to *A. alternata* infection. Highly resistance (HR), resistance (R), moderate resistance (MR), susceptible (S) and highly susceptible (HS) respectively represented no lesion observed, lesion area occupying less than 5%, 5 - 10%, 11 - 25%, and more than 25% of inoculated leaves, respectively.

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**Figure 1.** Amplification products of S361 in  $F_2$  individuals. 1, Yunyan 84; 2, 82-3041; 3 to 12, different accession of susceptible  $F_2$  individuals; 13 to 22, different accession of resistant  $F_2$  individuals.

### DNA extraction and RAPD analysis

Tobacco genomic DNA was extracted from young leaves as stated by Zhang et al. (2006). RAPD analysis was performed as described by Zhang et al. (2005). 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 0.2 mmol/L dNTPs, 2.5  $\mu$ L 10  $\times$  reaction buffer, 2 mmol/L of  $MgCl_2$ , 1U of Taq DNA polymerase (Shanghai Sangon), 20 ng of each primer (Shanghai Sangon) 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 5 min. The amplifications added with 2  $\mu$ L loading buffer were electrophoresed in 1.0% agarose gel containing 0.5  $\mu$ g/mL ethidium bromide, and were visualized and photographed on an UV transilluminator.

### Linkage analysis

Recombination rate (cM) was calculated as follow: recombinator / (no. of individual of  $F_2$  generation $\times$ 2)  $\times$ 100%, in which 2 stands for diploid genome (Liu et al., 2002).

## RESULTS AND DISCUSSION

One thousand and forty-two  $F_2$  plants derived from 82-3041 and Yunyan 84 crosses were inoculated with *A. alternata*. Resistance or susceptible reaction of each individual plant was significant, making the phenotypic identification easier. Among the 1042  $F_2$  plants inoculated, 780 resistant plants and 262 susceptible plants were identified. Statistical analysis ( $\chi^2$ ) showed that distribution at 3:1 segregation ratio of the resistance trait was significant, indicating that the resistance of Yunyan 84 was controlled by one gene or by closely linked genes.

The BSA method was used to screen markers co-segregated with brown-spot resistant gene as described by Michelmore et al (1991). From the over 800 random primers screened, one of them, S361 (5'-CATTCGAGCC-3'), reproducibly produce polymorphism S361<sub>650</sub> (about 650 bp) among parents, the resistant and susceptible pools. This RAPD primer was further used to test the 20 individuals in the resistant and susceptible pools. A poly-

morphic fragment was obtained from plant Yunyan 84 and resistant plants, but no corresponding band was obtained from 82-3041 and susceptible plants (Figure 1). The results confirm primarily that brown-spot resistant gene is linked with the RAPD marker S361<sub>650</sub>.

The amplified products of S361 showed that 43 plants have no S361<sub>650</sub> in 780  $F_2$  resistant plants, but 19 plants have S361<sub>650</sub> in 262  $F_2$  susceptible plants, that is, there are 62 recombinants in 1042  $F_2$  plants. The genetic distance between S361<sub>650</sub> and brown-spot resistant gene was about 2.98 cM, calculated as described in materials and methods.

Using tightly linked molecular markers to target genes to screen progenies is one of the most effective methods to carry out artificial selection. Identification and utilization of new resistant genes are important for breeding new tobacco cultivars with enhanced resistance to *A. alternata*. Random amplified polymorphic DNA (RAPD) is one of the simplest and fastest molecular marking technologies (Gabriel et al., 2005; Zhang et al., 2008), and bulked segregator analysis (BSA) is also one of the effective methods in finding a linked marker of a gene (Michelmore et al., 1991). In recent years, many important quality trait genes of main crops were marked (Zhu et al., 2004). Research on molecular markers of brown-spot resistant gene has not yet been reported. This study described one RAPD marker tightly linked to brown-spot resistant gene using the RAPD and BSA technique.

In the study, the 3:1 ratio of segregation of resistance trait showed that the resistance of Yunyan 84 was controlled by one gene or by one group of closely linked genes. The RAPD maker S361<sub>650</sub> in the study can be regarded as a marker tightly linked to a quality-character, and can be used in molecular-assisted breeding procedures. Molecular-assisted breeding of brown-spot resistant tobacco plants can avoid most inoculation procedure of *A. alternata* to cut down on workload, can identify the resistance of plants in early stage of breeding, increasing selection veracity and efficiency, and can accelerate reasonable and rapid utilization of the resistant gene, shortening the breeding cycle.

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## REFERENCES

- Adachi Y, Tsuge T (1994). Coinfection by different isolates of *Alternaria alternata* in single black spot lesion of Japanese pear leaves. *Phytopathology*. 84: 447-451.
- Bai D, Reeleder R, Brandle JE (1995). Identification of two RAPD markers tightly linked with the *Nicotiana debneyi* for resistance to black root rot of tobacco. *Theor. Appl. Genet.* 91: 1184-1189.
- Gabriel S, Nir H, Galina S, Leonid K (2005). Diversity assessment of leaf phenology variation in *Quercus ithaburensis* Decne by RAPD. *Isr. J. Plant Sci.* 53: 75-78.
- Guo YF, Zhu X C, Kong FY, Shi JK, Wang N (1997). Comparing two origins of resistance to brown spot disease in tobacco. *Chinese Tobacco Sinica*. 4: 1-6.
- Liu GF, Lu GD, Zeng LR, Wang GL (2002). Two broad-spectrum blast resistance gene, *Pi9(t)* and *Pi2(t)*, are physically linked on rice chromosome 6. *Mol. Genet. Genom.* 267: 472-480.
- Michelmore RW, Paran I, Kesseli RV (1991). Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. *Proc. Natl. Acad. Sci. USA.* 88: 9828-9832.
- Rei YH, Xu ML, Huang XY (1997). Flue-cured tobacco. In: Rei YH (eds): Tobacco collection in the Yunnan province. Science and Technology publishing company of Yunnan, Yunnan. pp. 38-42.
- Yi YH, Ruffty RC, Wernsmann EA (1998). Identification of RAPD markers linked to the wildfire resistance gene of tobacco using bulked segregant analysis. *Tobacco Sci.* 42: 52-57.
- Zhang HY, Li FS, He LL, Zhong HQ, Yang QH, He SC (2008). Identification of Sugarcane interspecies hybrids with RAPDs. *Afr. J. Biotechnol.* 7(8): 1072-1074.
- Zhang HY, Liu X, He Z, Zheng CM (2005). Random amplified DNA polymorphism of *Nicotiana tabacum* L. cultivars. *Biologia Plantarum.* 49: 605-607.
- Zhang HY, Liu XZ, Li TS, Yang YM (2006). Genetic diversity among flue-cured tobacco (*Nicotiana tabacum* L.) revealed by amplified fragment length polymorphism. *Botanical Studies.* 47: 223-229.
- Zhu JQ, Zhang HY, Liu Z, Liao JH, Li SJ, Ye XY, Wang LH, Zhao J, Yang ZR (2004). Mapping the nuclear fertility restorer gene in rice with simple sequence length polymorphism (SSLP). *High Technol. Lett.* 10: 94-96.