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Full Length Research Paper

An AFLP marker linked to turnip mosaic virus resistance gene in pak-choi

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Pak-choi is one of the most important vegetable crops in China. Turnip mosaic virus (TuMV) is one of its main pathogen. Screening the molecular marker linked to the TuMV resistance gene is an efficient method to improve pak-choi breeding. In this paper, a dominant gene, *TuRBCH01*, has been mapped. 180 F_2 individuals were inoculated with TuMV-C5 and tested by direct ELISA. The 3:1 ratio of F_2 hybrids segregation proved a single dominant allele for TuMV resistance. Amplified fragment length polymorphism (AFLP) technique and bulked segregant analysis (BSA) method were used to study the F_2 population. An AFLP marker (EccMctt3) linked to TuMV resistance gene with 7.8cM map distance was identified.

Key words: Pak-choi, TuMV resistance, AFLP, BSA.

INTRODUCTION

Pak-choi (*Brassica rapa* L. ssp. *chinensis*, 2n = 20) originated in China and does not form a head with darker green leaves and pronounced white midrib. Pak-choi is one of the most important vegetable crops in China for the largest planting area and total yield (Cao et al., 2006), but outbreak and spread of virus are decreasing its yield and quality greatly. Turnip mosaic virus (TuMV) is the most important pathogen of pak-choi.

TuMV is a member of the genus *Potyvirus* (type species Potato virus Y) in the family *Potyviridae* and the only potyvirus known to infect brassicas (Walsh and Jenner, 2002). TuMV was first described in *Brassica rapa* in 1921 (Gardner and Kendrick, 1921; Schultz, 1921) and has been widely studied. It has been known that TuMV is difficult to control by chemicals, and the natural plant resistance is the most effective method to control it (Hughes et al., 2002). TuMV, however, has many difference isolates and every strain has specific resistance gene. Two strains of TuMV were used as material to distinguish in 1963 (Yoshii). Four strains, C1-4, and C5 strain was found from Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) in 1980 (Provvidenti, 1980) and 1985 (Green and Deng, 1985) respectively. Liu et al. (1990a, b) identified and screened 19 TuMV isolates from 10 regions of China and defined seven strains (Tu1-7) with Green's identification method (Green and Deng, 1985). According to Liu's reports, TuMV-Hu1 is a member of Tu2 (Chinese cabbage *chinensis* strain) and TuMV-C5, which was used as virus material in this paper. Many resistance genes against specific TuMV isolates in *Brassica napus* and *B. rapa* have been identified (Walsh et al. 2002). Most of them are dominant, such as *TuRB01* (Walsh et al., 1999), *TuRB03* (Hughes et al., 2003), *TuRB04-05* (Jenner et al., 2002), *ConTR01* (Rusholme et al., 2007).

Among which, *TuRB01* is a single dominant resistance gene and the first TuMV resistance gene in *Brassica*.

The molecular markers of TuMV resistance genes provide a powerful tool to facilitate the TuMV-resistant *Brassica* varieties breeding program through marker-assisted selection (MAS). Zhang et al. (2008) reported four QTLs controlling TuMV-C4 resistance in Chinese cabbage. Zhang et al. (2006) reported two EST-PCR-RFLP markers linked to TuMV-C3 resistance gene in Chinese cabbage. Han et al. (2004) reported a pair of recessive gene markers linked to TuMV-C5 resistance gene in Chinese cabbage. But no molecular marker linked to resistance to TuMV has been reported in pak-choi.

This paper describes one molecular marker linked to resistance to TuMV-C5 in pak-choi, using the bulked seg-

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regant analysis (BSA) approach and amplified fragment length polymorphism (AFLP) technique.

MATERIALS AND METHODS

Materials

Q048 (P_1) is used as the resistant parent and A168-5D (P_2) is used as the susceptible parent. The F_2 (180 individuals) population was constructed by the progeny of Q048×A168-5D. TuMV-Hu1, a strain of TuMV-C5, was used as inoculation virus. The materials were obtained from the Protected Horticultural Research Institute, Shanghai Academy of Agricultural Sciences. The seedlings of 10 P_1, 10 P_2, 10 F_1 and 180 F_2 individuals were grown in an insect-proof glasshouse at 20 ± 2, during the experiments.

Disease assays

TuMV-Hu1 was prepropagated before inoculation. At the two to three true-leaf stages, 10 P_1, 10 P_2, 10 F_1 and 180 F_2 individuals were mechanically inoculated TuMV-Hu1 as described by Suh et al. (1995). The resistance evaluation was done by visual observation and direct enzyme-linked immunosorbent assay (ELISA). ELISA analysis was carried out using AGDIA (Agdia Incorporated, Elkhart Indiana, USA) ELISA Kit protocol. The resistance was evaluated by visual observation at weekly intervals up to 4 weeks. Phenotypes were classified into four types as described by Cao et al. (1990) (0, Resistance with no detectable infection; 1, Few mosaic leaves; 2, Systemic mosaic infection; 3, Systemic infection with necrosis). At the end of the 4-week period, both inoculated and uninoculated leaves were identified by the direct ELISA(-, Negative reaction for ELISA test; +, Positive reaction for ELISA test). Data of resistant and susceptible phenotypes were analysed by chi-square.

DNA bulking and DNA extraction

The BSA approach was used to compare two pooled DNA samples of individuals (Michelmore et al., 1991). In order to perform BSA for identification of markers closely linked to the TuMV resistance gene, we selected 10 resistant and 10 extreme susceptible F_2 individuals to construct resistant bulk (RB) and susceptible bulk (SB), respectively. The young leaves were selected to extract genomic DNA using the CTAB method (Murray and Thompson, 1980; Rogers and Bendich, 1988).

Amplified fragment length polymorphism (AFLP) analysis

The AFLP procedure was performed as described by Vos et al. (1995) with minor modifications. The genomic DNA (250 ng) was digested using 0.24 U *EcoR*I and 0.24 U *Mse*I for 3 h at 37℃ in 13.5 µl. Two specific adaptors to *EcoR*I and *Mse*I were ligated by T_4-ligation enzyme for 12 h at 20℃ in 26 µl including 13.5 µl restriction products, 1×T_4-buffer, 0.5 U T_4 ligase, 2 µmol ATP, 2.5 pmol *EcoR*I adaptors and 25 pmol *Mse*I adaptor. Pre-amplification was performed with E00 and M00 primers each containing one selective nucleotide (*EcoR*I and *Mse*I +A,C,G or T) in a 20 µl reaction mixture consisting of 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 U *Taq* polymerase, 1×PCR buffer, 150 ng Eoo and Moo primer, 3µl restriction ligation mix. The cycling parameters were as following: 24 cycles of 30 s at 94℃, 30 s at 56℃ and 60 s at 72℃. Selective amplification was performed in a volume of 20 µl, including 6 µl 20 × diluted pre-amplification product, 120 ng E and M primer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs,1.5 U *Taq* polymerase and 1×PCR buffer. The cycling parameters were: 12 cycles of 30 s at 94℃, 30 s at 65 - 56℃ (with a 0.7℃ -decrease each cycle) and 60 s at 72℃, then 24 cycles of 30 s at 94℃, 30 s at 56℃ and 60 s at 72℃. A total of 240 primer combinations were used as the selective amplification primers and combined with 15 E primers containing 3 selective nucleotides CAC, CAG, CAT, CCA, AAC, AAG, ACA, ACC, ACG, ACT, AGA, AGC, AGG, AGT, ATC, and 16 M primers containing 3 selective bases ACT, AGA, AGG, ATC, ATT, CAA, CAC, CAG, CAT, CCA, CTA, CTC, CTG, CTT, GAC, TAC, respectively. All primers were synthesized by Shanghai Sangon Biotechnology Co., Ltd (Shanghai, China). The PCR reactions were performed in an ASTEC PC818A Thermal Cycler (Astec Co. Ltd., Japan). The selective amplification products were denatured for 5 min at 94℃ with a half volume of formamide-loading buffer and cooled at -20℃. The AFLP samples were run out on 6% polyacrylamide gels on a DYY-12 DNA Sequencer (Beijing LiuYi Instrument Factory, China) and dyed by the silver-staining protocol (Carlos et al., 1994).

Marker scoring and linkage analysis

Among the 240 AFLP primer combinations, the polymorphic primer pairs were identified between the two parents and the F_1 hybrid. For each polymorphic primer, the AFLP bands of P_1, P_2 and 180 F_2 individuals were treated as dominant markers and scored as either present (1) or absent (0). Clearly distinguishable bands ranging from 100bp to 1500 bp were used in the genetic and marker analysis. Linkage between DNA markers and the TuMV resistance locus was determined by analyzing the data using MAPMAKER version 3.0 software (Lander et al., 1987). All markers were positioned to linkage groups with a logarithm of odds (LOD) threshold of 4.0.

RESULTS

Genetic analysis of resistance

Phenotypes were classified into four types according to the level of disease (Table 1). Data of type 0 was resistant phenotype and data of type 1, 2, 3 was susceptible phenotype. The result evaluated by visual observation coincided with that by ELISA test. P1 and F1 were all resistance and P2 plants were highly susceptibility to TuMV-Hu1, respectively. Data of resistant and susceptible phenotypes of F_2 population (145 resistant and 35 susceptible) were analysed by chi-square test, which is very close to the expected segregation of 3:1 with significant ($\chi^2 = 2.96 < \chi^2_{0.05}$). F_2 hybrids segregated for TuMV resistance in a 3:1 ratio for goodness of fit the expected Mendelian model based on the action of a single dominant allele.

Bulked segregant analysis

According to the polymorphism between P_1, F_1 and P_2, 36 AFLP polymorphic primer pairs were selected in 240 primer combinations. RB and SB were combined with 10 F_2 resistant plants ($R_{1\text{-}10}$, type 0) and susceptible plants ($S_{1\text{-}10}$, type 3), respectively. The 36 AFLP polymorphic primer pairs were used to screen P_1, P_2, F_1, RB and SB. Only two primer pairs provided a clear polymorphism in P_1, F_1, RB and $R_{1\text{-}10}$, but absent from P_2, SB a nd $S_{1\text{-}10}$

Table 1. Resistance evaluation of P_1, P_2, F_1 and F_2 plants to TuMV-Hu1.

Plant (number tested)	P_1 (10)	P_2 (10)	F_1 (10)	F_2 (180)			
Level of disease	0	3	0	0	1	2	3
Number of each level plants by visual observation	10	10	10	145	6	11	18
ELISA test	-	+	-	-	+	+	+

0 = No detectable infection; 1 = Few mosaic leaves; 2 = Systemic mosaic infection;
3 = Systemic infection with necrosis.
- = Negative reaction for ELISA test; + = Positive reaction for ELISA test.

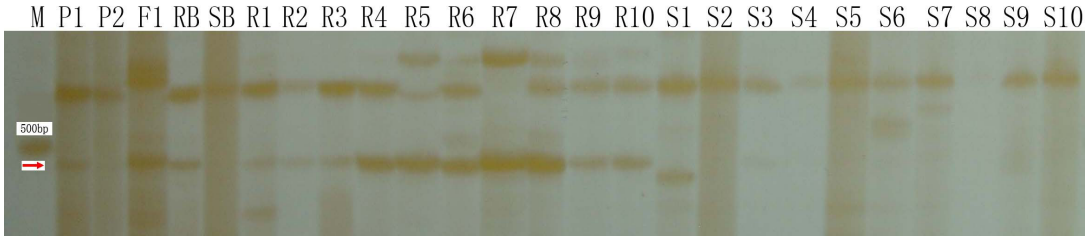

Figure 1. The polymorphism of *EcoR*I-ACC and *Mse*I-CTT at about 480bp. M, DNA marker (the unit is bp); P_1, resistant parent; P_2 susceptible parent. F_1 is the progeny of P_1 and P_2. RB, resistant bulk; SB, susceptible bulk; $R_{1\text{-}10}$, F_2 resistant plants; $S_{1\text{-}10}$, F_2 susceptible plants. The red arrow indicates the polymorphism band.

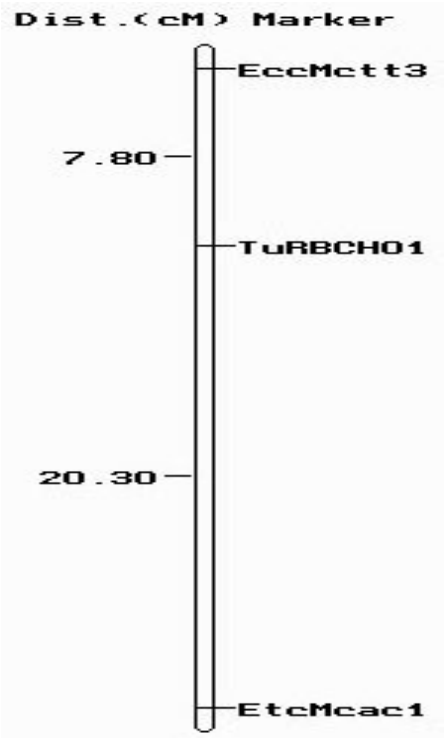

Figure 2. Map of two AFLP-defined loci EaccMctt3 and EatcMcac1 linked to the TuMV-Hu1 (a strain of TuMV-C5) resistance gene, *TuRBCH* 01 (TuMV RESISTANCE in *Brassica rapa* ssp. chinensis 01), in pak-choi. Map distances are in centiMorgans (cM) on the left-hand side of the linkage group.

(Figure 1). One primer pair (*EcoR*I-ACC, *Mse*I-CTT) amplified an about 480-bp band, which is 3rd polymorphism band, and was named EaccMctt3. The other primer pair (*EcoR*I-ATC, *Mse*I-CAC) amplified an about 900-bp band, which is 1st polymorphism band, and was named EatcMcac1.

Linkage map of resistance gene marker

Thirty-six AFLP polymorphic primer combinations were employed to amplify P_1, P_2 and 180 F_2 plants. All clearly distinguishable polymorphism bands ranging from 100bp to 1500bp were treated as dominant markers that P_1 was present (1) and P_2 was absent (0) and scored. A total of 42 dominant markers linked to the TuMV resistance locus were mapped in the same linkage group (data no shown). In the linkage group, the TuMV resistance locus (*TuRBCH01*) was positioned between AFLP markers EaccMctt3 (7.8cM) and EatcMcac1 (20.3 cM) (Figure 2). In the polymorphism band patterns of *EcoR*I-ACC/*Mse*I-CTT, the 3rd polymorphism band (EaccMctt3) included 132 presents and 48 absents. The ratio (132:48) was very close to the expected segregation of 3:1 with highly significant ($\chi^2=0.27<\chi^2_{0.05}$) to prove that TuMV-Hu1 resistance gene was a single dominant allele.

DISCUSSION

Visual observation, ELISA test and AFLP technique were all used to evaluate the resistance in pak-choi following

inoculation with TuMV-Hu1 (a strain of TuMV-C5) in this paper. Both genetic evaluation and marker analysis have shown that TuMV-C5 resistance gene in pak-choi (*Brassica rapa* ssp. *chinensis*) is a single dominant allele. TuMV resistance in rutabaga (*Brassica napus*) and in Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) was due to a single dominant gene (Shattuck and Stobbs, 1987) and two dominant genes (Niu et al., 1983; Leung and Williams, 1983), respectively. The number of resistance genes to TuMV strains C1, C2, C3, C4 and C5 in Chinese cabbage was reported in detail by Suh et al. (1995) and a single dominant gene was involved in TuMV-C5 resistance. But no report has related to the problem in pak-choi except Cao et al. in the last dozen years and it was stated that TuMV-C4 resistance in pak-choi was incomplete dominant (Cao et al., 1995), but maternal effect was significant. So the AFLP markers linked to TuMV-C5 resistance gene in pak-choi were firstly detected and we proposed that the resistance gene was named *TuRBCH 01* (*TuMV* resistance in *Brassica rapa* ssp. *chinensis* 01), the same nomenclature as *TuRB01-05* (Walsh et al., 1999; Jenner et al., 2002; Hughes et al., 2003).

Polymorphic AFLP loci are example of dominant markers (Bench and Åkesson, 2005) and allow high-resolution genotyping of DNA fingerprinting (Mueller and Wolfenbarger, 1999), the 3rd polymorphism band of *Eco*RI-ACC/*Mse*I-CTT, EaccMctt3, linked to *TuRBCH01* indicate that 131 AFLP locus individuals with a band (the presence allele) are either homozygous (1/1) or heterozygous (1/0) and 49 without the band are homozygous for the absence allele (0/0). So EaccMctt3 can be used as DNA fingerprint to identify whether pak-choi individuals are resistant or susceptible to TuMV-C5.

The BSA method provides a rapid and simple alternative technique to identify two AFLP markers (*Eco*RI-ACC/*Mse*I-CTT and *Eco*RI-ATC/*Mse*I-CAC) linked to specific TuMV-C5 resistance gene from 36 primer combinations. In linkage group, the two AFLP markers were positioned both sides of *TuRBCH01* and the map distances are 7.8 and 20.3 cM respectively. Other two maps previously include *TuRB01* (3.6 and 10.9 cM) and *TuRB03* (7.6 and 15.5 cM), but no map or definite distance of *TuRB04*, *TuRB05* and *ConTR01* etc.

The AFLP primer pair, *Eco*RI-ATC/*Mse*I-CAC, was employed to study the TuMV-CDN1 gene in *Brassica napus* by Hughes et al. (2003) which called EtcMcac1 and linked to *TuRB03* with 20.3 cM interval. *TuRBCH01* and *TuRB03* with the same primer pair and interval may be either the same resistance locus as *TuRB01* and *TuRB01b* (Walsh et al., 2002) or the members of a resistance gene cluster, which need further study.

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