

Full Length Research Paper

Inheritance and identification of SCAR marker linked to bacterial wilt-resistance in eggplant

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In the present work, the combinations (F_1) were crossed between highly resistant and susceptible to bacterial wilt eggplant parents and its F_2 , BC_1 segregation population plants were inoculated with race 1 of *Ralstonia solanacearum* in greenhouse. In this paper, we reported that the inheritance of bacterial wilt resistance in eggplant was controlled by a single dominant gene showing Mendelian inheritance model. In addition, a 762 bp molecular marker linked to a bacterial wilt-resistant gene of eggplant was screened by the bulked segregant analysis (BSA) method and sequence characterized amplified region (SCAR) marker linked to bacterial wilt-resistance gene was also obtained. The genetic distance between this marker and the resistance gene is 3.33 cM

Key word: Eggplant, bacterial wilt-resistance, molecular marker, inheritance.

INTRODUCTION

Ralstonia solanacearum is a soil borne bacterium that causes a serious disease known as bacterial wilt (BW) in hundreds of plant species, including many crops such as tomato, potato, tobacco, pepper, eggplant, banana, ginger, cowpea and peanut (Hayward, 1991). Wilting disease caused by this pathogen is an enormous risk for plants in tropical and temperate regions (Hayward, 1991). Because of the soil-borne nature of the pathogen, conventional management strategies of BW like crop rotation, adjusting the date of planting, cultural methods and soil treatment are not effective, especially for its broad host range. The most successful strategy is to breed resistant cultivars or graft plants using resistant rootstocks. BW also can be controlled by the application of fertilizers and cost-effective soil amendments, such as addition of compost, manure, urea, calcium oxide or solarization, to change soil pH and reduce survival and activity of plant pathogens (Gorissen et al., 2004). Applications of chemical pesticide are not only harmful to human health and the environment, but also not effective on BW controlling. Recently, biological control has been investigated, but which is still in its early development (Hayward, 1991).

Until recently, few resistant eggplant cultivars have

been developed, due to the lack of resistant resources.

Resistance sources have been identified in some wild relatives species, such as *Solanum torvum* and *Solanum aethiopicum* (Gousset et al., 2005), while their resistance is greatly affected by environmental factors and the race and strain diversity of the pathogen, which makes it very difficult to utilize these resistance sources in different countries. Studies on inheritance of resistance to BW caused by *R. solanacearum* in tomato are complex and different results have been obtained using different materials. The resistant genes had been defined as recessive (Singh, 1961; Acosta et al., 1964; Mohamed et al., 1997), incomplete dominance (Graham, 1976; Yue et al., 1995; Shou et al., 2006) and dominance (Scott et al., 1988; Grimault et al., 1995). Many resistant genes had been defined as monogenic (Scott et al., 1988; Gowha and Shivasbankara, 1990; Grimault et al., 1995; Li et al., 2001) or polygenic (Acosta et al., 1964; Gilbert et al., 1974; Thurston, 1976; Gonzalez and Summers, 1995; Yue et al., 1995; Osiru et al., 2001). Some researchers reported these genes had additive (Graham and Yap, 1976; Yue et al., 1995; Hanson et al., 1998; Balatero et al., 2000) and non-additive effect (Mohamed et al., 1997; Balatero et al., 2000). However, the inheritance of bacterial wilt-resistance in eggplant is still unclear.

The molecular marker technology have been used to assist breeding on many crops, except eggplant breeding for the genetic control of important traits in eggplant is still unclear (Kole, 2007). Sequence characterized amplified

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region (SCAR) markers are more reproducible and easier to manipulate in marker-assisted selection (MAS) programs than other markers. Due to the co-dominant or dominant nature, SCAR marker can provide a valid tool for the accurate assessment of genotype at the linked locus. Furthermore, it is useful to map whole F_2 populations without losing genetic information and it can discriminate between different alleles identifying homozygous and heterozygous plants in segregating populations. As a result, SCAR can be considered to be an ideal marker for plant breeding programs. For example, Nedim et al. (2008) screened two SCAR markers linked to *Fusarium* wilt resistance gene in eggplant. In this study, the inheritance of bacterial wilt resistance in a segregating population was analyzed, derived from a pair cross between bacterial wilt resistant and susceptible eggplant inbred lines. Moreover, a SCAR marker associated with the bacterial wilt resistance genes was identified using the bulked segregant analysis (BSA) with the RAPD markers. Finally, the usefulness of the molecular marker for MAS was evaluated.

MATERIALS AND METHODS

Plant materials

Two eggplant inbred line differing in their resistance to BW, 'E-31' (highly resistant, round fruit, from Huizhou local variety, Guangdong province) and 'E-32' (highly susceptible, round fruit) were from our laboratory and their offspring, including F_1 (population of the hybrid 'E-31' × 'E-32'), F_2 , BC_1R (the population formed by backcrossing with the resistant parent 'E-31'), and BC_1S (the population formed by backcrossing with the susceptible parent, 'E-32') were used in this experiment. All plant materials were planted in the field of South China Agricultural University, Guangzhou, China.

Inoculation with *R. solanacearum* strain in eggplant

Eggplant seeds were sown and seedlings were grown in a ratio of 2:1 mixture of turfy soil and perlite in a greenhouse, with a minimum temperature of 20°C and a maximum temperature of 25°C under natural light. At the two- or three-leaf stage, seedlings were grown in a phytotron maintained at (30 ± 2) / (25 ± 2) °C day/night (12 h day length), 90% relative humidity.

High virulent *R. solanacearum* strain (race1) was extracted from susceptible disease eggplant. A single colony of the virulent type was grown at 30°C for 48 h on TZC medium. The inoculum was cultured in liquid medium (casein hydrolysate 3 g, peptone 5 g, glucose 10 g pH 7.0) by shaking in a water bath at 30°C for 24 h. After incubation, the population in suspension was determined using a spectrophotometer and adjusted to 10^8 cfu ml⁻¹. At the five- or six-leaf stage, the seedlings were inoculated, the plant roots were wounded before inoculation and then the roots were put into the bacterial suspension prepared for 20 min and culture was then continued. After being inoculated for 15 days, the disease symptom was investigated. The susceptible plants died, but the resistant ones survived 1 month later after the inoculation with the bacterium.

Evaluation of bacterial wilt resistance in eggplant

Scale for evaluation of the symptoms of BW referred to the standard. (Winstead and Kelman, 1952) Disease severity was visually

evaluated and recorded on a scale of 0-4 for single plant (0 = healthy, 1 = one or two leaves wilted, 2 = three or more leaves wilted, 3 = all the leaves wilted, and 4 = dead). 0 - 2 grade for resistance, 3 - 4 grade for susceptibility.

Scale for evaluation of resistance to disease of plants by disease index (DI): resistant (R) $DI \leq 10$, moderately resistant (MR) $10 < DI \leq 20$, moderately susceptible (MS) $21 < DI \leq 40$ and highly susceptible (HS) $DI > 40$.

DNA extraction

Genomic DNA of each sample was extracted by CTAB methods (Doyle and Doyle, 1990).

Bulked segregant pool preparation and RAPD analysis

Bulked DNAs were prepared from equal volumes of standardized DNA of 10 resistant and 10 susceptible F_2 plants. 600 RAPD primers (from S81 to S680, Shanghai Sangon Corporation) were used to screen two parents, resistant and susceptible bulks. Primers that detected polymorphism among two parents, the resistant and the susceptible pooled DNAs, were then tested on the resistant and susceptible F_2 individual plants of 'E-32' × 'E-31', previously characterized for their resistant/susceptible phenotype.

PCR was performed in a 25 µL reaction mixture containing PCR buffer (10 ×) 2.5 µL, $MgCl_2$ (25 mmol L⁻¹) 1.5 µL, dNTP (10 mmol L⁻¹) 0.5 µL, primer 30 ng, DNA 20-50 ng, *Taq* polymerase 2.5 U; Reactions for the PCR were subjected to 95°C predenaturation for 5 min, 94°C 1 min, 36°C 1 min, 72°C 2 min, 35 cycles, 72°C 10 min; The PCR products were electrophoresed on 1.2% agarose gel.

SCAR primers design and PCR analysis

The special RAPD marker bands were purified, then cloned into a pGEM-T vector (Promega Corporation,) according to the manufacturer's instruction and sequenced. SCAR primers were designed based on the sequence of the cloned fragment. The sequence of primer were as follows: P₁ 5'-G ACTGCGTACC AATTCAGT T-3' and P₂ 5'-GATGAGTCCT GAGT AACACGATG-3'. Reactions for the PCR were subjected to cycle of 94°C for 5 min, 35 cycles each at 94°C for 30 s, 50°C for 45 s and 72°C for 1.5 min and then one cycle of 72°C for 10 min. The PCR products were electrophoresed on 1.0% agarose/ ethidium-bromide gel.

The SCAR primers designed were used to amplify DNA obtained from the 'E-31' and 'E-32' parental lines, as well as the F_2 , F_3 , BC_1R , BC_1S . Total 210 individual plants of F_2 , 159 resistant and 51 susceptible in F_2 . All 70 individual plants of BC_1R showed resistant and 37 out of 70 plants in BC_1S showed resistant, 33 showed susceptible. 115 out of 150 in F_3 plants showed resistant and 35 showed susceptible.

Data analysis

Data were analyzed by the χ^2 test to ascertain the goodness of fit between the expected ratio for the BW resistant genes and the segregation of the phenotypic data. Map units were computed by applying the Kosambi function (1944) with a LOD threshold of 3.0.

RESULTS

Inheritance of bacterial wilt-resistance of eggplant

The parents inbred lines 'E-31', 'E-32' resistant to the bac-

Table 1. Evaluation of the resistance to bacterial wilt in parents.

Parent	Identified by irrigating method			Assessment in field			Resistance evaluation
	Disease rating	Range	Disease index	Disease rating	Range	Disease index	
E-31	0.2	0 - 1	1.5	0.1	0 - 1	1.7	R
E-32	4.9	4 - 5	93.5	4.7	4 - 5	80.5	S

Table 2. The reaction to bacterial wilt in F₁, F₂ and BC₁ populations

Material	Generation	Number of resistant plants	Number of susceptible plants	Disease index	Ratio of R to S (R:S)	χ^2 $\chi^2_{0.05}=3.84$
E-32 × E-31	F ₁	150	0	2.3		
E-31 × E-32	F ₁	150	0	2.4		
E-31 × E-32	F ₂	109	41		3:1	0.32
(E31 × E32) × E-32	BC ₁ S	78	72		1:1	0.17
(E31×E32) × E-31	BC ₁ R	150	0		1:0	

terial wilt were tested by inoculating *R. solanacearum* strain (race1) in greenhouse and field and the results showed that 'E-31' exhibited high resistance to bacterial wilt disease, while 'E-32' was susceptible (Table 1).

About 150 plants of each material were inoculated with *R. solanacearum* (race1) in greenhouse (Table 2); all plants of F₁('E-32'×'E-31','E-31'×'E-32') and BC₁ with R parent as recurrent parent were resistant to *R. solanacearum* (race1). However, in the BC₁ with S parent as recurrent parent, 78 of 150 plants were classified as resistant plants, and 72 plants as susceptible plants. The observed segregation did not deviate significantly from the expected 1:1 Mendelian ratio for a monogenic trait ($\chi^2 = 0.17 < \chi^2_{0.05} = 3.84$). Out of 150 F₂ individuals, 109 plants showed resistant to the bacterial, 41 plants were susceptible. The resistant and susceptible plants were in the ratio of 3 to 1 ($\chi^2 = 0.32 < \chi^2_{0.05} = 3.84$). These results indicated that the resistance to bacterial wilt of 'E-31' is conferred by dominant mono-gene and cytoplasm had no effect on the resistance.

Screening the molecular marker linked to bacterial wilt-resistance of eggplant

Bulked segregant analysis (BSA) was used to screen RAPD markers linked to the bacterial wilt-resistant gene, about 119 F₂ segregation plants of 'E-32'×'E-31' were analyzed by RAPD. Firstly, the primers were detected polymorphism among two parents, the resistant and the susceptible pooled DNAs, 442 of 600 primers could be amplified bands in four pooled DNAs, but only 71 primers could be amplified different polymorphism bands between the resistant and the susceptible plants.

So these 71 primers were used to amplify 119 F₂ plants

to screen the marker linked to resistant to bacterial wilt. The primer S401 (GTTGGTGGCT) amplified a 762 bp special fragment linked to bacterial wilt-resistance (Figure 1). This fragment was present in 88 R individuals of F₂ segregation plants, but not in 27 S and 4 R individuals of F₂ segregation plants. The segregations fit a 3:1 ratio ($\chi^2 = 0.03 < \chi^2_{0.05} = 3.84$).

SCAR and linkage analysis

The fragment was cloned and sequenced, named *Rs*₋₇₆₂, it was a new sequence, had no similarity to any gene or EST in Genbank after blast (www.ncbi.nlm.nih.gov) and the sequence had been submitted to GenBank (GenBank accession No: EU547499).

Based on the sequence of the marker, two primer P₁/P₂ were designed to identify the 210 F₂ individuals, 70 BC₁R individuals, 70 BC₁S individuals and 150 F₃ individual plants (Table 3). The RAPD marker was successfully converted into SCAR marker. The SCAR marker was present in 153 bacterial wilt resistance plant and one susceptible plants of F₂, absent in 50 susceptible plants and 6 resistant plants of F₂ (Figure 2), present in all 70 BC₁R plants, present in 37 resistant plants of BC₁S, and absent in 33 susceptible plants of BC₁S. In order to confirm this marker stability, it was amplified in all 150 F₃ individual plants. The result showed that the marker was absent in 34 susceptible plant and 4 resistant plants of F₃, present in 111 out of 115 resistant plants and one susceptible plant of F₃ (Figure 3). Therefore, the marker could be stably inherited and is linked to bacterial wilt resistance in eggplant. The genetic distance between this marker and the resistance gene is 3.33 cM. It could be applied for marker assisted breeding of eggplant.

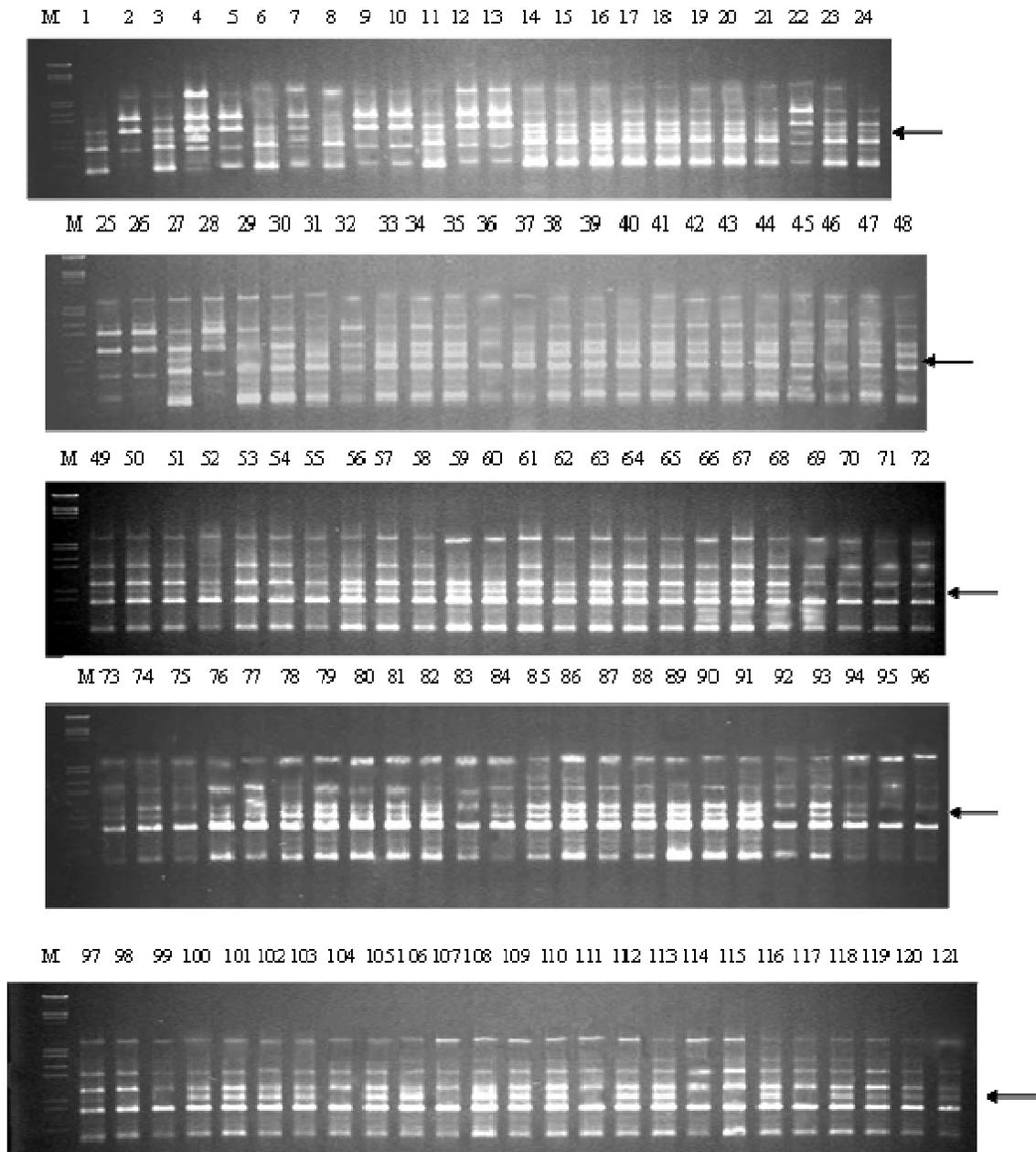


Figure 1. The PCR amplification of S401 in parents and F₂ segregation individual. Lane M: λ DNA (*EcoRI*+*HindIII*); lane 1: E-31; lane 2: E-32; lanes 3 - 121: the F₂ segregation individual plants. Arrow show the special fragment.

Table 3. The identification of the SCAR marker in F₁, F₂, F₃ and BC₁ populations.

Population	No. of total plants	Absent		Present	
		No. of resistant Plant	No. of susceptible plant	No. of resistant Plant	No. of susceptible plant
F ₂	210	6	50	153	1
BC ₁ R	70	0	0	70	0
BC ₁ S	70	0	33	37	0
F ₃	150	4	34	111	1

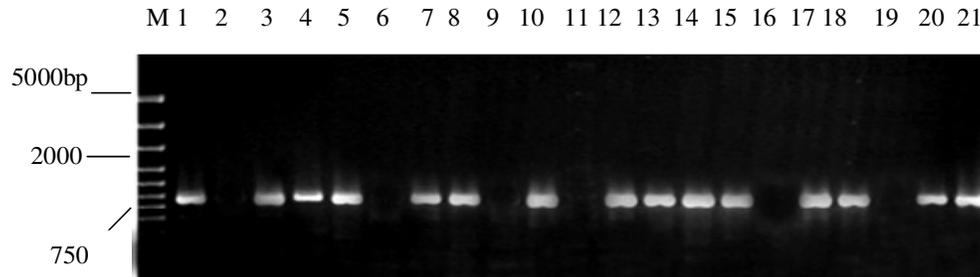


Figure 2. The identification of SCAR marker in F_2 population. Lane M: λ DNA (*EcoRI*+*HindIII*); lane 1: E-31(R); lane 2: E-32(S); lane 3: E-32×E-31 (R)); lanes 6, 9, 11, 16, and 19: susceptible plants of F_2 ; lanes 4, 5, 7, 8, 10, 12-15, 17, 18, 20, and 21: resistant plants of F_2 .

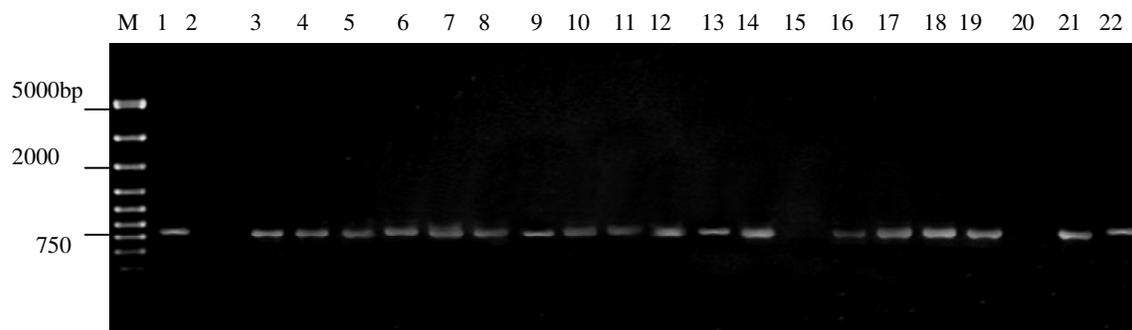


Figure 3. The identification of SCAR marker in resistant plants of F_3 . Lane M: λ DNA (*EcoRI*+*HindIII*); lane 1: E-31(R); lane 2: E-32(S); lanes 3 - 22: resistant plants of F_3 .

DISCUSSION

The inheritance of resistance to BW caused by *R. Solanacearum* in eggplant is complex and different results have been obtained using different materials. Various degrees of varietal resistance have been reported (Messiaen, 1975; Mochizuki and Yamakawa, 1979b; Che et al., 1997). Depending on the varieties used, resistance to *R. Solanacearum* is controlled by one dominant gene (Chadha, 1993; Chaudhary, 2000; Zhu et al., 2004; Yang et al., 2006; Ajjappalavara et al., 2008) or one recessive gene (Chaudhary, 2000; Gopalakrishnan et al., 2005; Li et al., 2006; Tian et al., 2007; Sun et al., 2008), or dominant polygene (Chaudhary, 2000; Li et al., 2002) or recessive polygene (Feng et al., 2003). The result of our work was similar to the studies of Swaminathan and Srinivasan (1972), Gopinath and Madalageri (1986), Zhu et al. (2004), Yang et al. (2006) and Ajjappalavara et al. (2008), which also mentioned single gene inheritance for bacterial wilt resistance. In the present work, it was observed that the cytoplasmic factors could not interfere with the bacterial wilt, the resistance was only related to nuclear gene, which was contrary with the result of Gousset et al. (2004), which found that bacterial wilt resistance could be interfered by cytoplasmic factors. These results indicated that several mechanisms of resistance were probably

available within eggplant germplasm and the resistance mechanism in eggplant appeared to be similar with tomato, which was the limitation of the spread of the pathogen within the stem: the more resistant a plant, the lower the stem colonization (Grimault et al., 1994; Gopalakrishnan et al., 2005). It was observed that in resistant cultivars particular features of the root cortical cells (small and tightly packed), as well as high phenolic content in the roots could both prevent the entry and further multiplication and spread of the bacteria.

Up till now, many molecular markers related to resistant BW have been reported, such as the RAPD marker located at 4.33 cM from the monogenic dominant resistance gene (Zhu et al., 2005), two AFLP markers related to monogenic recessive resistance (Li M et al., 2006; Sun et al., 2008) or one 400 bp RAPD marker related to polygenic dominant resistance (Li et al., 2002), but the function of these resistant markers were still not identified. In the present study, the resistant SCAR marker obtained had been identified, it may be a functional marker linked to the resistant gene and a useful tool for MAS in eggplant breeding in the future.

At the same time, many BW resistant loci and molecular markers have been found in tomato plants. Using F_2 and F_3 population derived from 'L285' (resistant) × 'CLN286' (susceptible), Danesh and Young (1994) found 3 QTLs.

Thoquet et al. (1996) found 7 QTLs using the F₂ and F₃ population derived from *Lycopersicon esculentum* cv. 'Hawaii 7996' (partially resistant) × *Lycopersicon pimpinellifolium* 'Wva700' (susceptible), while Carmeille et al. (2006) found 4 QTLs with a F_{2:3} and a population of inbred lines (RIL). Balatero et al. (2002) found 80 molecular markers, including 72 AFLP markers, 7 resistance gene analogs, 1 SSR marker, during the construction of a tomato linkage map of 12 linkage groups. Yui et al. (1999) found 4 RAPD markers, in which RA₁₂₋₁₃ and RA₁₂₋₂₉ had close linkage to the resistant gene. Most of the loci and markers are on the chromosome 6; the other chromosomes such as chromosome 4, 7, 8, 10, 11 and 12 also contribute to the control of the BW resistance (Mangin et al., 1999; Wang et al., 2000). These reports may be useful to explore bacterial wilt resistant marker in eggplant. The recent structure of the genetic diversity of *R. Solanacearum* into several distinct phylotypes (Prior and Fegan, 2005; Fegan and Prior, 2005) will contribute to better control of the interactions between the resistances and the bacterial strains used in breeding programs and hence to better understanding of the genetic control of the resistance(s).

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