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RAPD Markers Linked to Late Blight Resistance in Tomato

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Abstract

Identification of marker is the prerequisite for marker assisted selection (MAS) and is also very effective for gene pyramiding. Bulked Segregant Analysis (BSA) technique was used to identify RAPD markers linked to the late blight disease (caused by *Phytopthora infestans* (Mont. de Bary) resistance in tomato using F_2 population generated by crossing tomato inbred lines, NC 85L-1W(2007) which is resistant to late blight and NC 839-2(2007)-1 which is susceptible to it. A total of 250 F_2 plants, and 10 plants each of the parents and F_1 were used for BSA. Transgressive segregation was observed for late blight resistance. The segregation of susceptible and resistance perfectly fit the expected ratio of 3:1, that means resistance is governed by single recessive gene. Only 34 RAPD primers (17.26%) were found polymorphic between parents. Sixteen RAPD primers (47%) out of 34 gave polymorphic bands between resistant and susceptible bulks of the late blight. Four RAPD primers, namely MRTOMR-026, MRTOMR-031, MRTOMR-038 and MRTOMR-046 were identified as linked markers to loci related to late blight disease. Among those, two were linked to susceptible and two to resistance. Because of low reproducibility and dominant nature of RAPD, these markers need to be converted to SCAR markers.

Key words: bulked segregant analysis, resistant bulk, susceptible bulk, *Solanum lycopersicum*

Introduction

Tomato (*Solanum lycopersicum* L., 2n=2x=24) is a tropical vegetable and used both as fresh and processed form. It is the second most consumed vegetables after potato in the world (FAOSTAT 2010). Breeding efforts of tomato have been focused mainly on fruit yield and foliar diseases. The most important foliar disease of tomato is the late blight (Panthee & Chen 2010) caused by *Phytopthora infestans* (Mont.) de Bary, which can destroy tomato field within few days of infestation (Fry & Goodwin 1997) in conducive environment for the pathogen development. The most conducive environment for the disease development is wet and cloudy weather with cool temperature. Airborne nature of the pathogen helps to spread out in a larger area quickly. Late blight is the eighth most serious diseases of tomato in terms of crop loss per acre in USA (Davis *et al.* 2000). Advances have been made on resistance breeding to combat this disease in tomato (Foolad *et al.* 2008). Both vertical and horizontal resistances have been reported in tomato. Single dominant resistance gene (R) to late blight is commonly found in wild species. Four race specific R genes, *Ph-1*, *Ph-2*, *Ph-3* and *Ph-5* conferring resistance to it have been identified in *Solanum pimpinellifolium. Ph-1* is a completely dominant gene, identified in *S. pimpinellifolium* and mapped to the distal end of the chromosome 7 (Foolad *et al.* 2008). LA-3707, a selection of *S. pimpinellifolium* from Asian Vegetable Research and Development Center (AVRDC), contributed the *Ph-3* gene, and Richter's Wild Tomato was the source of a partially dominant gene, *Ph-2* (Gardner & Panthee 2010b). *Ph-2* gene was mapped in the chromosome 10. The *Ph-3*, another partially dominant gene is more

durable than *Ph-1* and *Ph-2*. The newly identified R gene, *Ph-5* was found superior to all the other R genes for late blight (Foolad *et al.* 2008). Quantitative trait loci (QTLs) have also been identified from the population developed by crossing cultivated tomato with wild tomatoes (Brouwer *et al.* 2004).

A few resistant varieties of tomato have been developed through the introgression of resistant genes either from cultivated or from wild species of tomato (Panthee & Gardner 2010, Gardner & Panthee 2010a). However, these varieties may not be adaptable to all tomato growing regions and the resistance may not be long lasting. Therefore, research towards the development of new resistant varieties is being continuously undertaken. More than eight years is required to develop a resistant variety through conventional breeding i.e. by using phenotypic selection. Molecular markers have now become a very useful tool for breeders to select the desirable genotypes. Molecular markers are useful for accelerating the breeding works more precisely with efficient selection. Markers linked to the gene(s) of interest, help to select plants genotypically that are genetically similar to the recurrent parents possessing the desired traits. However, due to the unavailability of PCR-based molecular markers tightly linked to late blight resistance, marker assisted selection (MAS) has not been in routine in late blight resistance breeding. Identification of linked marker(s) is the prerequisite for MAS and is also very effective for gene pyramiding.

Earlier, use of near isogenic lines (NIL) was the common means of identifying genes of interest. NIL are not available for most of the target regions and it takes long time to develop NILs. Alternatively, Michelmore *et al.* (1991) developed a very rapid and simple PCR based method to identify the gene of interest called BSA and identified RAPD markers linked to a disease resistant genes in lettuce. For BSA, any kind of mapping populations e.g. Recombinant Inbred Lines (RIL), Backcross (BC), F_2 or Double Haploid (DH) that are segregating for a trait of interest can be used. For dominant marker, e.g. RAPD, the F_2 population is considered best, because of the double number of segregating loci in F_2 than in the BC (Mackay & Caligari 2000).

In BSA, two extreme phenotypes, i.e. low and high of a particular trait (e.g. resistance and susceptible) from a segregating population are compared using bulk DNA from these two contrasting individuals. DNA from individuals similar in trait of interest are bulked and assumed that the bulks are homozygous for the targeted loci and heterozygous for the rest of the loci (Giovannoni *et al.* 1991, Michelmore *et al.* 1991, Quarrie *et al.* 1999). DNA markers are then used to screen the parents and the bulks. If polymorphism is found between bulks, this marker (polymorphic band) is expected to be associated with gene of interest.

Many disease resistance genes have been identified in tomato using random amplified polymorphic DNA (RAPD) following the BSA approach (Stevens *et al.* 1995, Chagué *et al.* 1996, De Giovanni *et al.* 2004). Once it is identified, this marker is then converted into co-dominant markers, such as cleaved amplified polymorphic sequence (CAPS), sequence characterized amplified region (SCAR) which are more reliable than RAPDs. Chunwongse *et al.* (2002) identified the Amplified Fragment Length Polymorphism (AFLP) markers linked to *Ph-3* using BSA.

In this study, BSA technique was used to identify RAPD markers linked to late blight resistance in tomato using F_2 population. An F_2 population is the earliest segregating generation available for mapping genes from the crosses and provides the greatest genetic window around the locus (Michelmore *et al.* 1991). R gene was tagged based on the segregating population that were naturally infested with late blight.

Methodology

Plant materials

Two tomato inbred lines, NC 85L-1W(2007) (referred to hereafter NC 85L) and NC 839-2(2007)-1 (referred to hereafter NC 839) were crossed to produce F_1 population in greenhouse. NC 85L, used as a female is resistant to late blight (resistant parent, RP) and NC 839, used as male is susceptible to late blight (susceptible parent, SP). The F_1 plants were selfed to obtain F_2 seeds. A total of 250 F_2 plants, and 10 plants each of RP, SP and F_1 plants were grown. Nine F_2 plants were blind (deformed plants), therefore 241 F_2 plants were used. The fruit of NC 85L is mini roma type with dark red fruit color (Table 1). The resistance source of late blight in NC 85L traces back to the L3707 and Ritcher's wild tomato (*S. pimpinellifolium*).

Table 1. Parental description along with their pedigrees and coefficient of parentage

Parent	Maturity	Fruit type, shape, color	Disease reaction	Pedigree	Common pedigree	COP
NC 85L-1W(2007)	Early	Mini roma type, dark red	Late blight resistance	051(x)-18//0463/9722(x)-18	0179(x)-1-18-4, 215E-1(93), 9722(x)-18, 051, 03220, L3707	0.227
NC 839-2(2007)-1	Average	Grape type, light red	Septoria leaf spot resistance	051(x)-18//CB25(x)-18-3/9722(x)-18/0464		

COP = Coefficient of Parentage

Field evaluation

A total of 280 plants, consisting of 10 plants each of RP, SP, and F_1 plants and 250 F_2 plants were planted in the research plot of Mountain Horticultural Crops Research and Extension Center (MHCREC), Mills River, USA during summer, 2009. This research plot was a hot-spot for late blight and natural inocula were observed at high pressure for screening late blight segregating population in the summer of 2009. Weather condition in 2009 was favorable for disease development (Figure 1). All these plants were evaluated for resistance to late blight with natural inocula. Experimental site was in Mills River, Henderson, North Carolina, located at an altitude of 630 m above sea level with latitude of 35.42721° N and longitude of 82.55888° W.

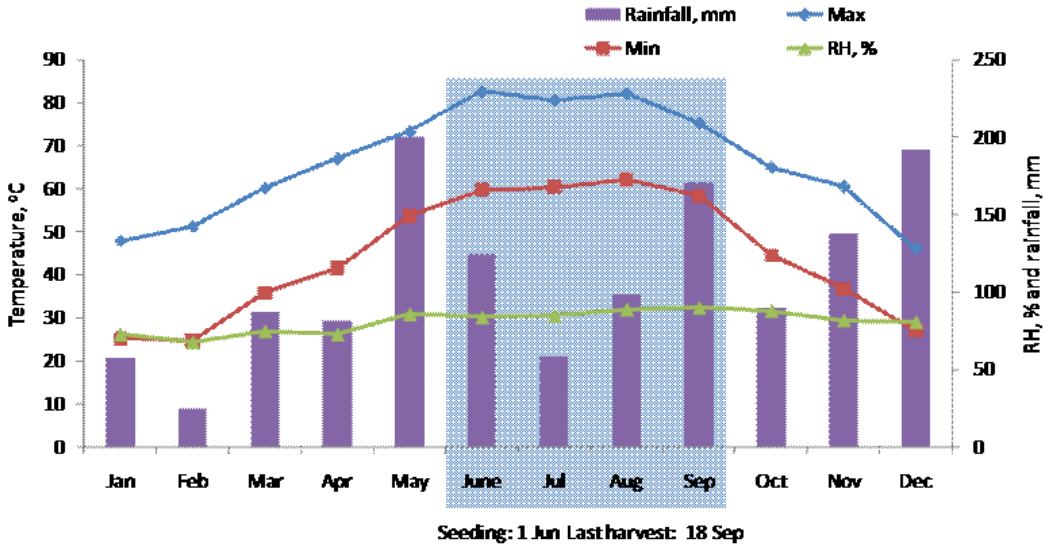

Fig. 1. Monthly weather conditions during tomato growing period (shaded area) in Mills River, North Carolina, 2009. Min = Minimum temperature (°C), max= Maximum temperature (°C), RH= Relative humidity (%)

Initially, seeding was performed on June 1, 2009 in trays (30.5 × 45.7) cm containing peat moss and vermiculite. The trays were then kept in glasshouse with temperature set at 21.1°C. Twelve days old seedlings were transplanted in a 50-cell tray (12.5 x 24.4 cm). Six-week-old seedlings were then transplanted in the field having loam type soil with row to row distance of 1.5 m and plant to plant distance of 45 cm. The bed was raised and covered with black plastic. Other recommended cultural practices were followed as described in Vegetable Crop Handbook, Southern US 2010. Fruits were harvested from September 9 to 18, 2009.

DNA extraction, quantification and dilution

DNA was extracted from all the individual F_2 plants, parents and F_1 following the method of Fulton *et al.* (1995). Approximately 100 mg of young leaves from 2-3 week old tomato seedlings was collected from greenhouse in 1.5 ml Eppendorf tube. This tube was then dipped into liquid nitrogen and samples were ground by glass rod. After adding 200 µl microprep buffer (Fulton *et al.* 1995), samples were incubated at 65°C water bath for about 60 min and chloroform/ isoamyl (24:1) solution was added (about 600 µl) to each tube. Samples were then centrifuged at 10,621 g for 5 min. Aqueous phase was pipetted off into new

micro-centrifuge tube and 2/3rd times the volume of cold isopropanol was added to precipitate DNA. Samples were finally centrifuged at 10,621 g for 5 min and the obtained DNA pellets were washed with 70% ethanol. Dry DNA pellets were resuspended in 1x TE buffer (100 µl, Fisher) and stored at -20°C. Concentration of DNA in different samples was estimated by spectrophotometer (NANO Drop 1000, Thermo Scientific, USA). Working solutions of DNA samples with a concentration of 20 ng/ µl were prepared from the original DNA samples in 1x TE buffer.

RAPD

A total of 197 10-mer RAPD primers were screened using 20 ng DNA template of two parental lines. Primers polymorphic to parental lines were then used to screen resistant and susceptible bulks. Amplification reactions were performed in 10 µl reaction volume containing 1x PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, Fisher), 200 µM each of dNTPs, 0.2 µM primer and 1 U Taq polymerase. About 15 µl mineral oil was overlaid on the reaction mixture in each tube. DNA amplifications were performed in thermal cycler (Eppendorf, New York) using the following cycling condition: one cycle of 92°C for 3 min, 45 cycles of 92°C for 60 sec, 42°C for 1 min and 72°C for 60 sec; one cycle of 72°C for 8 min followed by holding at 4°C.

Bulked segergant analysis

Bulked segregant analysis was done following the method of Michelmore *et al.* (1991). Two DNA bulks, called resistant bulk (RB) and susceptible bulk (SB) were prepared from F_2 individuals. RB consisted of 8 individuals with disease score of 0 and SB consisted of 8 individuals with the score of 4.5 and 5. DNA was extracted separately from each individual of F_2 population. Later, DNA bulk was prepared by pooling equal volume (50 µl) of DNA of each of eight resistant and susceptible F_2 plants for RAPD analysis. PCR was performed with polymorphic primer for the bulks and parental DNA samples using the same reaction condition as described above.

Gel electrophoresis

All PCR products were analyzed in 2% Agarose gel which was mixed with 1.86 µl of ethidium bromide solution (Fisher) of 10 mg/ml concentration in 1x TBE buffer (40 mM Tris-borate, pH 8.0, 1 mM EDTA) with a 100-bp ladder (Fisher). Electrophoresis was performed at 135 V for 2 hours. Gels were rinsed with water to enhance contrast and photographed under UV light on GelDoc system.

Data scoring and analysis

Disease severity was scored on 85 day-old plants. Individual disease rating scores were based on visual assessment of symptom severity. Following scoring criteria was developed based on Winstead and Kelman (1952), Tu and Poysa (1990), Danesh *et al.* (1994) and used in this study:

0 = no disease symptoms; 0.5 = Less than 10% leaf area with symptoms; 1 = 10-20% leaf area with symptoms; 1.5 = 20-30% leaf area with symptoms; 2 = 30-40% leaf area with symptoms; 2.5 = 40-50% leaf area with symptoms; 3 = 50-60% leaf area with symptoms; 3.5 = 60-70% leaf area with symptoms; 4 = 70-80% leaf area with symptoms; 4.5 = 80-90% leaf area with symptoms; 5 = 90-100% leaf area with symptoms.

Categories from 0 to 1.5 were considered resistant, from 1.5 to 3 as moderately resistant and from 3 to 5 as susceptible. For inheritance study, all these segregating plants were grouped into two, one resistant group with score from 0 to 2 and susceptible group with score from 2 to 5. Scores of parental lines and F_1 were averaged. Frequency of F_2 populations under different score categories was estimated for segregation analysis using SPSS v.17.0 (IBM Corporation, New York, USA). Skewness was estimated using SPSS v.17.0. Frequency data were analyzed by the χ^2 to test the goodness of fit at an expected ratio of 1 resistance and 3 susceptible lines using SAS v.9.1.

The pedigrees of these two lines were traced back to estimate the coefficient of parentage (COP), which was estimated between parents based on the assumption that all ancestors and parental lines were homozygous and a line derived from a cross obtained one-half of its genes from each parent. The computer software KIN (Tinker & Mather 1993) was used to calculate the COP. RAPD fragments were scored as 1 for presence and 0 for absence. Band size was estimated based on the 100-bp ladder

(Bioline USA Inc, MA, USA). Simple statistics based on the DNA bands were calculated using MS Excel 2007.

Results and Discussion

Disease reaction

Frequency distribution of late blight resistance among tomato plants in F_2 population is given in Figure 2. Distribution was slightly right skewed with -0.71 skewness. Disease scores of some F_2 individuals were either higher than susceptible parent or lower than resistant parent. Average score of resistant parent was 1.1 and of susceptible parent was 4.2. Transgressive segregation was observed towards resistance as well as susceptibility. This indicated that resistant parent also had some role in susceptibility and susceptible parent contributed towards resistance.

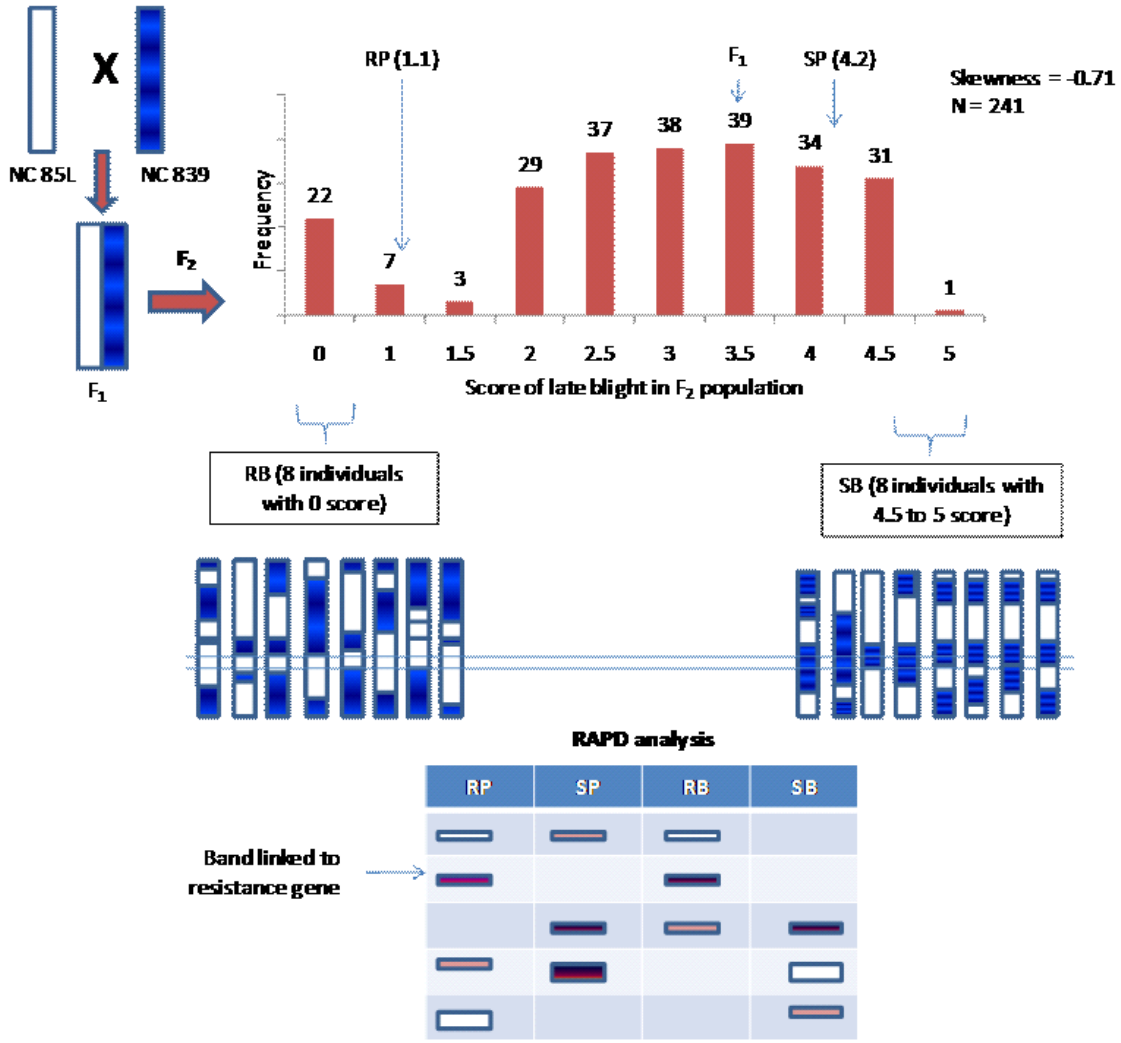

Fig. 2. Frequency distribution of 241 F_2 individuals of NC 85L-1W(2007) x NC 839-2(2007) based on the score of infestation of late blight in tomato, Mills River, 2009 and bulked segregant analysis method adopted in this study and schematic representation of RAPD band linked to resistance gene. The average phenotypic values of the parents and F_1 are shown by arrow. RP = late blight resistance parent, NC 85L-1W(2007). SP = late blight susceptible parent, NC 839-2(2007). RB = Resistant Bulk. SB = Susceptible Bulk

The average score of F_1 was 3.5, which indicated that the resistance was controlled by incomplete dominance. Some of F_1 individuals were affected as severely as the susceptible parents. The total resistant (with scale from 0 to 2) F_2 plants were 61 and susceptible (with scale from 2 to 5) 180. The segregation of resistance and susceptibility perfectly fitted 1:3 ratio ($\chi^2 = 0.0124$, P-value = 0.911). The distribution of resistant plants supported that the resistance to late blight pathogen in the present study was governed by single recessive gene.

Distinction of other traits between parents

Two parents were contrasting phenotypically for fruit type and color (Table 1). Fruit quality and shape of NC 839 were superior to NC 85L. Pedigree analysis of these parents showed that, six parents were common. The coefficient of parentage between them was 0.23 indicating some dissimilarity between these lines. Although variation was found between these lines for morphology and pedigree, polymorphic SSR markers could not be found. Parents were screened by 157 SSR, two COS and 23 M-13 tail SSR primers. Most of them showed monomorphic banding pattern and some of them did not amplify the genomic DNA of these parents. Three systems of fragment analyses, agarose gel, polyacrylamide gel and capillary gel electrophoresis were attempted, which could not detect any polymorphism indicating similarity between these parents for SSR loci.

RAPD analysis

Out of 197 RAPD primers used to screen parental lines, 34 RAPD primers (17.26%) were found polymorphic (Table 2). A total of 176 bands with maximum band size of 1500 bp and minimum band size of 100 bp were amplified using 34 primers. Among these bands, 84 were found polymorphic between parents. Average number of bands per polymorphic RAPD primers was 4, ranging from 2 to 8 bands. The number of polymorphic bands ranged from 1 to 4 with an average of 2. All these 34 polymorphic RAPD primers were used to screen the resistant and susceptible bulks.

Table 2. RAPD primers polymorphic between resistant and susceptible parents of NC 08135 F_2 population of tomato screened for late blight resistance

Primer	Sequence	% GC	Amplified bands	Band size, bp		Polymorphic band	% polymorphic	Tm
				Max	Min			
MRTOMR-001	TCGAGCACT	50	2	450	400	1	50	30
MRTOMR-003	AGGGGCTGC	70	5	600	150	1	20	34
MRTOMR-014	CGTCTCCAG	70	7	600	150	3	43	34
MRTOMR-022	AGGGCCAGC	80	8	1000	100	6	75	36
MRTOMR-023	GACCACGAA	60	3	650	100	2	67	32
MRTOMR-026	AGGCACCGT	60	5	1000	300	3	60	32
MRTOMR-027	CCTGATGCA	50	3	650	200	3	100	30
MRTOMR-029	GCCATACGG	70	2	900	650	2	100	34
MRTOMR-031	GGACGTCGC	80	5	600	200	2	40	36
MRTOMR-033	GCTCGCGGC	90	5	1000	150	2	40	38
MRTOMR-038	TACCTTCGCC	60	6	1100	150	3	50	32
MRTOMR-039	CAGCACCACC	70	7	650	100	3	43	34
MRTOMR-040	CAGTCCGCG	70	3	500	180	1	33	34
MRTOMR-046	CCATGCGCTA	60	5	600	100	4	80	32
MRTOMR-063	CGAGTGACC	60	6	550	100	2	33	32
MRTOMR-078	TGCCATGTG	60	4	700	200	1	25	32
MRTOMR-100	GACGGCCCCA	80	7	1500	200	3	43	45
MRTOMR-110	ATGACGACCT	50	3	800	400	1	33	31
MRTOMR-112	CATACACCTC	50	4	1500	650	2	50	25

MRTOMR-117	CCGAACAATC	50	6	950	300	5	83	28
MRTOMR-118	TGCTTGGGGG	70	4	1500	500	3	75	39
MRTOMR-121	GGCGTCGTAA	60	3	1100	550	2	67	36
MRTOMR-128	AGACCCGGTC	70	3	600	300	1	33	38
MRTOMR-130	AGGTCTCTCG	60	3	700	250	1	33	32
MRTOMR-133	TTCAGCCACA	50	3	900	300	2	67	32
MRTOMR-140	TGCCAACGCC	70	5	800	250	1	20	42
MRTOMR-141	CATTGGTGCT	50	2	900	450	1	50	31
MRTOMR-142	TTGCGCTTGT	50	2	800	400	1	50	37
MRTOMR-146	CGTTACCGGG	70	5	2000	450	2	40	35
MRTOMR-161	TGTCTCCCTG	60	5	1500	700	1	20	32
MRTOMR-171	GAGGCCAGCG	80	4	700	200	1	25	43
MRTOMR-178	GCCATCCGAA	60	2	800	650	1	50	35
MRTOMR-181	CATGCGCTCC	70	3	800	400	1	33	39
OPK6	CACCTTTCCC	60	4	1000	600	1	25	31
P#72	GAGCACGGGA	70	4	600	250	1	25	39

bp = base pair. Tm = Melting temperature

Table 3. RAPD markers polymorphic between resistance and susceptible bulks of tomato to late blight

Primer	Amplified bands	Size, bp		Polymorphic		% Polymorphic	
		Max	Min	Between parents	Between bulks	Between parents	Between bulks
MRTOMR-014	7	1500	300	3	2	43	29
MRTOMR-022	7	1200	300	1	0	14	0
MRTOMR-026	7	1500	250	1	2	14	29
MRTOMR-031	7	2000	450	3	1	43	14
MRTOMR-038	4	1500	300	2	1	50	25
MRTOMR-040	10	1600	280	4	5	40	50
MRTOMR-046	9	2000	100	6	5	67	56
MRTOMR-050	4	1000	400	1	0	25	0
MRTOMR-063	11	1500	150	2	1	18	9
MRTOMR-076	6	1500	350	1	2	17	33
MRTOMR-110	6	1200	180	1	0	17	0
MRTOMR-112	5	1200	300	1	0	20	0
MRTOMR-121	6	100	380	2	0	33	0
MRTOMR-130	4	750	200	1	0	25	0
MRTOMR-146	9	1500	250	4	4	44	44
MRTOMR-147	3	900	500	2	0	67	0

RAPD markers and bulked segregant analysis

Sixteen RAPD primers (47%) out of 34 were polymorphic between resistant and susceptible bulks of late blight (Table 3). A total of 105 RAPD bands were observed among four DNA samples from two parents and two bulks. Total polymorphic bands between parents (RP

and SP) and between bulks (RB and SB) were 35 and 23 respectively. On an average, each primer amplified 7 loci. Average polymorphic bands per primer for parental lines was 2 and for bulks 1. The largest band size was of 2000 bp and smallest was of 100 bp. The polymorphism shown by MRTOMR-046 was the largest (Table 2). The bands generated by MRTOMR-147 were mostly polymorphic between parents but none of these bands could distinguish the bulks. Four RAPD primers, namely MRTOMR-026, MRTOMR-031, MRTOMR-038 and MRTOMR-046 were identified as linked markers to loci related to disease reaction (Table 4). Among those, two were linked to susceptibility (Figure 3) and two to resistance (Figure 4). The bands size of 1100 bp amplified by MRTOMR-026 and 800 bp amplified by MRTOMR-046 were found only in susceptible parent i.e. NC 839 and susceptible bulk (SB). Other two primers, MRTOMR-031 and MRTOMR-038 produced 550 bp and 1100 bp sized bands respectively that were present only in resistant parent (RP) i.e. NC 085L and resistant bulk (RB).

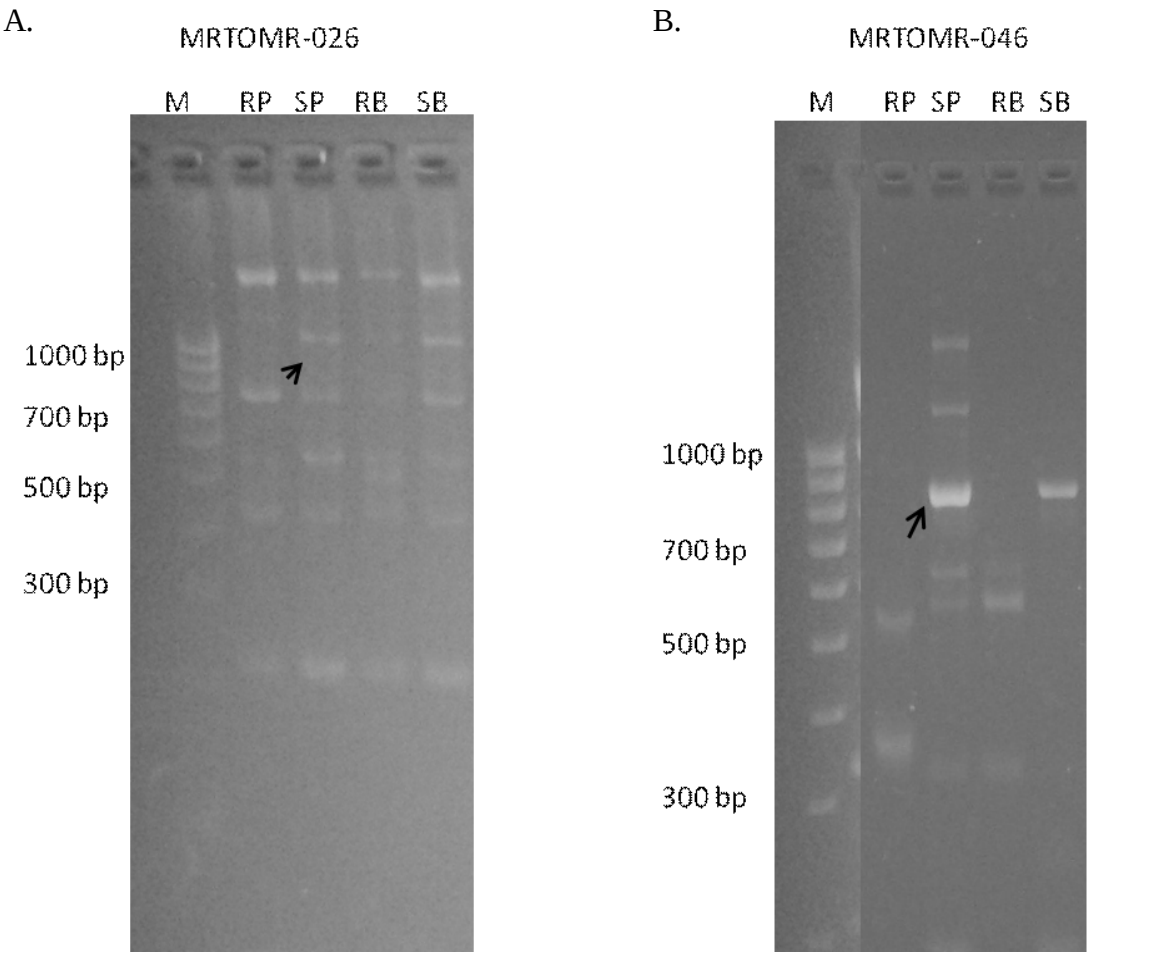

Fig. 3. Electrophoretic pattern of DNA fragments generated by RAPD marker (A. MRTOMR-026, B. MRTOMR-046). Polymorphic band (i.e. linked to susceptible) between parents and between resistant and susceptible bulks are indicated by arrow. RP = Resistant parent, NC 085L. SP = Susceptible parent, NC 839. RB = Resistant bulk. SP = Susceptible bulk. M = Marker

Table 4. Polymorphic bands of RAPD markers linked to either resistance or susceptible conferring genes in tomato to late blight

Name	PBN	Size, bp	RP	SP	RB	SB	Marker type
MRTOMR-026	2	**1100**	0	1	0	1	N
	5	500	0	0	1	0	-
MRTOMR-031	3	1200	1	0	0	0	-
	6	**550**	1	0	1	0	P
MRTOMR-038	2	**1100**	1	0	1	0	P
	3	750	0	1	1	1	-
MRTOMR-046	1	2000	0	1	0	0	-
	2	1200	0	1	0	0	-
	3	**800**	0	1	0	1	N
	4	650	0	1	1	0	-
	5	600	0	1	1	0	-
	6	550	1	0	0	0	-
	7	380	1	0	0	0	-
	8	350	0	1	1	0	-
	9	100	0	1	0	1	-

PBN = Polymorphic band number. RP = Resistant parent (NC 085LW). SP = Susceptible parent (NC 839-2). RB = Resistant bulk. SB = Susceptible bulk. P = Positive. N = Negative. 1 = Presence. 0 = Absence. Bold band size is specific marker band linked to either resistance or susceptible genes to late blight of tomato

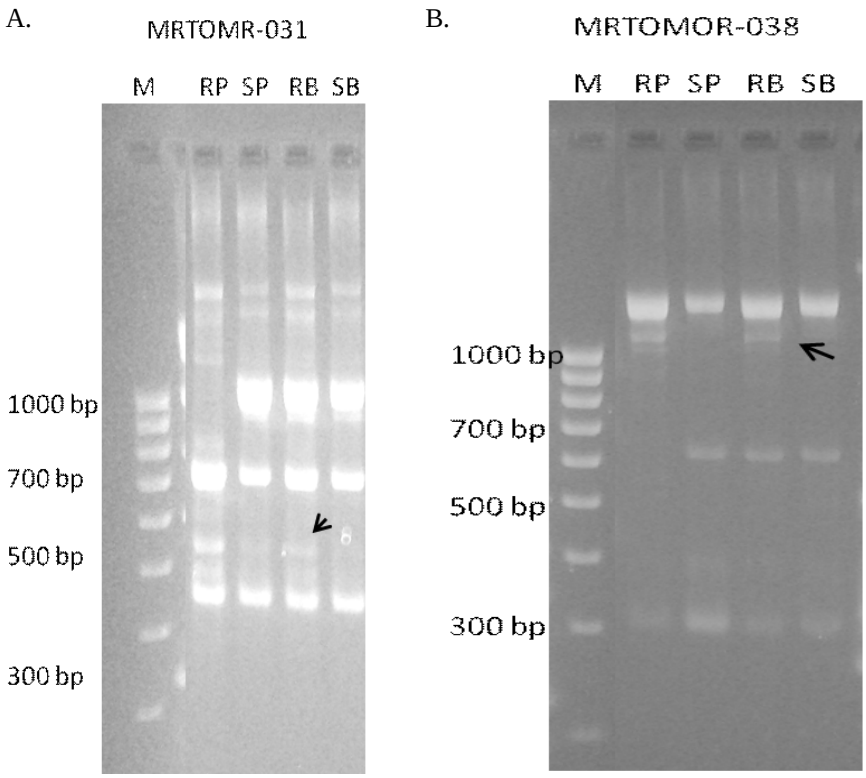

Fig. 4. Electrophoretic pattern of DNA fragments generated by RAPD markers (A. MRTOMR-031, B. MRTOMR-038). Polymorphic band (i.e. linked to resistance) between parents and between resistant and susceptible bulks are indicated by arrow. RP = Resistant parent, NC 085L. SP = Susceptible parent, NC 839. RB = Resistant bulk. SP = Susceptible bulk. M = Marker

Eleven primers were found to have bands that were unlinked to the loci. These primers distinguished only parents and not the bulks, therefore, defined as unlinked markers (Figure 5A). The amplified bands of eight RAPD primers were only found in bulks but not in either parent (Fig. 5B).

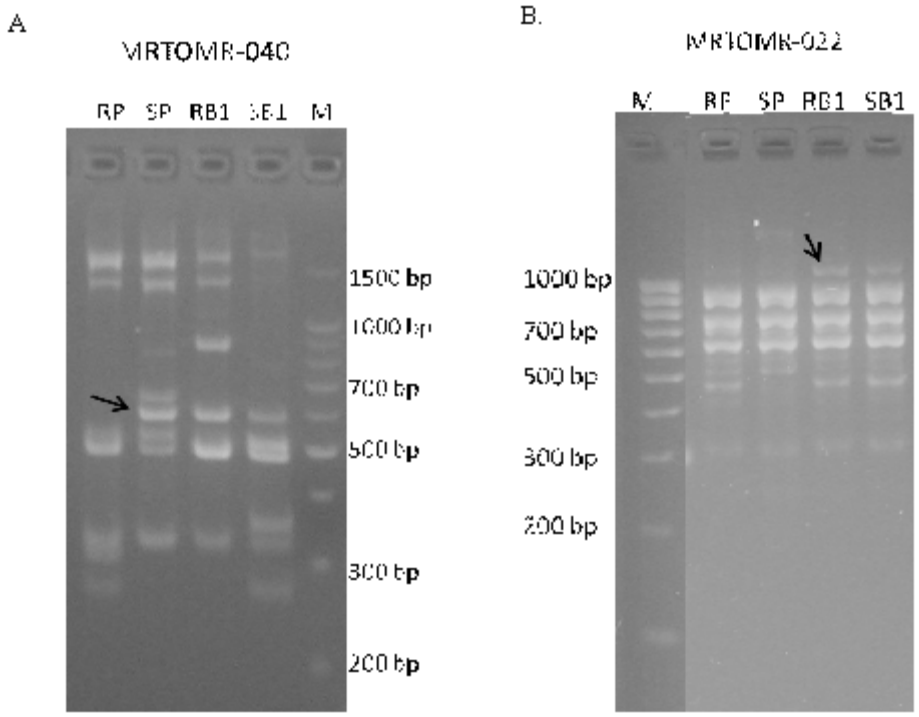

Fig. 5. A. RAPD marker (MRTOMR-040) showing polymorphic band (indicated by arrow) only to parents, i.e. band with unlinked loci. B. RAPD marker (MRTOMR-022) showing band (indicated by arrow) only in two bulks. RP = Resistant parent, NC 085L. SP = Susceptible parent, NC 839. RB = Resistant bulk. SP = Susceptible bulk. M = Marker

Inheritance study of late blight using 241 F_2 individuals showed that late blight resistance was governed by partial recessive gene. Similar result was also reported by Elsayed (2010). He also reported that resistance to late blight was controlled by partial recessive gene. In his study he used NC 1 CELBR and NC 2 CELBR as resistant parents to late blight having *Ph-2* and *Ph-3* genes respectively. Among the *Ph* genes resistant to late blight in tomato, *Ph-2* and *Ph-3* are partially dominant genes (Foolad *et al.* 2008). In our study, resistant parent, NC 85L also has *Ph-2* and *Ph-3* genes, derived from L3707 and Ritcher's wild tomato, respectively. Different results on the inheritance of late blight might be mainly due to different environments and isolates. The expressions of *Ph-2* gene has been reported to be dependent on environmental conditions, crop physiological stage and the pathogen isolate (Moreau *et al.* 1998). *Ph-3*, though superior to *Ph-2* does not exhibit strong resistance to some isolates (Foolad *et al.* 2008).

Two RAPD markers linked to resistant allele and two RAPD markers linked to susceptible allele were identified based on the field screening of F_2 population by 197 RAPD primers,. Through the bulking of two extreme phenotypes of F_2 population it was possible to rapidly tag the markers associated with chromosomal segment that has a role on reaction to late blight pathogen. With BSA technique consisting eight individuals in each bulk, four primers gave different band sizes that were found to be linked to late blight resistance. Chunwongse *et al.* (2002) identified AFLP marker linked to *Ph-3* gene using BSA and Qiu *et al.* (2009) identified one RAPD marker which was at distance of 5.8 cM from the target region of late blight resistance.

The probability of declaring an unlinked polymorphic marker linked to a gene is related with the size of the bulk. Both types of population and the markers should be considered during preparing the bulks (Michelmore *et al.* 1991). If the size of bulks is small, the frequency of false positives will increase. Michelmore *et al.* (1991) suggested that, few individuals per bulk were enough to identify the linked markers. The probability of finding an independent marker linked to the gene with bulks size of n is reported to be $2(1/4)^n[1-(1/4)^n]$ (Michelmore *et al.* 1991). Eight individuals were used and based on this formula, the proportion of false positives is about $3x10^{-5}$.

In principle, BSA and NIL are related and many advantages of BSA over NIL are discussed by Michelmore *et al.* (1991). Tagging of resistance gene using BSA is very fast which facilitates to screen new alleles of resistance for a particular disease. This is important because resistant gene once tagged may not be effective for a long time either because of recombination in the host genome or mutation in the pathogen. The BSA approach is also considered useful to fill the gaps in the maps.

Two parental lines used in this study are closely related to each other and SSR screening showed similar result. However, some RAPD markers distinguished these parents. RAPD is multilocus-marker therefore some primers identified here might be from the overlap regions of the chromosome. For example, MRTOMR-026 produced polymorphic band size of 1100 bp between bulks' band and MRTOMR-046 produced polymorphic 800 bp band. The band produced by MRTOMR-046 might be the part of the band generated by MRTOMR-026. These linkages should be verified by mapping the markers. Sequence of amplified region would help if they were from the same region and could be Blast searched in the web to see the similarity and location in the chromosome. A number of disadvantages associated with RAPD, for example, including annealing in multiple sites, dominant nature, sensitive to reaction conditions may limit its use directly in MAS. Therefore, candidate RAPD marker is generally converted to co-dominant SCAR or CAPS markers that are more useful for MAS (Chague *et al.* 1996).

BSA has been used in a number of crop species to tag various traits including quantitative traits. After identifying useful markers by BSA in tomato, MAS is now possible to apply during the selection of resistance to verticillium wilt, tomato spotted wilt virus, root-knot nematodes, powdery mildew, and fusarium wilt. De Giovanni *et al.* (2004) identified RAPD marker linked to the *ol-2* gene which is resistant to powdery mildew. A single RAPD marker, OPU_{31500} was detected in the susceptible bulk and converted to a CAPS marker. The distance between marker and *ol-2* gene was also estimated through linkage analysis. Czech *et al.* (2003) have used MAS for developing TSWV resistant tomato lines using PCR markers. Smiech *et al.* (2000) used BSA in F_2 segregating population and found five primers that distinguished resistant and susceptible bulks.

In the present study, the disease scores were used that were based on the natural infestation. Different levels of infestation rate were observed in F_2 population which indicated that inoculum pressure was enough to screen the population. Use of natural inocula saves cost and time. Screening of target population in target environment can be more successful in the long run to get durable resistant genotypes. Use of hot spot (area naturally favorable for pathogen development) for specific disease helps to increase disease pressure and is cost effective way of resistance breeding and particularly useful in horizontal resistance breeding. Horizontal resistance system is generally considered suitable for long term cultivation of resistance varieties. Spaner *et al.* (1998) mapped loci affecting resistance to powdery mildew, leaf rust, stem rust, scald and net blotch in barley using field-scored data of disease severity under natural infestation. Naturally infested population had been used in a number of crop species. For example, two QTLs for glume botch resistance in wheat were identified using composite interval mapping from naturally inoculated populations (Schnurbusch *et al.* 2003). Spaner *et al.* (1998) mapped the loci affecting resistance to powdery mildew, leaf rust, stem rust, scald and net blotch in barley using field-scored data of disease severity under natural infestation. Natural infestation was also used by Frei *et al.* (2005) to identify QTLs resistance to thrips in common bean.

Four potential RADP primers associated with reaction to late blight resistance in tomato were identified. Two primers gave positive bands in resistant genotypes so those primers could be very useful in MAS. Because

of low reproducibility and dominant nature of RAPD, these markers should be converted to co-dominant SCAR markers to identify the tightly linked markers, so that MAS could be applied using a single marker. MAS is cost effective and more precise, because it does not require pathological evaluation and genotyping can be done at any growth stage. Identified markers linked to resistance may also have utility in gene pyramiding.

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