Indian Journal of Agricultural Sciences **88** (4): 559–62, April 2018/Article

Marker assisted screening of F_2 population for late blight (*Phytophthora infestans*) resistance in indeterminate tomato (*Solanum lycopersicum*) under protected condition

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Received: 07 February 2017; Accepted: 22 December 2017

ABSTRACT

Late blight (LB) (*Phytophthora infestans* (Mont.) de Bary) is a highly destructive disease of tomato (*Solanum lycopersicum* L.) worldwide and attacks all the aerial parts of the plant including leaves, stems and immature green fruits causing 100% yield loss under congenial environmental conditions. The objective of this study was to identify molecular markers associated with *Ph*-2 and *Ph*-3 gene conferring LB resistance in tomato. Two co-dominant markers (TG422 and TG328) were associated with *Ph*-2 and *Ph*-3, which were cleaved amplified polymorphic sequences (CAPS) type. The tomato line PAU IND 2372 was crossed with the late blight resistant line LBR 21 to develop F_2 population. A total of 540 plants were screened for the target loci and tagged. From 540 F_2 plants, 112 were resistant for *Ph-2* and 106 for *Ph*-3 gene and 281 plants were positive for both *Ph*-2 and *Ph*-3 gene. The pattern of segregation of susceptibility or resistance was investigated using the chi-square ($\chi 2$) test.

Key words: Markers assisted selection, *Phytophthora infestans,* Resistance breeding, *Solanum lycopersicum*

Tomato (*Solanum lycopersicum* L.) plants are generally regarded as highly susceptible to late blight [*Phytophthora infestans* (Monte) de Bary] (Becktell *et al*. 2005). In India, prior to 2006, the disease was restricted to northern parts but since 2008 epidemics on tomato from south and east India had been reported (Chowdappa *et al*. 2013, Tripathi *et al*. 2017). In the absence of resistant cultivars, farmers often depend on fungicide to control diseases. Various fungicides have been commonly used to control late blight in tomato production worldwide. However, chemical control using fungicides has become ineffective against resistant strains, because they were treated too frequently, at 5-12 day intervals. High reliance on pesticides poses health hazards to farmers and their families, the environment, and consumers. Intensive pesticides use also can substantially increase production costs, which increases farmer financial risks and pass the accrued higher costs to consumers (Wilson and Tisdell 2001). Earlier populations of *P. infestans* were supposed to be A1 mating type (Gototh *et al*. 2005). Recent reports from south India recorded A2 mating type which is metalaxyl resistant, mtDNA haplotype Ia and had RG57 and SSR fingerprints almost identical to the 13_A2 clonal lineage reported in Europe (Chowdappa *et al*. 2014).

Resistant cultivars are among the cheapest, simplest, and most environmentally safe ways to manage disease. Host resistance is an important component in late blight management due to its long term economic benefits for small scale farmers. It also minimizes changes in the population structure of *P. infestans* and decreasing the likelihood of development of fungicide resistance (Hakiza 1999, Mukalazi *et al*. 2001).

Effective selection for disease resistance in segregating populations requires accurate, cost effective screening methods that permit rapid testing of thousands of plants. Common disease screening techniques include field testing under natural disease pressure, and net house screening procedures in which plants are inoculated with specific pathogen strains. Many disease resistance genes have been mapped in tomato, and molecular markers linked to these genes are available for marker assisted selection (MAS). Pyramiding known resistance genes and searching for new resistance genes may contribute towards sustainable solutions to the reduced effectiveness of known resistance genes and the formation of more aggressive pathogen isolates (Foolad *et al*. 2008, Fry 2008, Nowicki *et al*. 2012).

Disease resistance alone is insufficient to ensure farmer adaptation; commercial cultivars also must possess high yield potential, early maturity, fruit quality and nutrient content. Therefore, disease resistance breeding must be

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conducted with selection for important horticultural and fruit characters. The present study was conducted to search for late blight resistance in indeterminate tomato grown under poly house using marker assisted screening.

MATERIALS AND METHODS

The resistant line LBR- 21 containing the *Ph-2 and Ph-3* genes (World Vegetable Centre, Taiwan) was crossed with the late blight susceptible advance breeding line PAU 2372 in Feb 2014. The resulting F_1 plants were self-crossed and produced F_2 population in Feb 2016. A total of 540 F_2 plants were grown in net house and were subjected to recombinant screening using markersTG422 and TG328. The selected F_2 recombinants were tested for late blight resistance. The experiment was carried out at research farm and breeding laboratory of the Department of Vegetable Science, Punjab Agricultural University, Ludhiana, Punjab, India during the spring and autumn seasons of 2014-15 and 2015-16.

Resistance against *P. infestans* was evaluated using a detached leaf assay (Thind *et al*. 1989). Diseased leaves were collected from the late blight affected tomato plants in the morning hours. These leaves were kept in polyethylene bags and brought to the laboratory. The infected leaves were placed in 30 × 40 × 5 cm plastic trays in order to multiply the primary inoculum. A single ply of facial tissue paper was plastered with water on the bottom of the tray to maintain adequate humidity for inoculum development. The trays were kept in a dark chamber at 18-20^{0}C for 24 h. After that, the inoculum was prepared by collecting sporangia from the infected leaves by adding distilled water. The sporangial suspension was kept in the dark at 11-12^{0}C for 90-100 min to release the zoospores (Nilson 2006). Uniform suspension was used to obtain an accurate sporangial count.

Tomato lines LBR-21 (resistant) and PAU IND 2372 (susceptible) as control were used in the study. Five leaves/plant were placed in a tray and each set was replicated for three times. Leaves were inoculated with sporangial suspension (4.5×10^4 sporangia/ml) by using an atomiser. The trays were covered with moist polythene sheet to ensure high relatively humidity and incubated at 16 ±1°C (16h light period with fluorescent tubes) as per Thind *et al.* (1989). The observations were recorded after 5 days as extent of leaf area damaged on a 0-5 rating scale (0: healthy; 1: one or two lesions on the leaves; 2: few lesions on the leaves; 3: 25% area of leaves covered with lesions; 4: 50% of the leaves covered with lesions and 5: 75% of the leaf areas covered with lesions (1-2 was regarded as resistant; 3 as moderately resistant and between 4-5 as susceptible).

Genomic DNA was extracted from young leaves of each plant by using CTAB method described by Fulton *et al*. (1995) and subjected to polymerase chain reaction (PCR). Conditions for PCR reactions were 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 50 mM of each dNTP, 0.1 mM of each forward and reverse primers, 20 ng of template DNA, and 1 unit of Taq DNA polymerase in a total volume of 20 µl. Sources of resistance genes and molecular markers linked to late blight resistance genes used in MAS are given in Table 1. Markers were visualized as size polymorphisms by 2% agarose gel electrophoresis.

Table 1 Sources of resistance genes and molecular markers linked to late blight resistance genes used in MAS

Disease	Gene	Chromosome	Marker	Anneali/g Temp	Restriction enzyme	Band size	Referenc
Late blight	*Ph-2*	10	TG422	55^{0}C	*Hinf* 1	245 (S) 275 (R)	Foolad and Panthee (2012)
	Ph-3	9	TG328	60^{0}C	*Apo 1*	274 (S) 243 (R)	Robbins *et al*. (2010)

The segregation ratios of resistant and susceptible plants in the F_2 progenies were subjected to Mendelian genetic models using the chi-square ($\chi 2$) test. The probability of the goodness-of-fit between the expected and observed segregation ratios was obtained using the $\chi 2$ test (Pearson1922).

RESULTS AND DISCUSSION

This study describes the integrated application of molecular markers and detached leaf screening method to develop late blight resistant tomato progenies**.** The disease scoring data revealed that the resistant parent LBR-21 showed 100% resistance to late blight while the susceptible genotype PAU IND 2372 showed 100% susceptibility. In detached leaf method, out of 540 F_2 plants, 296 were resistant and 212 were moderately resistant (Table 2). A total of 540 F_2 plants were subjected to DNA marker analysis for two co-dominant CAPS markers (TG422 and TG328) and plants were tagged. The markers were associated with *Ph*-2 and *Ph*-3 genes. From 540 F_2 plants, 112 were resistant for *Ph-2* and 106 for *Ph*-3 gene. As many as 281 plants were positive for both *Ph*-2 and *Ph*-3 gene (Fig 1) and 41 plants were found susceptible. The homogeneity chi-square value was within the acceptable limit for cross resulting in a good

Table 2 Screening of F_2 plants for late blight resistance using detached leaf method

Tomato line	Total plants tested	Plant under different grades of LB severity		
		R	MR	S
PAU 2372 × LBR 21	540	296	212	32
LBR 21 (Resistant control)	10	10	00	00
PAU IND 2372 (Susceptible control)	10	00	00	10

LB = Late blight, R= Resistant, MR= Moderately resistant, S= Susceptible.

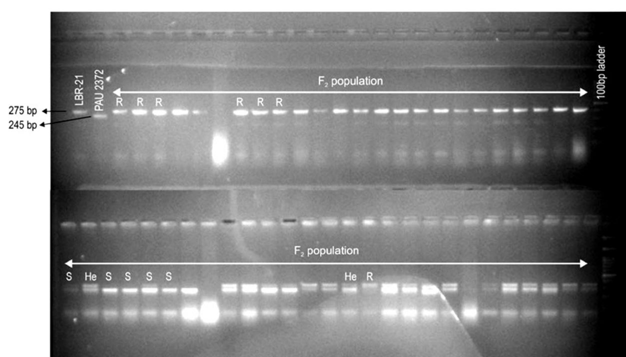

Fig 1 Agarose gel image of PCR product with TG422 primers for *Ph*-2 gene confirmation in F_2 population

fit for the expected 9:3:3:1 segregation ratios (Table 3). For single marker analysis, the ratios showed good fit 3:1 ratio.

Michalska *et al*. (2011) examined response over two dozen cultivars and breeding line of potato and reported that a detached leaflet assay could be useful for screening of potato for LB resistance. Practical considerations, such as low cost and minimal facility requirements for conducting detached leaflet experiments, the need for leaves or leaflets, as opposed to whole plants, the small amount of inoculums needed, minimal pathogen escaping into commercial growing environments, and being fast and non destructive make the detached leaflet assay attractive in particular for large scale screening of tomato breeding population. The detached leaflet assay may also be useful for screening breeding material to reduce the number of entries in field trials (Dorrance and Inglis 1997, Michalska *et al*. 2011).

Effective and inexpensive screening methods enable efficient selection of plants carrying desired resistance genes. Different screening methods were employed during advance generation including field evaluation under natural disease pressure, seedling inoculation with selected pathogen isolates, morphological marker and DNA markers linked to disease resistance genes. The *Ph-2* and *Ph-3* markers were advantageous because individual plants could be assayed for both *Ph-2* and *Ph-3*, and homozygotes versus heterozygotes could be distinguished. Tomato breeders continue to search for new late blight resistance genes in wild tomato species *S. pimpinellifolium* (Merk *et al*. 2012) and *S. habrochaites* (Brouwer and Clair 2004, Li *et al*. 2011), but introgession of new alleles from wild species into commercial cultivars requires many years. Farmers should be encouraged to adopt sanitation, fungicides, and other control tactics to help prolong the durability of late blight resistance.

Table 3 Segregation ratios for resistance to late blight in 540 F_2 population

Genetic constitution gene	Expected ratio	Plants observed (O)	Plant expected (E)	$(O-E)^2/E$
Ph2Ph2Ph3Ph3	1	34	33.75	0.00
Ph2Ph2Ph3ph3	2	58	67.5	1.33
Ph2ph2Ph3Ph3	2	56	67.5	1.95
Ph2Ph2ph3ph3	1	34	33.75	0.00
Ph2ph2Ph3ph3	4	142	135	0.36
Ph2ph2ph3ph3	2	63	67.5	0.30
Ph2ph2Ph3Ph3	1	40	33.75	1.16
Ph2ph2Ph3ph3	2	72	67.5	0.30
Ph2ph2ph3ph3	1	41	33.75	1.56

ACKNOWLEDGEMENT

The authors are thankful to the Head, Department of Vegetable Science, PAU, Ludhiana, Punjab for providing necessary facilities to conduct the experiment and Department of Science and Technology, Government of India New Delhi for sponsoring the study under WOSA scheme.

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