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Validaton of markers for late blight resistance in Tomato (*Solanum lycopersicum* L.)

Manisha Mangal[*] *and Zakir Hussain*

Abstract: *Marker assisted breeding has been widely and successfully used for selecting desirable traits including disease resistance by identifying genetic markers that are linked to specific genes/alleles or combination of multiple resistance genes/allele. Late blight, caused by the oomycete Phytophthora infestans (Mont.) de Bary, is a devastating disease frequently leading to severe crop losses. A number of workers have reported various molecular markers linked to the genes for resistance to late blight in tomato. Many markers have not been not validated across tomato genotypes, thus greatly reducing their utility in crop improvement programs. During present investigations 40 markers comprising 15 SSRs, 2 dominant markers, one SCAR marker and 22 CAPS markers reported by different workers were screened on five tomato lines out of which one line CLN3241H was carrying gene for resistance to late blight (Ph3) and four were susceptible lines. Twenty markers including twelve CAPS markers, two dominant markers, six SSRs and one SCAR markers, located in close vicinity of Ph3 genes could be validated on the lines under study*

Key words: *Late blight, Cleaved Amplified Polymorphic Sequences, MAS, Tomtato,*

Tomato, *Solanum lycopersicum* L., is the second most consumed vegetable crop. A large number of commercial cultivars of tomato have been developed through traditional breeding, however, with the advent of molecular markers, marker-assisted breeding has been carried out for several economically important traits, in particular disease resistance as well as for testing hybrid purity, and marker assisted backcross breeding. Late blight, caused by the oomycete *Phytophthora infestans* (Mont.) de Bary, is a devastating disease to both cultivated tomato (*Lycopersicon esculentum*) and potato (*Solanum tuberosum*), frequently leading to severe crop losses (Fry and Goodwin 1997). Intensified epidemic outbreaks of the disease have occurred throughout the world since the 1980s. Breed-ing for LB resistance is an economical and environmen-tally friendly strategy that provides an attractive alterna-tive to chemical control Wild relatives of tomato show various levels of LB resistance and therefore can be used as potential resources for breeding tomatoes with LB resistance. In tomato, both qualitative and quantitative LB resistances have been reported. Three major LB resistance genes, *Ph-1*, *Ph-2* and *Ph-3* have been identified in the wild species *Solanum pimpi-nellifolium* (Bonde and Murphy 1952; Gallegly and Mar-vel 1955; Peirce 1971; Moreau *et al.* 1998; Chunwongse et al. 2002). The *Ph-1* gene has been mapped to chromosome 7 and confers resistance only to *P. infestans* race T0 (Bonde and Murphy 1952; Gallegly and Marvel 1955; Peirce 1971). The *Ph-2* gene, conferring incomplete LB resistance, was identified in *S. pimpinellifolium* line WVa 700 and is located on the distal part of the long arm of chromo-some 10 (Gallegly and Marvel 1955; Moreau et al. 1998). Resistance conferred by *Ph-1* and *Ph-2* was overcome by different *P. infestans* isolates from Taiwan, Indonesia, Nepal and The Philippines (AVDRC 1995, 1998, 1999). This prompted further screening of tomato germplasm for new LB resistance genes. As a result, *S. pimpinellifolium*

* Division of Vegetable Science, ICAR-IARI, Pusa Campus, New Delhi-110012, *For correspondence: manishamangal@rediffmail.com*

L3708 was found to be highly resistant to a wide range of *P. infestans* isolates that overcome *Ph-1* and *Ph-2*-related resistance (Black *et al.* 1996a, b). Genetic study indicated that LB resistance in L3708 was conditioned by a single partially dominant gene, *Ph-3*, which was mapped to the long arm of chromosome 9 (Black *et al.* 1996a; Chun-wongse *et al.* 2002). A number of workers have reported various molecular markers linked to the genes for resistnace to late blight in tomato. Many markers have not been not validated across tomato genotypes or are not polymorphic within tomato breeding populations, thus greatly reducing their utility in crop improvement programs. Therefore the objective of the present study was to validate the available molecular markers which have been reported to be linked to late blight resistance in tomato so that the reproducible markers could be identified for MAS in future crossing programmes.

PLANT MATERIAL

The germplasm used in this study for validation of molecular markers linked to late blight resistance consisted of 5 lines procured from AVRDC, Taiwan viz. CLN3451D,CLN3126a-7, C3070JLN, CLN3241H-27, CLN3125P out of which line CLN3241H-27 was having gene for resistance to late blight. All the germplasm was maintained at the research farm of Division of Vegetable Science, IARI, New Delhi, India. Young, healthy and uninfected leaves from each genotype were collected and brought to the laboratory in liquid nitrogen (-196^{0}C) where they were kept in deep freezers at -80^{0}C for further use.

DNA EXTRACTION

Genomic DNA was extracted from young leaf tissue following the C-TAB procedure (Murray and Thompson 1980). DNA quality and quantity were assessed on a 1% (w/v) agarose gel stained with ethidium bromide (Sigma Aldrich Chemical Pvt. Ltd, Bangalore, India) and also by using a NanoDrop® ND-1000 spectrophotometer.

Selection of the primer: Markers used for validation of different genes are given in table 1.

POLYMERASE CHAIN REACTION (PCR) ANALYSIS

All the markers were amplified by PCR in 15µl volumes with 50ng genomic DNA, 1.0 U *Taq* DNA polymerase (Hi media Laboratories, Mumbai, India), 1.0 µM of each primer, 0.6 ul of 10 mM dNTP mix (Hi media Laboratories, Mumbai, India), and 1.5 ul of 10X PCR buffer having 17.5 mM $MgCl_2$ (Hi media Laboratories, Mumbai, India). Amplification conditions used for *Ph3* genes were, one cycle of 94°C for 3 min; 10 cycles of 94°C for 0.5 min, 55–45°C decreasing by 1°C per cycle for 1 min, and 72°C for 1 min; 30 cycles of 94°C for 0.5 min, 45°C for 1 min, and 72°C for 1 min; and a final cycle of 72°C for 5 min. Amplified products were resolved on 3.0% agarose gels with Tris/Acetate /EDTA (TAE) stained with ethidium bromide, at a constant voltage of 60 V for 3 h using a horizontal gel electrophoresis system (BioRad, USA) and visualized and photographed under UV light in a gel documentation unit (Alpha imager, Cell bioscinces, Santa Clara, CA).

RESULT AND DISCUSSION

Availability of reproducible and reliable markers can greatly aid in breeding for resistance to various diseases. During present investigations 40 markers comprising 15 SSRs, 2 dominant markers, one SCAR marker and 22 CAPS markers reported by Brower and Clair, 2004; Moreau et al., 1998 ; Zhu et al., 2006 and Zhang et al., 2013; were screened on five tomato lines out of which one line CLN3241H was harbouring gene for resistance to late blight (*Ph3*) and four were susceptible lines.

Brouwer and Clair (2004) developed near-isogenic lines between susceptible cultivated tomato *Lycopersicon esculentum* and resistant *L. hirsutum* and evaluated the NILs and sub-NILs for disease resistance and eight horticultural traits at three field locations. Resistance QTLs viz. lb4, lb5b, and lb11b were detected in all three sets of NIL lines. Genotypes were determined using restriction fragment length polymorphisms (RFLPs) and PCR-based cleaved amplified polymorphic (Konieczny and Ausubel 1993) markers. For each QTL interval, they converted one central and two flanking RFLP

Table 1
Primers for detection of polymorphisms for late blight

Marker	*Marker type*	*Forward primer*	*Reverse primer*	*Enzyme*	*Reference*	*Results*
SSR43	SSR	CTCCAAATTGGGCAATAACA	TTAGGAAGTTGCATTAGGCCA	-	Zhu *et al.*, 2006 (Ag sc. China)	Not validated in present study.
SSR66		TGCAACAACTGGATAGGTCG	TGGATGAAACGGATGTTGAA			
SSR104		TTCCATTTGAATTCCAACCC	CCCACTGCACATCAACTGAC			
SSR306		ACATGAGCCCAATGAACCTC	AACCATTCCGCACGTACATA			
SSR320		ATGAGGCAATCTTCACCTGG	TTCAGCTGATAGTTCCTGCG			
SSR333		GTTCCCGCTTGAGAAACAAC	CCAATGCTGGGACAGAAGAT			
TOM59		TAACACATGAACATTAGTTTGA	CACGTAAAATAAAGAAGGAAT			
TOM184		CAACCCCTCTCCTATTCT	CTGCTTTGTCGAGTTTGAA			
TOM236		GTTTTTTCAACATCAAAGAGCT	GGATAGGTTTCGTTAGTGAACT	-		
SSR19		CACAGAACTTGACCAAACAA	GCTGTCACTAACATAGGGGA	-	Zhang *et al.*, 2013	All the markers validated in present study.
s06214-SSR15		AAAACGGTACTCTCTCTGTCTC	CCAGCAGTTGTACTAGGCTC			
s06214-SSR12		TGGAGGCTACTGACATTTTT	AGCAAAGAGGTCTATTTTGG			
s06214-SSR03		GCAACAAAGTTTTAGCATCC	GCTTCCATTTTGTCTCAACT			
s06214-SSR01		GCATCTCTACGGAGTAAGGA	CTGGAATACCGGTACGTATG			
TES0562		GAGGAACAGGTCTAGCAGGCA	ATAACCGTGATTGTCCCGAA			
sc02760_2	Dominent	GATAGAGCGGCTGTGA	GGGTAGTCTCCGATAA			
P55		CAAGGCTGCACTCTTACCAT	AATGTGCCAAATGCCTAATG			
RGA2M1	SCAR	GTTTATTTGCTCACTCGG	AGATACATAGGAGGGGATT			
P15	CAPS	GCTGATTCGGACTTACTG	ACTTTGCAGGGAGGGAAC	*HhaI*	Zhang *et al.*, 2013	All the markers validated in present study.
P19		AGGCATACTGAAGGTGGC	TTGGCTCTTATTGGTCTT	*ApoI*		
P24		GCTCTAAGGCTGCTCGTT	TGGACCAGTCAAGGGAGA	*HincII*		
P27		GGGAGGACACGATTACAC	GATGATGGTATTCGGTAG	*TaqI*		
P31		TCTGCTTAGACCGACAAT	ATCCTACCTCATCCTCGT	*HinfI*		
P60		AGGTGAGACTTTGCAGGAG	TGCCGACATCTCCATAACC	*RsaI*		
HBa		GACGGAAGACGGTGAT	TCATCGCAGTAGAGGC	*TaqI*		
Indel_3		AAGGTTTATTCGGGTTCA	TCACTCCTTTGTCTACGC	*DdeI*		
Indel_4		ACAGATAGTTGAGTCGGTAT	ATTCGGATAGCCTACTGG	*HpaII*		

contd. table 1

Marker	*Marker type*	*Forward primer*	*Reverse primer*	*Enzyme*	*Reference*	*Results*
T0156		GCGGTTGATTCACATCGTAA	CCTGTAGCACCCAAAGGATG	*DpnII*		
TG328		GGTGATCTGCTTATAGACTTGGG	AAGGTCTAAAGAAGGCTGGTGC	*BstNI*		
TG591		AAGGCAAAGGAAGTTGGAGGTCA	AGAGGTTGCAACTCGTGGATTGAG	*AciI*		
TG233 *(Ph2)*		CATGCCTTTTTCTTGGGATG	TGGAACCCCTTTAACTGTGC	AluI	Moreau *et al.*, 1998	Not validated
TG400		TCCAAATCCACCACCTATCC	AGCATTGCTCCCTGCTAAAG	HinfI	Brower and Clair, 2004	
TG 609		CTTTGAGCCGAAACTTTCTTTTAG	ATGATCATTAACACTGATGCAAGG	TaqαI		Not validated in present study.
TG 427		GTTCTCCTCTTCAACTGGGTATTC	TGTGGGAACTCTGTAATGAATTTG	RsaI		
TG 503		AGATTACACGGCCTAATTTTCAAC	TTTTCACCAGCTTAAAGTTCCTTC	RsaI		
TG 358		CAACTTTTCCAGGTTCATTTTCTC	ACACCTACATGCTACTAAGGGGTC	HhaI		
TG 185		GTTTGTCATCAAGCCATAGAACCG	ATAACGATGTGGTCTTTCTGAAGC	TaqáI		
TG 194		CAGATGAAAGAAAAGCCAAAAGAG	AATGCTCAGAAGGGAAACATAAAG	MnlI		
TG 393		CACGCAAAGACACTACATAACCTC	CAAATTTATCTGTTGGGTGTCCTG	Tsp509I		
TG 400		GCTAATTGAAGTCAAAGAGCACAC	ACCTGTTGTTTGCTTGGTTATATG	HaeIII		

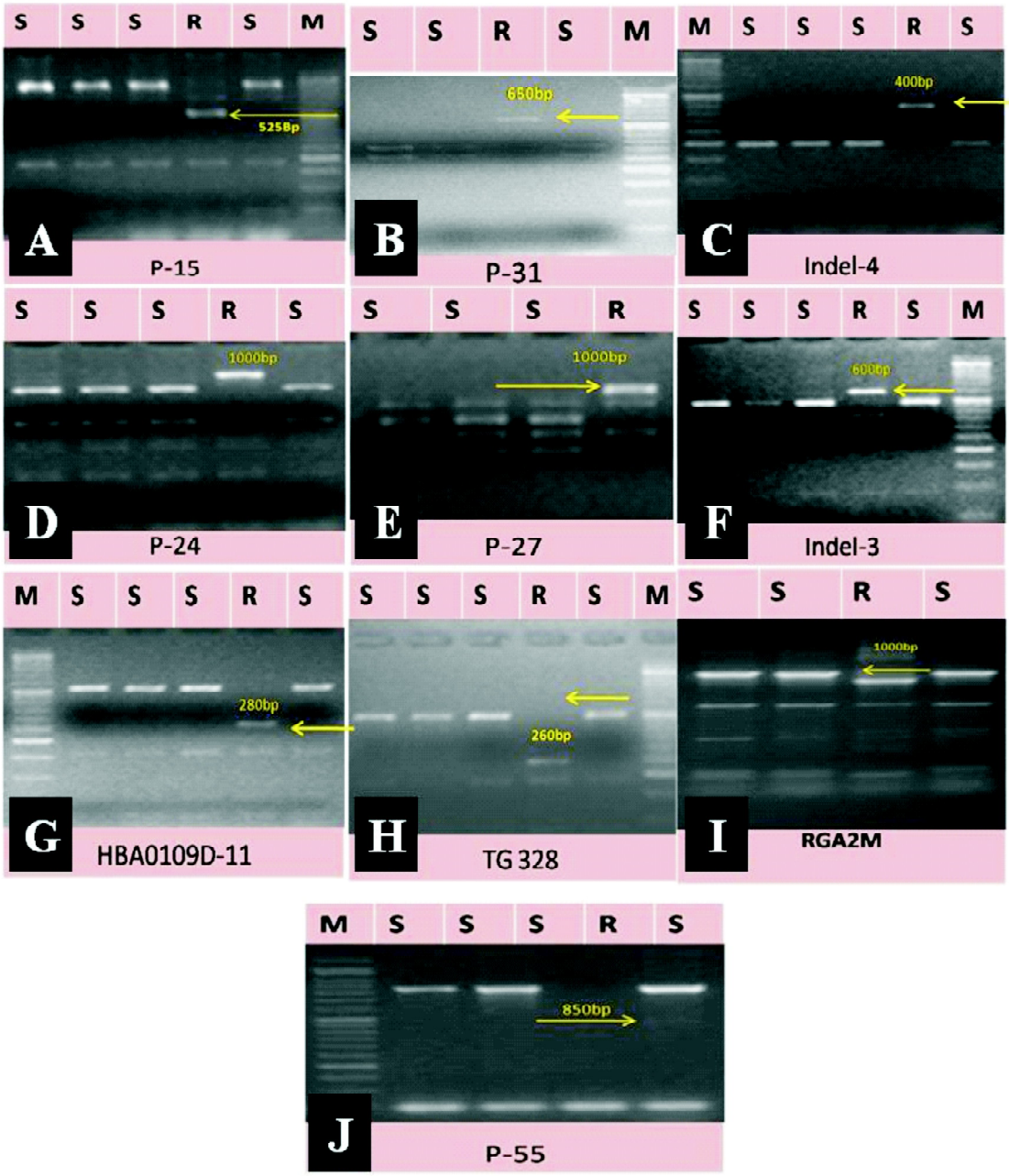

Figure 1: Validation of markers linked to Ph3 gene for resistance to late blight in tomato . A- H : CAPS markers; I : SCAR marker and J: dominant marker.

Lines used in Fig 1A-IG: CLN3451D,CLN3126a-7, C3070JLN,CLN3241H-27, CLN3125P and in Fig 1H-II: CLN3126a-7, C3070JLN,CLN3241H-27, CLN3125P

markers in to CAP markers. CAP markers were developed for TG15, TG609, and TG427 on NIL4; TG503, TG358, and TG185 on NIL5; and TG194, TG400, and TG393 on NIL11. In the present investigations all these CAPS markers were tested on the five lines under study. Amplification was observed in all the lines however no polymorphism was detected between susceptible and resistant lines suggesting that the source of resistance in the resistant line under study viz. CLN3241H has not been derived from *S.hirsutum*.

A partial dominant gene *Ph-2* was found in the wild relative *S. pimpinellifolium*, that mapped to chromosome 10 (Moreau *et al.*, 1998) and molecular

markers TP105 and TG233 have been found to be closely associated with *Ph-2*, however in the present investigations marker TG233 did not show polymorphism under the lines under study indicating an absence of *Ph2* gene in the lines under study.

Zhu *et al.* (2006) studied the inheritance of late blight resistance and identified simple sequence repeat (SSR) markers associated with resistance allele in tomato *in* an F2 progeny of 241 plants derived from a cross between susceptible inbred line and a resistant accession CLN2037E. They observed that the resistance is dominant and inherited as monogenic trait. Genetic mapping and linkage analysis showed that the late blight resistance gene *Ph-ROL* was located on chromosome 9 with a genetic distance of 5.7 cM to the SSR marker TOM236. In order to validate the marker TOM 236 we tested this marker on the lines under study, however Tom 236 did not reveal any polymorphism between resistant accession CLN3241H and for susceptible accessions.

The resistance (*R*) gene *Ph-3*, derived from *Solanum pimpinellifolium* L3708 which provided resistance to multiple *P. infestans* isolates has been widely used in tomato breeding programmes (Zhang *et al.* 2014). The *Ph-3* gene has been assigned to the long arm of chromosome 9. Zhang et al. (2013) developed a high-resolution genetic map covering the *Ph-3* locus using an F2 population of a cross between *Solanum lycopersicum* CLN2037B (containing *Ph-3*) and *S. lycopersicum* LA4084. They mapped *Ph-3* in a 0.5 cM interval between two markers, Indel_3 and P55. In the present study twelve CAPS markers, two dominant markers, six SSRs and one SCAR markers linnked to the Ph3 gene in the map constructed by Zhang et al., 2013 were used to study the polymorphism between resistant line CLN3241H and four were susceptible lines. All the markers could be validated in the resistant line.

In conclusion, the study validated some of the highly efficient markers for identification of late blight resistant loci and will enable breeders to better exploit these markers for pyramiding of late blight resistant loci in the pursuit of stable and broad spectrum resistance to multiple diseases.

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