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Marker assisted detection of TYLCV and late blight resistance in tomato (*Solanum lycopersicum*)

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ABSTRACT

Marker assisted breeding relies on the identification of genetic loci linked to desirable genes/alleles and is being utilized for selecting desirable traits including disease resistance in a wide variety of crops. The tomato (*Solanum lycopersicum* L.) production worldwide has lately been threatened by outbreaks of *tomato yellow leaf curl virus* transmitted by the whitefly (*Bemisia tabaci)* and *late blight* caused by oomycete pathogen *Phytophthora infestans* (Mont.) de Bary. Resistance genes against both pathogens are available and widely used in tomato breeding throughout the world. Molecular markers for resistance alleles greatly enhance selection of resistant plants. For effective breeding programme, the validation of markers linked to resistance genes in tomato was carried out for tomato improvement. For this purpose, available markers for the tomato yellow leaf curl disease resistance gene loci *Ty-2, Ty3, Ty3a* and late blight resistant loci *Ph3* were validated. The assay correctly predicted the genotypes of tomato breeding lines harbouring resistance allele at each loci. Further a multiplex assay was also standardized to simultaneously detect the presence of resistant/susceptible alleles at *Ty2,Ty3* and *Ty3a* loci. The present study carried out in the Division of Vegetable Science, ICAR-IARI, New Delhi, demonstrates the feasibility of combining two SCAR markers (TG0302 and P6-25) for detecting simultaneously three important resistance gene loci in tomato genotypes and thereby improving the cost and efficiency of the assay.

Keywords: Late blight, MAS, SCAR, Tomato, Tomato leaf curl disease

Tomato (*Solanum lycopersicum* L.) cultivation and production is often threatened due to severe crop losses caused by various pathogens including viruses, bacteria, fungi, and nematodes. Genomic tools particularly molecular markers linked to important traits are being successfully used for breeding tomato. Biotechnological advancements have led to the discovery, mapping, cloning and sequencing of more than 40 genes conferring resistance to major biotic stresses in tomato (Grube *et al.* 2000) which in turn lead to pyramiding of resistance genes through MAS and hastening the introgression of multiple genes into one genotype. Tomato leaf curl virus (ToLCV) causes one of the most destructive diseases in tomato which takes a heavy toll of the crop. Characteristic geminate virus particles have often been found in the plants infected with ToLCV (Thomas *et al.* 1986) and the disease symptoms appear to be somewhat similar to those caused by tomato yellow leaf curl virus (TYLCV) (Fauquet 2005).Warm and sub-tropical regions throughout the world favour the spread of this virus and hence are witnessing severe reduction in tomato production due to the disease (Cohen and Lapidot 2007). Major symptoms of the disease include stunting of infected plants, curling of leaves and abortion of flowers leading to poor fruit set and yield. Crop losses up to 100% have also been encountered due to the disease in cases where infection occurred at young stage (Varma and Malathi 2003).

Late blight is another devastating disease of tomato, the causal organism for which is omycete *Phytophthora infestans* (Mont.) de Bary. The *Ph3* resistance locus has been introgressed into cultivated tomatoes from the line L3708 of *S. pimpinellifolium*. Marker assisted selection (MAS) has immense potential for cultivar improvement in tomato breeding programmes wherein genetic markers can be applied for selection of single resistance genes as well as for combining multiple resistance genes (Foolad and Sharma 2005). In this study, we tested different allele specific molecular markers for the *Ty2, Ty3, Ty3a* and *Ph3* resistance loci in our available germplasm so that the reproducible markers could be used for MAS for gene pyramiding in our commercial tomato cultivars.

MATERIALS AND METHODS

Plant material: *The* 15 genotypes of tomato used in this study were grown at the research farm of ICAR-IARI,

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Table 2 Primers used for validation of *Ty2*, *Ty3* and *Ph3* alleles in the 15 genotypes under study

R-gene	Marker	Chr.	Forward primer	Reverse primer	Fragment size	References
Ty-2	T0302	11	TGGCTCATCCTGAAGCTGA-TAGCGC	AGTGTACATCCTTGCCATTGACT	~ 900/791	Garcia *et al.* (2007)
Ty-3	P6-25	6	GGTAGTGGAAATGATGCT-GCTC	GCTCTGCCTATTGTCCCATATATA-ACC	~450/319	Ji *et al.* (2008)
Ty3	FLUW 25F	6	CAAGTGTG-CATATACTTCATA(T/G)TCACC	CCA TAT ATA ACC TCT GTT TCT ATT TCG AC	~ 640/475	Salus *et al.* (2007)
Ph3	RGA2M1	9	GTTTATTTGCTCACTCGG	AGATACATAGGAGGGGATT	~ 1000/1200	Zhang *et al.* (2013)

New Delhi (Table 1). The study was performed in the year 2017–18 and markers were revalidated in 2018–19. Phenotypically healthy uninfected and young leaves from each genotype were collected in liquid nitrogen (-196^{0}C) and stored in deep freezers at -80^{0}C till further use.

DNA extraction: Genomic DNA was extracted from all the leaf samples following the C-TAB method (Murray and Thompson 1980). Qualitative evaluation of isolated DNA was done using 1% (w/v) Agarose gel electrophoresis (Sigma Aldrich Chemical Pvt Ltd, Bengaluru, India) while quantitative evaluation was done using NanoDrop® ND-1000 spectrophotometer.

Selection of the primer: The sequences of primers used for validation of different genes (Table 2) were custom synthesized from SBS Genetech Co. Ltd, Beijing, China.

Polymerase chain reaction (PCR) analysis: PCR reactions were performed in a total volume of 15µl containing 50ng genomic DNA, 1.0 U *Taq* DNA polymerase (Geno Biosciences Pvt Ltd Noida, India), 1.0 µM of each primer, 0.6 ul of 10 mM dNTP mix (Geno Biosciences Pvt Ltd, Noida, India), and 1.5 ul of 10X PCR buffer having 17.5 mM $MgCl_2$ (Geno Biosciences Pvt Ltd, Noida, India). All the primers were amplified using touchdown protocol of PCR in an Eppendorf Mastercycler. Thermocycler conditions used for amplification of Ty genes were: denaturation at 94°C for 3 min; 10 cycles of 94°C for 0.5 min, 63–53°C (touchdown 1°C/cycle) for 1 min, and 72°C for 1 min followed by 30 cycles of 94°C for 0.5 min, 53°C for 1 min, and 72°C for 1 min; and a final cycle of 72°C for 5 min. Amplification conditions used for *Ph3* genes were, denaturation at 94°C for 3 min; 10 cycles of 94°C for 0.5 min, 55–45°C (touchdown 1°C/cycle) for 1 min, and 72°C for 1 min followed by 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. PCR products were separated on a horizontal gel electrophoresis system (BioRad, USA) at a constant voltage of 60 V for 3 h using 3.0% agarose gels with Tris/Acetate/EDTA (TAE) stained with ethidium bromide. Amplified products were observed and photographed in a gel documentation unit (Alpha imager, Cell Biosciences, Santa Clara, CA). On the agarose gel, 15 genotypes of tomato were loaded in the same order as they are mentioned in the Table 2.

Table 1 *Ty-2*, *Ty-3*, *Ty3a and Ph3* alleles in a representative set of tomato germplasm used in the study

Genotype	Known resistance	Markers			
		Ty3 (FLUW-25)	*Ty3/Ty3a* (P-625)	*Ty2* (TG0302)	*Ph3* (RGA2M1)
Tanya	Susceptible to all	-	-	-	-
TMB486	Ty1	-	Ty3a	-	-
CLN24980	Ty2	-	-	+	-
CLN3024A	Ty2	-	-	+	-
CLN3205B	Ty3	+	Ty3	-	-
ClN3552B	Ty3	+	Ty3	-	-
CLN3212C	Susceptible to all	-	-	-	-
CLN3150A-5	Ty2	-	-	+	-
CLN3451D	Ty3a	+	Ty3a	-	-
CLN3126a-7	Ty3a, ty2	+	Ty3a	+	-
C3070JLN	Ty3, Ty2	+	Ty3	+	-
CLN3241H-27	Ty3, ty2, Ph3	+	Ty3	+	+
CLN3125P	Ty3, ty2	+	Ty3	+	-
F9-158	Susceptible to all	_	-	-	-
FLA46-4	Susceptible to all	_	-	-	-

RESULTS AND DISCUSSION

Availability of reproducible and reliable markers can greatly aid in breeding for resistance to various diseases. SCAR and CAPS markers linked to begomovirus resistance loci located on chromosome 6 of *S. chilense* were reported by Ji *et al.* (2006, 2007). Salus *et al.* (2007) developed PCR primers FLUW-25 for the selection of *ty3/Ty3* alleles of *Ty3* locus. During the later studies, it was revealed that the sequence for *Ty3* allele derived from *S.chilense* line LA2779 was different from those derived from LA1932 (Maxwell *et al.*2007) and hence the two different introgressions were designated as *Ty3* from LA2779 and *Ty3a* from LA1932. Ji *et al.* (www.plantpath.wisc.edu), further designed a set of PCR primers, viz. P6-25F2/P6-25R5 which could detect *Ty3* and *Ty3a* alleles as well as a newly discovered allele from *S. chilense* LA1969. These primers gave 450-bp fragments in TYLCV resistant lines derived from either the *S. chilense* LA2779 source, Gc9, or the Ih902 line (Vidavsky and Czosnek 1998), while a 320-bp *ty3* fragment was amplified from breeding lines which lacked both these resistant alleles. In addition, a 630-bp *Ty3a* fragment was obtained from lines derived from *S. chilense* LA1932 and Gc171. Heterozygous hybrids were detected on the bases of presence two fragments corresponding to a *ty3* fragment(320 bp) and either of *Ty3* (450 bp) or the *Ty3a* (630 bp) fragment.

The *Ty2* locus was fine mapped to a 300 Mb region of chromosome 11 (Yang *et al.* 2014), allowing sourcing of new, tightly linked markers for this locus. *Ty2* linked marker (T0302) was shown to amplify a fragment that could differentiate a *Ty2* carrying line from the other lines with a co-dominant banding pattern in earlier studies (Prasanna *et al.* 2014). Several laboratories have tried combining multiple primers for different genes in a single reaction. Combining PCR primers targeting different resistance genes in one multiplex reaction reduces the workload and costs for marker-assisted selection. Therefore, in the present investigation, the three markers namely, FLUW-25, P-625 and T0302 were tested on 15 genotypes harbouring various resistance QTLs for TLCV using unipelx and multiplex assay.

In our study marker TG 0302 could clearly distinguish allele *ty2* from *Ty2* on the basis of presence of 800bp fragment in susceptible genotypes or 900bp fragment in resistant genotypes. Likewise, marker FLUW 25 was found to detect the *Ty3* introgressions on the basis of an amplicon of 640bp, but it could not detect *Ty3a* introgressions. However, Marker P6-25 could specifically distinguish between *ty3*, *Ty3* and *Ty3a* alleles on the basis of fragment sizes of 320bp, 450bp and 630 bp respectively, and, hence, it was found to be an efficient marker for selection of introgression from *S. chilense* LA2779 or *S. chilense* LA1932 lines. Further, the line TMB486 which has known resistance QTL for *Ty1* locus gave a 630bp band which is specific for *Ty3a* allele with marker P6-25 in both uniplex and duplex assays. Since *Ty1* allele was originally introgressed from *S. chilense* LA1969 and both the loci *Ty1* and *Ty3* loci are known to be allelic, therefore, this may be a reason for occurence of *Ty3a* specific band in this line. Ji *et al.* (www.plantpath.wisc.edu) reported that this primer pair detects another PCR fragment size of 660 bp with three commercial hybrids which on sequencing showed 100% identity with the fragment from *S. chilense* LA1969 but they did not suggest the size of fragment in lines having *Ty1* loci. The current study also demonstrated the feasibility of combining TG0302 and P6-25 markers for a duplex assay for simultaneously detecting three important resistance gene loci, viz. *Ty2, Ty3* and *Ty3*a for breeding tropical tomato.

The *Ph-3* locus that has been derived from *Solanum pimpinellifolium* L3708, provides resistance to multiple isolates *of P. infestans* and therefore has been widely used for tomato improvement programmes (Zhang *et al.* 2014). This gene has been mapped to 0.5 cM region on the long arm of chromosome 9 between the markers, Indel_3 and P5. We have used a SCAR marker RGA2M1 shown to be located within this region by Zhang *et al.* (2013) for detection of *Ph3* gene. This marker produced an amplicon of approximately 1000bp in the resistant line, whereas susceptible lines amplify a larger fragment of about 1200bp. The present study validated four highly efficient markers for identification of TYLCV and late blight resistant loci and these will enable breeders to exploit these markers for pyramiding of major *Ty* and late blight resistant loci in the pursuit of stable and broad spectrum resistance to multiple diseases.

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