Validation of molecular markers for multiple disease resistance in tomato (Solanum lycopersicum)

ZAKIR HUSSAIN1, SUMAN LATA2, MANISHA MANGAL3, B S TOMAR4, R K YADAV5, GOKUL GOSAVI6, ASHWANI KUMAR7, PAWAN YADAV8, MONIKA9 and S K YADAV10

ICAR-Indian Agricultural Research Institute, Pusa Campus, New Delhi 110 012, India

Received: 6 September 2018; Accepted: 31 December 2018

ABSTRACT

Marker assisted breeding has been successfully used for selecting disease resistance by identifying genetic markers linked to resistance genes/allele. In tomato, availability of molecular markers linked to genes conferring resilience against Tomato leaf curl virus (ToLCV) reaction, late blight, Tomato Mosaic Virus (ToMV) and Tomato gray leaf spot were utilised to develop pyramided MAS derived lines for multiple disease resistance. For this purpose, markers for the tomato leaf curl disease (ToLCD) resistance gene loci Ty-2, Ty-3 and Ty-3a, late blight resistant loci Ph-3, ToMV resistant allele Tm22 and Tomato gray leaf spot resistant gene Sm were validated using PCR assay. The assay correctly predicted the genotypes of tomato breeding lines harbouring resistant as well as susceptible alleles at each loci. Duplexing PCR assay combining two SCAR markers (T0302 and P6-25) for detecting simultaneously 3 important resistance gene loci (Ty-2, Ty-3 and Ty-3a) in tomato genotypes and thereby improving the cost efficiency of the PCR assay. Further, we have validated the Tomato leaf curl New Delhi virus (ToLCNDV) infection in tomato leaves by Reverse transcription (RT) PCR with ToLCNDV genome specific AC4 primers.

Key words: Late blight, Marker-assisted selection (MAS), Tomato, Tomato gray leaf spot, Tomato leaf curl virus (ToLCV), Tomato Mosaic Virus (ToMV)

Tomato production is often threatened due to severe losses caused by various pathogens including viruses, bacteria, fungi, and nematodes. Molecular breeding using molecular markers are now being widely used in tomato (Foolad and Sharma 2004). There are more than 40 genes that confer resistance to major classes of tomato pathogens which can be pyramided through MAS, for the introgression of more than one resistance genes into 1 genotype.

In India, tomato leaf curl disease (ToLCD) is one of the most devastating diseases of tomato. Symptoms of ToLCD include stunting, yellowing, leaf curling and flower abortion which results in significant yield loss (Chakraborty et al. 2009). Most of tomato cultivars in India are susceptible to ToLCD. However, several wild species of tomato including Solanum chilense, Solanum habrochaites and Solanum peruvianum (Ji et al. 2007a) have been found promising for ToLCD resistance. Currently, 6 resistance genes i.e. Ty-3and Ty-3 on chromosome 6, Ty-2 on chromosome 11, Ty-4 on chromosome 3, ty-5 on chromosome 4 and Ty-6 on chromosome 10 are available for commercial breeding against ToLC (Zamir et al. 1994, Ji et al. 2007b). Ty-3and Ty-3 were demonstrated to be allelic (Verlaan et al. 2013). Ty-2 originated from S. habrochaites B6013 (Ji et al. 2009a) mapped to a 300 kb interval on chromosome 11 (Yang et al. 2014). Ty-4 (Ji et al. 2009b) and Ty-6 (Hutton and Scott 2013) have also originated from S. chilense accessions. Recessive resistance against TYLCV was identified in S. peruvianum and designated as ty-5 (Hutton et al. 2012).

The Ph-3 gene with a coiled-coil nucleotide-binding leucine-rich repeat (NBS–LRR), conferring incomplete resistance against a wide range of Phytophthora infestans isolates has been introgressed into cultivated tomatoes from S. pimpinellifolium (L3708) (Zhang et al. 2014).

Tomato Mosaic Virus (ToMV) disease is another important disease of tomato which causes significant yield losses. Based on molecular studies the Tm22 allele derived from Solanum chilense has proved most efficient in providing resistance against ToMV (Lanfermeijer et al. 2003). Tomato gray leaf spot is another devastating disease of tomato caused by Stemphyli um lycopersici. The Sm gene has incomplete dominance and considered as effective source of resistance against 4 species of Stemphyli um (Scott and Gardner 2006).

Present address: 1,3,4,5Principal scientist (drzakirhussain24@gmail.com, manishamangal@iari.res.in, bst_spu_iari@rediffmail.com, rkyadavneh@gmail.com); 2Scientist (sumanlata3@gmail.com), 6,7,8,9SRF (gosavi.gokul@gmail.com, ashwinikumar1500@gmail.com, pawanyadav0626@gmail.com, ICAR-IARI); 10(satish.yadav1@icar.gov.in), ICAR-NBPGR.
In this study, different allele specific molecular markers for the Ty-2, Ty-3, Ty-3a, Ph-3, Tm-2 and Sm resistance loci were validated in available germplasm and F₁ hybrids (Table 1) so that the reproducible markers could be used for Marker-assisted selection (MAS) for gene pyramiding in commercial tomato cultivars. In addition, we have confirmed the ToLCNDV symptoms in the field grown tomato leaf samples using one of ToLCNDV gene specific primer AC4, using RT PCR.

Table 1 List of representative set of tomato breeding lines

| Genotype | R/S |
|----------|-----|
| EC904111 | R   |
| EC814916 | R   |
| EC904112 | R   |
| EC814915 | R   |
| EC814917 | R   |
| Pusa Rohini | S |
| Pusa Ruby | S |
| Pusa-120 | S |
| Pusa Sadabahar | S |
| Pusa Sheetal | S |
| H-86 | S |
| F₁ (Pusa Ruby × EC814916) | R/S |
| F₂ (Pusa Ruby × EC814916) populations | R/S |
| Rohini × EC814916 | R/S |
| H-86 × EC814916 | R/S |
| Pusa-120 × EC814916 | R/S |
| Pusa Rohini × EC814916 | R/S |
| Pusa Sheetal × EC814916 | R/S |

Table 2 Molecular markers used for validation of Ty-2 Ty-3 Ty-3a Ph-3 Tm-2 and Sm alleles in the genotypes under study and their primers sequences

| Resistance Gene | Marker | Resistant allele (bp) | Susceptible allele (bp) | Primer sequence (5´-3´) | Reference |
|-----------------|--------|-----------------------|-------------------------|--------------------------|-----------|
| Ty-2 | T0302 | 900 | 791 | F.P: TGGCTCATCCTGAAGCTGATAGGC | Yang et al. (2014) |
| Ty-2 | TG36 | 520 | 450 | R.P: AGTGTACATCCTTGCCATTGACT | Schmitz et al. (2002) |
| Ty-3 | P6-25 | 450 | 320 | F.P: AACCACCAAAAGGATGCCC | Jensen et al. (2007) |
| Ty-3 | TY3-SCAR1 | 519 | 269 | R.P: TTGAGGATAAGATGATGC | Dong et al. (2016) |
| Ph-3 | TG328 | 260 + 240 | 500 | BstNI | Robbins et al. (2010) |
| Sm | CT-55 | 200 + 140 | 330 + 200 + 140 | DdeI | Ji et al. (2009) |
| Tm-2 | NCTm-019 | 270 + 600 | 870 | HaeIII | Panthee et al. (2013) |

MATERIALS AND METHODS

The breeding lines and F₁ hybrids used in this study (Table 1) were maintained at the research farm of IARI, New Delhi, India. Young and healthy leaves from each genotype were collected for genomic DNA extraction following the C-TAB method (Murray MG and Thomson 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).

Markers used for validation of different genes (Table 2) were custom synthesized (G- Biosciences, USA). PCR was carried out in 10 µl volumes with 40 ng genomic DNA, 0.5 U Taq DNA polymerase (G- Biosciences, USA), 1.0 µM of each primer, 0.5 µl of 10 mM dNTP mix (G-Biosciences, USA), and 1.0 µl of 10× PCR buffer having 17.5 mM MgCl₂ (G- Biosciences, USA). All the primers were amplified using touchdown PCR in an Eppendorf Mastercycler (Germany). For amplification of Ty genes the annealing temperatures (Tₐ) were 53°C for 1 min followed by 72°C for 1 min and a final cycle of 72°C for 5 min for 35 cycles. Amplification conditions used for Ph-3 genes were, one cycle of 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 57°C for 30 seconds and 72°C for 1 min and final extension at 72°C for 10 min. Amplification conditions for Tm-2 and Sm genes were one cycle of 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 55°C for 30 seconds and 72°C for 1 min and final extension at 72°C for 10 min. Amplified products were resolved on 2.5% agarose gels and observed under UV light (Alpha imager, Cell biosciences, Santa Clara, CA).

Leaf samples for RNA isolation were collected from field, 4 month after transplanting. Total RNA was isolated from the tomato leaves using Spectrum plant total RNA isolation kit (Sigma, USA) following manufacturer instructions. To remove any DNA contamination, RNA samples were subjected to column DNase-I treatment.
(Sigma, USA). 1 µg of RNA was taken for cDNA synthesis (Applied biosystem cDNA synthesis kit). Concentration of cDNA was checked using Nanodrop spectrophotometer (Thermo Scientific, USA). RT PCR was done to detect the AC4 transcripts in the cDNA samples. AC4 primers (F CTTAGAAGCTCCGCTTTTGTGATGT, R GGGTCTCCGCAATCCATGTTCTCTCA) specific to the ToLCD viral genome were procured (Sigma Aldrich Chemical Pvt. Ltd, Bangalore, India). The reaction was set up with single strand cDNA (template) 2 µL (100 ng), Buffer (10×) 5 µL, dNTP (10mM) 1.5 µL, AC4 Forward primer 1 µL (20 pmol), AC4 Reverse primer 1 µL (20 pmol), Taq polymerase 1 µL (3U), final reaction volume of 50 µL. The amplification was performed in a Thermal cycler (Eppendorf, Germany) at temperatures (Tm) 60°C for 30 seconds followed by 72°C for 30 seconds. Final extension at 72°C for 10 min. Amplified products were visualized on gel.

RESULTS AND DISCUSSION

Validation of markers for Ty-2, Ty-3 and Ty-3a genes: The markers for ToLCD resistance were identified from wild species like S. chilense (Ty-1, Ty-3, Ty-4 and Ty-6), S. habrochaites (Ty-2) and from S. peruvianum (ty-5) (Ji et al. 2009b, Hutton et al. 2012, Hutton and Scott 2013, Verlaan et al. 2013, Yang et al. 2014). Availability of reproducible and reliable markers can greatly aid in molecular breeding for important agronomic traits like resistance to various diseases. The resistant donors, susceptible lines and F1 population (Table 1) derived from donor lines (S. chilense and S. habrochaites) were screened for the introgression of Ty-2 and Ty-3 genes. For genotyping of Ty-2 gene, we have used (TG36) a CAPS marker identified by Schmitz et al. (2002). It is clear from Fig. 1a that marker (TG36) (Schmitz et al. 2002) is capable to discriminate resistant donor line, susceptible var. (Pusa Ruby) and F1 hybrids by amplifying 520 bp and 450 bp fragments. In case of heterozygotes both fragments were amplified (Fig 1a).

Dong et al. (2016) designed gene based SCAR marker Ty-3 SCAR1 that could be effectively used for the selection of Ty-3 locus (ty-3/Ty-3 loci). In our genotypes marker Ty-3 SCAR1 (Fig. 1b) was found to detect the Ty-3 introgressions on the basis of an amplicon of 519 bp and ty-3 susceptible allele with 269 bp amplicon. It was reported that the sequences for Ty-3 alleles were different for lines derived from S. chilense LA2779 and LA1932 (Maxwell et al. 2007) and the 2 different introgressions were designated as Ty-3 from LA2779 and Ty-3a from LA1932. Jensen et al (2007) designed a set of PCR primers, viz. P6-25F2/P6-25R5 which could discriminate Ty-3 and Ty-3a introgressions and a newly discovered introgression from S. chilense LA1969. These primers gave 450-bp fragments in lines derived from either the S. Chilense LA2779, while a 320-bp ty-3 fragment was amplified from susceptible breeding lines. In addition, a 630-bp Ty-3a allele was found in lines derived from S. Chilense LA1932. Respective heterozygous alleles were detected in hybrids.

We have used marker P6-25 which specifically distinguished between ty-3, Ty-3 and Ty-3a alleles on the basis of fragment sizes of 320 bp, 450 bp 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 (Fig 1c). Since Ty-1 allele was originally introgressed from S. Chilense LA1969 and both the loci Ty-1 and Ty-3 are known to be allelic, therefore, this may be a reason for occurrence of Ty-3a specific band in this line. For genotyping of Ty-2 gene, we have also used (T0302) a tightly linked SCAR marker identified by Yang et al. (2014). Marker (T0302) (Yang et al. 2014) is capable to discriminate resistant donor line, susceptible var. (Pusa Ruby) and (F1 hybrids) by amplifying 900 bp and 800 bp fragments in a duplex assay with P6-25 (Jensen et al. 2007) (Fig 1c). In case of heterozygote both fragments were amplified (Fig 1c).

Testing of markers for Ph-3, Tm2 and Sm genes: For Ph-3 gene a CAPS marker TG328 developed by Robbins
et al. (2010) was used. This marker produced an amplicon of approximately 260/240 bp in the resistant line, whereas susceptible lines amplified a longer fragment of about 500 bp (Fig 2a). To validate Tm2<sup>2</sup> allele for ToMV resistance, NCTm-019 marker developed from Tm2<sup>2</sup> locus by Panthee et al. (2013) based on restriction site differences. The resistant line gave a fragment of 270+600 bp and in heterozygotes 3 fragments were produced (270+600 bp and 870 bp) upon restriction of amplified product with Hae III (Fig 2b). For confirmation of Sm gene linked to tomato gray leaf spot resistance in the germplasm lines (Table 1) recessive CAPS marker CT-55 developed by Ji et al. (2009) was used. The resistant line generated a fragment of 200 bp and 140 bp, while an additional fragment of 330 bp amplified in susceptible line along with 200 bp and 140 bp fragment (Fig 2c).

Screening for Tomato leaf curl New Delhi virus (ToLCNDV): ToLCNDV is the most predominant begomovirus in northern India. The present study was undertaken to know the ToLCNDV status in the tomato lines (Ty-2 and Ty-3 donor lines, susceptible var. and F<sub>1</sub> hybrids) (Table 1) grown in the IARI field. ToLCNDV genome specific AC4 primers were used to detect viral titre by RT PCR in the cDNA samples of above mentioned lines. An amplicon of 180 bp specific for AC4 marker was detected which indicates the presence of virus in all the samples tested under study (Fig.3). The presence of virus also in donor lines indicates that Ty genes provide immunity but not resistance.

Multiplexing reduces the workload and costs for marker-assisted selection (Maxwell et al. 2007). Therefore, in the present investigation 6 markers, viz. TG36, T0302, SCAR1, P-625, Ph-3, Tm2<sup>2</sup>, and Sm were tested on genotypes harbouring various resistance alleles for ToLCV, late blight, ToMV and grey leaf spot using uniplex and multiplex assay. 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. T<sub>y</sub>-2, T<sub>y</sub>-3 and T<sub>y</sub>-3a for breeding tropical tomato. The present study validated 4 highly efficient markers for identification of ToLCV, late blight, ToMV and grey leaf spot resistant loci and these will enable breeders to exploit these markers for pyramiding of resistant loci in the pursuit of stable and broad spectrum resistance to multiple diseases.

**ACKNOWLEDGEMENTS**

The author acknowledges the ICAR-CRP project ‘Molecular breeding for improvement of ToLCV tolerance in tomato’ for financial support.

**REFERENCES**

Anbinder I, Reuveni M, Azari R, Paran I, Nahon S, Shlomo H, Chen L, Lapidot M and Levin L. 2009. Molecular dissection of Tomato leaf curl virus resistance in tomato line TY172 derived from Solanum peruvianum. Theoretical and Applied Genetics 119(3): 519–30.

Chakraborty S. 2009. Tomato leaf curl viruses from India, pp. 339–47. (Eds) Mahy B and Regenmortel M V. Desk Encyclopedia of Plant and fungal virology. Academic press.

Dong P, Siddique M I, Zhao, M and Kang B C. 2016. Gene-based markers for the Tomato yellow leaf curl virus resistance gene Ty-3. Plant breeding and biotechnology 4(1): 79–86.

Foolad M R and Sharma A. 2004. Molecular markers as selection tools in tomato breeding. International Symposium on Tomato Diseases 695: 225–40.

Hutton S F and Scott J W. 2013. Fine-mapping and cloning of Ty-3 and Ty-3<sub>a</sub> and mapping of a new TYLCV resistance locus Ty-6. Tomato Breeders Round Table. University of Florida.

Hutton S F, Scott J W and Schuster D J. 2012. Recessive resistance to Tomato yellow leaf curl virus from the tomato cultivar Tyking is located in the same region as Ty-5 on chromosome 4. Horticultural Science 47(3): 324–7.

Jensen K S, Van Betteray B, Smeets J, Yuanfu J, Scott J W, Mejia L, Havey M J and Maxwell D P. 2007. Co-dominant SCAR Marker for Detection of the Ty-3 and Ty-3a alleles at 25cM of chromosome 6 of Tomato. Tomato Genetics Cooperative 57: 6–25.

Ji Y, Schuster, D J and Scott J W. 2007. Ty-3, a begomovirus resistance locus near the Tomato yellow leaf curl virus resistance loci Ty-1 on chromosome 6 of tomato. Molecular Breeding 20(3): 271–84.
Ji Y, Scott J W and Schuster D J. 2009. Toward fine mapping of the tomato yellow leaf curl virus resistance gene Ty-2 on chromosome 11 of tomato. Horticultural Science 44(3): 614–8.

Ji Y, Scott J W, Hanson P, Graham E and Maxwell D P. 2007. Sources of resistance, inheritance, and location of genetic loci conferring resistance to members of the tomato-infecting begomoviruses, pp. 343–62. Tomato Yellow Leaf Curl Virus Disease. Springer, Dordrecht.

Ji Y, Scott J W, Schuster D J and Maxwell D P. 2009. Molecular mapping of Ty-4, a new tomato yellow leaf curl virus resistance locus on chromosome 3 of tomato. Journal of the American Society for Horticultural Science 134(2): 281–8.

Ji Y, Scott J W and Maxwell D P. 2009. CAPS marker linked to the tomato gray leaf spot (Stemphyllium sp.) resistance gene Sm. Report of Tomato Genetics Cooperative 59: 29–31.

Ji Y, Scott J W, Hanson P, Graham E and Maxwell D P. 2007. Sources of resistance, inheritance, and location of genetic loci conferring resistance to members of the tomato-infecting begomoviruses, pp. 343–62. Tomato Yellow Leaf Curl Virus Disease. Springer, Dordrecht.

Lanfermeijer F C, Dijkhuis J, Sturre M J, de Haan P and Hille J. 2003. Cloning and characterization of the durable tomato mosaic virus resistance gene Tm-2 from Lycopersicon esculentum. Plant molecular biology 52(5): 1039–51.

Maxwell D P, Martin C T, Garcia B E, Salus M S, Jensen K S, Havey M J and Mejia L. 2007. Markers for tomato chromosomes. www.plantpath.wisc.edu/Geminivirus Resistant Tomatoes.

Murray M G and Thompson W F. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic acids research 8(19): 4321–6.

Panthee D R, Brown A F, Yousef G G, Ibrahim R and Anderson C. 2013. Novel molecular marker associated with Tm2a gene conferring resistance to tomato mosaic virus in tomato. Plant Breeding 132(4): 413–6.

Robbins M D, Masud M A, Panthee D R, Gardner R G, Francis D M and Stevens M R 2010. Marker-assisted selection for coupling phase resistance to tomato spotted wilt virus and Phytophthora infestans (late blight) in tomato. Horticultural Science 45(10): 1424–8.

Schmitz G, Tillmann E, Carriero F, Fiore C, Cellini F and Theres K. 2002. The tomato Blind gene encodes a MYB transcription factor that controls the formation of lateral meristems. Proceedings of the National Academy of Sciences 99(2): 1064–9.

Scott J W and Gardner R G. 2006. Breeding for resistance to fungal pathogens, pp. 421–56. Genetic Improvement of Solanaceous Crops. CRC Press, Taylor & Francis Group.

Verlaan M G, Hutton S F, Ibrahem R M, Kormelink R, Visser R G, Scott J W, Edwards J D and Bai Y. 2013. The tomato yellow leaf curl virus resistance genes Ty-1 and Ty-3 are allelic and code for DFDGD-class RNA-dependent RNA polymerases. PLoS genetics 9(3): p. e1003399.

Yang X, Caro M, Hutton S F, Scott J W, Guo Y, Wang X, Rashid M H, Szinay D, de Jong H, Visser R G and Bai Y. 2014. Fine mapping of the tomato yellow leaf curl virus resistance gene Ty-2 on chromosome 11 of tomato. Molecular Breeding 34(2): 749–60.

Zamir D, Ekstein-Michelson I, Zakay Y, Navot N, Zeidan M, Sarfatti M, Eshed Y, Harel E, Pleban T, Van-Oss H and Kedar N. 1994. Mapping and introgression of a tomato yellow leaf curl virus tolerance gene Ty-1. Theoretical and Applied Genetics 88(2): 141–6.

Zhang C, Liu L, Wang X, Vossen J, Li G, Li T, Zheng Z, Gao J, Guo Y, Visser R G and Li J. 2014. The Ph-3 gene from Solanum pimpinellifolium encodes CC-NBS-LRR protein conferring resistance to Phytophthora infestans. Theoretical and Applied Genetics 127(6): 1353–64.