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Evaluation of tomato parental lines for leaf curl disease resistance and its validation through molecular markers

Rahul Chandel*, A. T. Sadashiva, T. H. Singh**

Dept. of Vegetable Science, College of Horticulture, University of Horticultural Sciences, Bagalkot 587104, Karnataka

ABSTRACT

The tomato leaf curl virus (ToLCV) is the most devastating viral disease of tomato. ToLCV is transmitted by whitefly (*Bemisia tabaci* Genn.) in a persistent and circulative manner. No viricides are available to combat the viral diseases and insect vectors had developed resistance against the various groups of insecticides. So, it becomes necessity to develop varieties and hybrids carrying genetic resistance. Fifteen entries comprised of eleven advanced breeding lines, two resistant and two susceptible checks were screened against tomato leaf curl Bangalore virus (ToLCBV) resistance in the greenhouse using natural vector whiteflies. Among fifteen entries five (IIHR-2852, IIHR-2919, IIHR-2913, IIHR-2902, and IIHR-2907) have shown a highly resistant reaction, two (IIHR-2853, Abhinav) have shown a resistant reaction, and other were either susceptible or highly susceptible in respect of disease reaction. Two molecular markers *Ty-2* and *Ty-3* linked to ToLCV resistance were validated with fifteen entries and a susceptible check as Punjab Chhuhara. Among these, two entries have shown the presence of both *Ty-2* and *Ty-3* genes for ToLCBV resistance. Punjab Chhuhara and 15-SB-SB did not show any presence of the *Ty-2* and *Ty-3* resistant alleles. Further, these resistance lines can be used to develop varieties/hybrids resistant to ToLCBV with better yield and quality attributes.

Key words: *Solanum lycopersicum*, Ty genes, *Ty-2*, *Ty-3*, marker assisted selection.

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of the most important and extensively grown vegetables around the world. It is grown for its edible fruits, which are consumed either as fresh, cooked or as processed products like juices, ketchup, sauce, puree and pickle, etc. Tomato fruit is a rich source of Vitamin 'A', Vitamin 'C', minerals and organic acids (Singh *et al*., 9). The Successful cultivation of tomato has been globally challenged by Tomato leaf curl virus disease. Despite the numerous efforts made by breeders towards the development of resistance varieties/hybrids in the past few decades, tomato leaf curl disease is still being the most devastating disease. Tomato leaf curl disease (ToLCD) was, first reported in India by Vasudeva and Samraj (10). The infected susceptible plants show stunted growth, severe curling of leaves, and reduction in the size of leaves and internodes, twisting and rolling of the leaves accompanied by dark green outgrowth or enations of the vein on the undersurface of the leaflets which can results in up to 100% yield loss (Abhary *et al*., 1; Muniyappa and Saikia, 6: Sastri *et al*., 7). The disease is caused by a Begomovirus, transmitted naturally by the whitefly vector (*Bemisia tabaci* Genn.) in a persistent circulative manner. There are two types of vector viz., indigenous and B. The B biotype is more dangerous due to its greater fecundity, strong pesticide resistance, broad host range and virulence. This has posed a serious problem for the breeders to take up any successful breeding programme to develop varieties / hybrids with stable resistance to ToLCV.

Large fruited domesticated tomato is highly prone to ToLCV. The wild species of tomato possess resistance to ToLCV and rigorous screening programs were carried out to know their response towards tomato leaf curl virus. So far six Ty genes were reported to be present in various wild species such as *Solanum chilense*, *S. peruvianum*, *S. cheesmaniae,* S. *pimpinellifolium*, and *S. habrochaites*. *Ty*-1 and *Ty*-3 were reported to have their origin from two accessions (LA 1969 and LA 2779) of *S. chilense*. A major gene *Ty*-2 has been known to originate from accession B6013 of *S. habrochaites.* In addition to this, three more genes *Ty*-4, *ty*-5 and *Ty*-6 were also identified. *Ty*-4 and *Ty*-6 both were identified in *S. chilense, whereas ty*-5, was suggested to be derived from the complex of *S. peruvianum* accessions.

A number of PCR based markers are present nowadays, which are linked to the Ty genes (*Ty*-1 to *ty*-5). These markers can effectively utilize for pyramiding of these genes into a single line/ genotype to attain maximum and stable resistance. The validation of molecular markers establishes the

*Corresponding author: rahulchandel02@gmail.com

**Division of Vegetable Crops, ICAR-IIHR, Bengaluru 560089

value of a marker among different lines which belongs to different backgrounds in which recombination would have occurred in varying degrees and chances were there that maker may have been got separated from the trait of interest. Also, this validation process is also required to assess the usefulness of published reports of linked markers developed using genetic material divergent from that in target breeding programmes. Hence, the present study was carried out to screen the parental lines and validation of the reported markers (*Ty*-2 and *Ty*-3) linked to ToLCV resistance in diverse set of genotypes.

MATERIALS AND METHODS

A total of fifteen entries, eleven advanced breeding lines among them five (IIHR-2852, IIHR-2853, IIHR-2898, IIHR-2902 and IIHR-2907) were outsourced from World Vegetable Center in Taiwan. Five advanced breeding lines (IIHR-2886, IIHR-2888, IIHR-2919, IIHR-5-3-7-5 and TLBR-6) were developed earlier at ICAR-IIHR and a line IIHR-2913 from Indian Institute of vegetable research, Varanasi along with two susceptible checks (Punjab chuhhara and 15-SB-SB) and two resistant checks (Arka Rakshak and Abhinava), were used for screening against ToLCV. These resistant entries included, Twelve ToLCV resistant parent (IIHR-2852, IIHR-2853, IIHR-2886, IIHR-2888, IIHR-2898, IIHR-2919, IIHR-5-3-7-5, IIHR-2913, TLBR-6, IIHR-2902 and IIHR-2907), two resistant commercial hybrids (Arka Rakshak, Abhinav); one breeding line 15-SB-SB. Tomato variety Punjab Chhuhara was used as susceptible check. The experiment was conducted in glasshouse at Indian Institute of Horticultural Research, Bengaluru. Disease reaction were given on the basis of disease severity index. The experiment for screening of genotypes against ToLCV was laid out in Complete Randomized Design (CRD) replicated twice. All recommended agronomical practices were followed as per the institute guidelines.

Maintenance of *B. tabaci* culture

The type culture of whitefly (*Bemisia tabaci* Genn.) was collected from the brinjal field of UAS, Bengaluru and was maintained on cotton and also on brinjal plants grown in insect proof screen house. Cotton and eggplant seedlings were grown in the small plastic pots or in polythene bags, which are the host plants for *Bemisia tabaci* and the whiteflies were reared on these cotton and eggplant in wooden insect proof screen house which was built under the glass house at Experimental plot (Block-8), Division of vegetable crops, Indian Institute of Horticultural Research (IIHR), Hessaraghatta, Bengaluru.

Maintenance of ToLCBV culture

Pre confirmed cultures of ToLCBV maintained in the Plant Virology lab at ICAR-IIHR, Bengaluru was used as initial source of ToLCBV inoculum. Seedlings of susceptible tomato variety Punjab Chhuhara were raised and allowed to feed by whiteflies in order to transmit ToLCV from infected plants collected from the field. ToLCV cultures were thus maintained continuously in the screen house.

Acquisition feeding studies

The 20 cm long plastic bottle with 7.5 cm diameter at one end and tapering towards the narrow mouth was used to prepare cage to ToLCV acquisition. The white flies were collected in to the bottle and the ToLCV infected tomato branch was inserted into the bottle through the narrow mouth and then closed with cotton plug. The 24 hours acquisition access feeding period was given. After the acquisition access period, the viruliferous whiteflies were taken and used for inoculation.

Raising of healthy tomato seedlings for inoculation feeding studies

Healthy seedlings of tomato at two cotyledonary leaf stage were used for conducting transmission studies. The seedlings were raised in plastic pots and they were kept in insect proof cages in the glasshouse and 10-12 days old seedlings were used for transmission studies.

Inoculation feeding and transmission studies

Mylar plastic tubes of 8 cm height and 5 cm diameter were used for conducting the transmission studies. Muslin cloth was fixed to the sides of plastic tube to avoid the accumulation of excess moisture inside the cage. Tomato seedlings of 10-12 days age (first two-true leaf stage) were inserted into the plastic tube. Ten viruliferous whiteflies were released from the top of the tube and enclosed with muslin cloth and tied with rubber band. After allowing two days inoculation feeding, the Mylar cages were removed and the plants were sprayed with imidachloprid and kept in the glasshouse for symptom development.

Screening of tomato genotypes for ToLCV resistance

Fifteen tomato entries were screened under glasshouse conditions individually against ToLCV disease by releasing the viruliferous whiteflies. Ten plants of each tomato genotypes/lines were screened at their two cotyledonary leaf stage along with susceptible check Punjab Chhuhara. The number of diseased and healthy plants was recorded after 30, 45 and 60 days of inoculation by counting leaf

curled plants per genotype and the per cent disease incidence was calculated by using the following formula.

$$\text{ToLCBV incidence (\%)} = \frac{\text{Total number of plants infected with ToLCBV}}{\text{Total number of plants observed}} \times 100$$

$$\text{Disease severity (\%)} = \frac{(1n_1 + 2n_2 + 3n_3 + 4n_4 + 5n_5)}{\text{Tn (Nc-1)}} \times 100$$

Where, 1 -5: Disease categories, n1 to n5: Number of plants in respective disease categories, Tn: Total number of plants, Nc: Number of categories

The following scale was employed for scoring the disease reaction on the different genotypes were categories as follows:

Symptoms	Disease severity (%)	Grade	Disease reaction
Symptoms absent	0%	1	Highly resistant
Very mild curling up to leaves 25 %	1-10%	2	Resistant
Curling, puckering of leaves up to 50%	11-20%	3	Moderately Resistant
Curling, puckering of leaves up to 75 %	21-40%	4	Susceptible
Severe curling, puckering of >75% of leaves	< 40%	5	Highly susceptible

DNA extraction

DNA was extracted using CTAB method (Doyle and Doyle, 4) from fresh tomato leaves collected from the 15 entries after screening. The molecular markers linked to ToLCV resistance used in the present study are listed in Table 1 and PCR mixture components and conditions of these molecular markers used are listed as follows-

PCR Reaction Mixture and Conditions for *Ty*-2 Marker

The PCR reaction was carried out in a total volume of 25 µl and standardized using 10x incomplete buffer, 25 mM MgCl2, 5 µM of each forward and reverse primer, 1 mM dNTPs, 1U Taq polymerase, 20 ng of template DNA and Distilled water. The amplification was carried out in an Eppendorf Mastercycler Thermal Cycler with the following conditions- Initial Denaturation 94°C for 5 min, 35 cycles of 94°C for 30 sec, 55°C for 1 min and 72°C for 2 min, Followed by final extension of 72°C for 8 min and Store at 4°C.

PCR Reaction Mixture and Conditions for *Ty*-3 Marker

The PCR reaction was carried out in a total volume of 25 µl and standardized using 10x incomplete buffer, 25 mM MgCl2, 5 µM of each forward and reverse primer, 1 mM dNTPs, 1U of Taq polymerase, 20 ng of template DNA and Distilled Water. The amplification was carried out in an Eppendorf Mastercycler Thermal Cycler with the following conditions- Initial Denaturation 94°C for 4 min, 35 cycles of 94°C for 30 sec, 53°C for 1 min and 72°C for 1 min, Followed by final extension of 72°C for 10 min and Store at 4°C.

RESULTS AND DISCUSSION

Tomato cultivation suffers to a great extent due to various biotic and abiotic stresses. The present study was focused on the evaluation of a diverse set of advanced breeding line for ToLCV resistance and validation of *Ty*-2 and *Ty*-3 linked molecular markers. Results as indicated in the (Table 2) revealed a wide range of resistance reaction to the tune of 0 to 83.33 % among the fifteen entries evaluated for ToLCV resistance under glasshouse conditions. Among the various entries, IIHR-2852, IIHR-2919, IIHR-2913, IIHR-2902 and IIHR-2907 were found to be highly resistant without any symptom (0.00) up to 60 days. Two entries have shown resistant reaction IIHR-2853 (19.17) and Abhinava (15.62) while the genotypes, IIHR-2886 (46.23), IIHR-2888 (40.07), IIHR-2898 (23.92), IIHR-5-3-7-5 (44.77), TLBR-6 (43.93) and Arka Rakshak (33.85) showed the susceptible reaction. Punjab Chhuhara (83.33) and 15-SB-SB (74.98) showed highly susceptible reaction against ToLCBV (Table 2). In the earlier studies (De Hoop, 2), it was reported that *Ty*-2, a dominant gene imparts higher level of resistance than other Ty genes. In this study it has been noticed that lines which have *Ty*-2 alone developed susceptible reaction within 30 days of inoculation, but one advanced breeding line IIHR-2853 which has only *Ty*-2 showed resistance. This might be due to the presence of some other resistant gene that is imparting some degree of resistance. Another resistance imparting gene *Ty*-3 is a partial dominant in nature. In this study, one entry Abhinava having *Ty*-3 in heterozygous

Table 1. Molecular markers used to validate ToLCV resistance in tomato.

Ty-2	TG0302	SCAR	F-TGGCTCATCCTGAAGCTGATAGCGC R- AGTGTACATCCTTGC CATTGACT	900(R)/ 800(S)	Garcia *et al*., 5
Ty-3	SCAR1	SCAR	F-GCTCAGCATCACCCTGAGACA R-TGCAGGAACAGAATGATAGAAAA	519 (R)/269 (S)	Dong *et al*., 3

Table 2. Artificial screening of parental lines for ToLCV resistance in tomato.

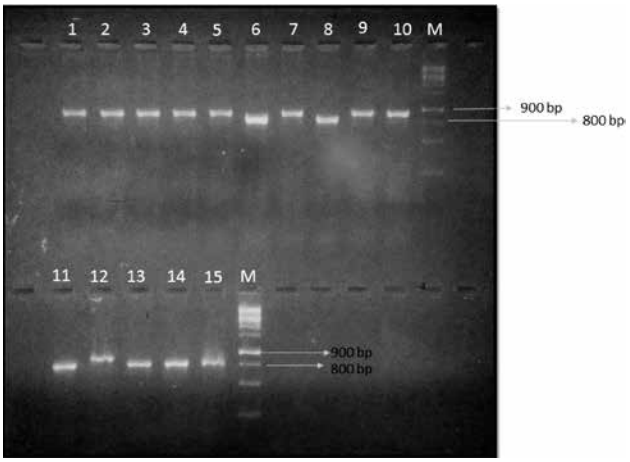
Sl. No.	Parents	PDI at 30 days	PDI at 45 days	PDI at 60 days	Mean PDI	DSI at 30 Days	DSI at 45 Days	DSI at 60 days	Mean DSI	Disease Reaction	*Ty2*	*Ty3*
1	IIHR-2852	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	HR	+	+
2	IIHR-2853	20.00	40.00	50.00	36.67	7.50	21.25	28.75	19.17	R	+	-
3	IIHR-2886	77.78	77.78	88.89	81.48	38.80	44.40	55.50	46.23	S	+	-
4	IIHR-2888	75.00	75.00	75.00	75.00	32.80	40.62	46.80	40.07	S	+	-
5	IIHR-2898	37.50	50.00	62.50	50.00	14.06	23.40	34.30	23.92	S	+	-
6	IIHR-2919	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	HR	-	+
7	IIHR-5-3-7-5	75.00	75.00	87.50	79.17	37.50	40.60	56.20	44.77	S	+	-
8	IIHR-2913	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	HR	-	+
9	TLBR-6	66.67	77.78	77.78	74.07	33.30	45.80	52.70	43.93	S	+	-
10	IIHR-2902	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	HR	+	+
11	IIHR-2907	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	HR	-	+
12	Arka Rakshak	62.50	75.00	75.00	70.83	23.43	37.50	40.62	33.85	S	+	-
13	Punjab chuhhara	100.00	100.00	100.00	100.00	75.00	87.50	87.50	83.33	HS	-	-
14	15-SB-SB	88.89	100.00	100.00	96.30	66.60	79.16	79.16	74.98	HS	-	-
15	Abhinava	0.00	50.00	62.50	37.50	0.00	18.75	28.12	15.62	R	-	+/-
	S.E(m)	0.79	0.65	0.60		1.52	1.34	1.48				
	C.D @ 5%	2.28	1.88	1.74		0.52	0.46	0.51				

R, resistant; MR, moderately resistant; S, susceptible; HR, highly resistant; Present (+); Absent (-); Heterozygous (+/-); PDI, percent disease incidence; DSI, Disease severity index

condition has also produced the symptom, which indicate the partial nature of this gene and to achieve maximum resistance *Ty*-3 gene should be present in homozygous condition

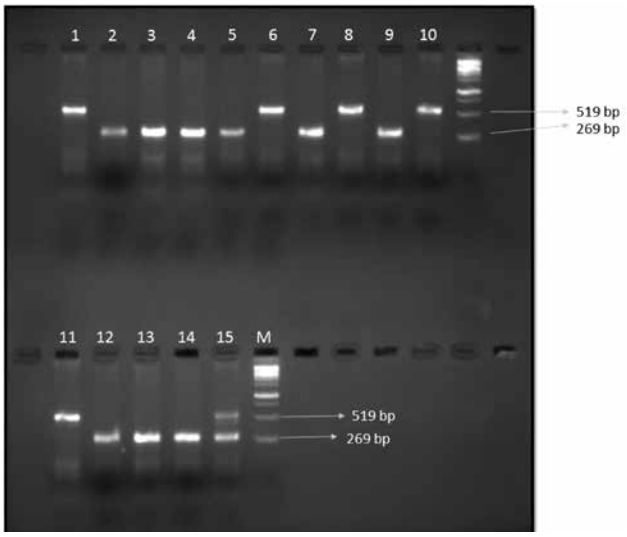
Fifteen entries used in the present study was also used to validate molecular markers linked to Ty-2 and Ty-3 gene using TG0302 primers and SCAR1 primers respectively. In TG0302, upon amplification a band size of 900 bp confer the presence of Ty-2 gene whereas band size of 800 bp shows the absence of Ty-2 gene (Fig. 1). Moreover, in SCAR1, the presence of Ty-3 gene was detected by the presence of resistant allele as 519 bp bands and susceptible allele the presence of 269 bp bands (Fig. 2). Presence of both the bands showed that the genotype was heterozygous for the Ty-3 gene. Among the evaluated 15 entries nine have shown the presence of Ty-2 gene and five entries have shown the presence of Ty-3 gene (Table 2). Two entries IIHR-2852 and IIHR-2902 only have shown the presence of both Ty-2 and Ty-3 genes Table 2.

This information will further help the breeders for rapid screening against ToLCV resistance at seedling stage and also favor the development of stable resistant genotypes by gene pyramiding through MAS. Development of molecular markers



Lane 1- IIHR-2852Lane 9-TLBR-6
Lane 2- IIHR-2853Lane 10- IIHR-2902
Lane 3- IIHR-2886Lane 11-IIHR-2907
Lane 4- IIHR-2888Lane 12-Arka Rakshak
Lane 5- IIHR-2898Lane 13-Punjab chhuhara
Lane 6- IIHR-2919Lane 14-15 SB SB
Lane 7- IIHR-5-3-7-5Lane 15-Abhinav
Lane 8- IIHR-2913

Fig. 1. Validation of *Ty*-2 resistant marker amplification in parents and checks.



Lane 1- IIHR-2852Lane 9-TLBR-6
Lane 2- IIHR-2853Lane 10- IIHR-2902
Lane 3- IIHR-2886Lane 11-IIHR-2907
Lane 4- IIHR-2888Lane 12-Arka Rakshak
Lane 5- IIHR-2898Lane 13-Punjab chhuhara
Lane 6- IIHR-2919Lane 14-15 SB SB
Lane 7- IIHR-5-3-7-5Lane 15-Abhinav
Lane 8- IIHR-2913

Fig. 2. Validation of *Ty*-3 resistant marker amplification in parents and checks.

(PCR based) tightly linked to ToLCV resistant genes, and their validation help the plant breeders to efficiently incorporate these resistant genes into elite tomato genotypes, thus accelerating the breeding of resistant cultivars (Shamprasad *et al*., 8). The strategy of pyramiding resistance genes through marker assisted selection is a valuable method due to an increased resistance and its durability. This will give the tomato breeders more options in providing tomato growers with durable resistance to the begomoviruses along-with their high production. Earlier *Ty*-2 was effectively showing resistance against mono-partite begomovirus, but in this study *Ty*-2 carrying entries also produced susceptible symptoms, while entries carrying *Ty*-3 alone or along with *Ty*-2 does not produced symptoms up to 60 days of inoculation. Thus, in future pyramiding of *Ty*-3 gene into desirable hybrids/varieties background can combat the monopartite and bi-partite viruses of tomato.

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