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Identification and validation of an ISSR marker linked to Tomato leaf curl New Delhi virus resistant gene in a core set of tomato accessions

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Abstract: Tomato leaf curl disease causes yield loss in tomato all over the world. Despite of various efforts no immune commercial varieties or F_1 hybrids are available in India. *Tomato yellow leaf curl virus* resistance linked genes have been identified and introgressed from several wild tomato species. Here, we aimed to identify PCR-based markers linked to gene(s) confering resistance to *Tomato leaf curl New Delhi virus* (ToLCNDV). This study covers the generation of a BC_1F_1 population derived from a reciprocal cross between a ToLCNDV tolerant accession of *Solanum habrochaites* LA1777 and susceptible cultivar 15SBSB (*Solanum lycopersicum* L.). Genetic analysis of 135 plants of BC_1F_1 population indicated that three dominant genes confer resistance to ToLCNDV in the accession *S. habrochaites* LA1777. Using Bulk-Segregant-Analysis, we identified an ISSR marker, which produced a 564 bp fragment in the tolerant wild accession and also in the tolerant bulk sample. The identified marker has been validated in a set of 18 diverse tomato accessions and can be used as a diagnostic marker to assist marker-assisted-breeding for ToLCNDV tolerance in tomato.

Keywords: Bulk-segregant-analysis, Inheritance, Marker-assisted-breeding, Molecular markers, Resistant gene, Tomato, *Tomato leaf curl virus*

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Introduction

Tomatoes undeniably occupy a significant position in world vegetable production due to its world-wide consumption. China, United States of America and India are three leading countries in terms of overall tomato production. However, in terms of average tomato production, Holland and Belgium have approximately 10 times better than India or China in (FAOSTAT 2010, Sahu *et al.,* 2012a). Thus, increasing its yield by protecting the crops with pathogen infection is the necessity for meeting demand of the growing population. One such pathogen is tomato leaf curl virus (ToLCV) which belongs to the family *Geminiviridae*, genus *Begomovirus*, and transmitted by the whitefly *Bemisia tabaci*. Begomoviruses are small, circular, single-stranded DNA plant viruses (Yang *et al.,* 2004), causing up to 100% crop losses in many countries (Polston and Anderson 1997). Tomato leaf curl disease (TLCD) symptoms consist of a more or less prominent upward curling of leaflet margins, reduction of leaflet area and yellowing of young leaves, together with stunting and flower abortion (Moriones and Navas-Castillo 2000). The management of ToLCV is difficult due to ability of vector (whitefly) populations to reach high numbers and their ability to develop pesticide resistance (Polston and Lapidot 2007). Molecular aspects of resistance have been shown to be involved during diverse host-virus interaction, but it needs further investigation (Sharma *et al.,* 2012). Therefore, enhancement of host genetic resistance seems to be the best answer against whitefly-transmitted viruses.

Traditionally the breeding for resistance to *Tomato yellow leaf curl virus* (TYLCV) implies introgression of the resistance traits into the domesticated tomato from wild tomato species such as *Solanum chilense*, *S. peruvianum*, and *S. habrochites*. Five different TYLCV resistance loci (*Ty*-1 to *Ty*-5) have been identified till date, which were originated from different wild tomato

accessions (Zamir *et al.,* 1994, Hansom *et al.,* 2000, Agrama and Scott 2006, Ji *et al.,* 2007, 2008, 2009, Anbinder *et al.,* 2009). Resistance gene *Ty*-1 was inrogressed from *S. chilense* accession LA1969 and found to be located on chromosome 6 at the vicinity of marker TG97 (Zamir *et al.,* 1994). Resistant gene *Ty*-2 identified from the *S. habrochaites* accession B6013 was mapped at the chromosome 11 between the markers TG393 and TG36 (Hanson *et al.,* 2000). Another major partial dominant gene, termed *Ty*-3, mapped on chromosome 6 was introgressed from *S. chilense* accessions LA1932, LA2779 and LA1938 and found to be resistant to tomato infecting virus (Agrama and Scott 2006) *Ty*-4 derived from *S. chilense* accessions was mapped on chromosome 3 between the markers C2_At4g17300 and C2_At5g60610 (Ji *et al.,* 2008). Further, Ty-5 has been identified in breeding line TY172 which was originated from *S. peruvianum* and mapped to chromosome 4 (Anbinder *et al.,* 2009). With the availability of PCR-based markers for the mapped ToLCV resistance loci (*Ty-1 to Ty*-5), it is promising to combined introgress these genes together in a single genotype to reach the maximum level of resistance (Ji *et al.,* 2008). However, most of these markers identified for ToLCV resistance loci have not shown tight linkage with resistance gene. Therefore, we need to identify more number of markers and these at the target genetic interval to establish tight linkage between marker and resistance gene loci for marker-assisted-selection. DNA marker technology has been used in commercial plant breeding programs and proven helpful for tagging and mapping of genes for rapid and efficient transfer of useful traits into agronomically desirable varieties and hybrids through marker-assisted breeding (Pilowsky and Cohen 1990, Sarkar *et al.,* 2011). The present work emphasized on the genetic analysis of *Tomato leaf curl New Delhi virus* (ToLCNDV) tolerance, along with identification and validation of ToLCNDV linked marker in a diverse set of tomato accessions.

Materials and methods

Screening of tomato accessions for ToLCNDV tolerance

In order to determine the infectivity of individual tomato accession, screening for ToLCNDV tolerance was performed according to method described elsewhere (Sahu *et al.,* 2010). The tomato accession LA1777 and 15SBSB, previously reported as ToLCNDV tolerant and susceptible, respectively were also selected for further study (Sahu *et al.,* 2010, 2012b). In brief, screening of twenty tomato accessions for tolerance and susceptibility to ToLCNDV infection was done under greenhouse conditions. Percentage infectivity at 21 days post-inoculation (dpi) and initiation of symptom appearance were selected as criteria for scoring. Tomato accessions were subsequently classified as tolerant (T, 1–20%), moderately tolerant (MT, 20.1–40%), susceptible (S, 40.1–60%) and highly susceptible (HS, 60.1–100%) (Sahu *et al.,* 2010).

Plant materials and virus inoculation

An inter-specific hybrid was developed between S. *habrochaites* (LA1777; ♂) and S. *lycopersicum* (15SBSB; ♀) in a reciprocal cross combination (unidirectional). The F_1 was further backcrossed with 15SBSB. The seeds of the BC_1F_1 population (135 lines) along with parents LA1777 (tolerant) and 15SBSB (susceptible) were sown in pots (6 inch diameter) filled with vermiculite and agropeat (2:1). Seeds of the 20 diverse tomato accessions obtained from various sources were also sown (Table 1). At the 15 days stage, these seedlings were uprooted and transplanted to the maintained pots in the green house of National Institute of Plant Genome Research (NIPGR). All the plants were inoculated with mixed culture of *Agrobacterium* harboring dimeric tandem repeats of DNA-A and DNA-B of ToLCNDV at two leaf stage (Seven days after transplantation). For molecular marker studies, sampling was done at 21 days after agroinoculation.

Genomic DNA isolation, DNA markers and PCR amplification

Genomic DNA was extracted from the leaves of different lines of tomato plants using Daryl's SDS extraction method (Pallotta *et al.,* 2003). The genomic DNA was quantified spectrophotometrically and concentration was calculated against the standard value of $1OD_{260} = 50\mu g/ml$. The quality of genomic DNA was checked by fractionating on 0.8% (w/v) agarose gel and was used for PCR amplification.

Sequences for designing different PCR markers were obtained from the Solanaceae Genomics Network (SGN; at http://www.sgn.cornell.edu), the National Center for Biotechnology Information (NCBI; at http://www.ncbi.nlm.nih.gov/). These markers were based on candidate gene, tomato genomic (TG), simple sequence repeat (SSR), conserved ortholog set (COS) and bacterial artificial chromosome (BAC) clone sequences deposited in these sites. Other DNA markers used in this study include Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeat (ISSR), Resistance Gene Analogs (RGAs) and Cleaved Amplified Polymorphic Sequences (CAPS).

Polymerase chain reaction was performed using the iCycle thermal cycler (BioRad) in a volume of 25 µl containing 50ng of template DNA, 10pmol of each of two primers, 0.2mM of each of the dNTPs, 0.5U of Taq DNA polymerase (Sigma, USA) and 1X PCR buffer containing 2mM of $MgCl_2$. The PCR profile included: an initial incubation at 94°C 5 min, followed by 35 cycles of denaturation at 94°C 30s, annealing at 50–60°C (depending upon the specific melting temperature of the primers) 45s, and 72°C 45s. A final elongation step at 72°C was carried out for 7 min following the completion of the above cycles. PCR products were resolved by electrophoresis either in 1.5–2.5% agarose gels or 3% metaphor agarose stained with ethidium bromide.

Detection of ToLCNDV DNA in plant by Southern bolt and RCA

Total genomic DNA of the 135 lines of BC_1F_1 population along with 20 diverse tomato accessions were prepared as described above and further subjected to electrophoresis in a 0.8% agarose gel and photographed. Viral DNA in ToLCNDV infected tomato accessions were identified by Southern hybridization and rolling circle amplification (RCA). In brief, 5 µg of total DNA was electrophoresed on 1% agarose gel in TBE [Tris-borate EDTA; 45 mM Tris-borate, 1 mM EDTA (pH 8)]. Samples were transferred to hybond-N^+ membrane (Amersham Bioscience, USA) and hybridized with a α_{32}P-dCTP labelled coat protein (CP) gene. Radiolabelling of probe was done according to the manufacturer's protocol by a high prime DNA labelling kit (Roche, Indianapolis, USA). RCA was performed by Illustra TempliPhi kit (GE Healthcare Life Sciences, USA) according to the manufacturer's protocol.

Results

Infectivity analysis for ToLCNDV tolerance in tomato accessions

In order to screen the tomato accessions for ToLCNDV tolerance, we performed the infectivity analysis on the basis of percentage infectivity at 21 days post-inoculation (dpi) and initiation of symptom appearance. It revealed that, out of 20 accessions only five were tolerant (HT and MT) while remaining were susceptible (HS and S) to ToLCNDV (Table 1). Although, CLN2116B, TLBRH 5, TLBRH 6 carry Ty-2 introgressions, but we observed different proportion of resistance and susceptible plants in the 75 plants inoculated. This may be due to the inoculation artefacts.

Table 1. Infectivity analysis of *Tomato leaf curl New Delhi virus* in tomato accessions.

Accessions	Source [$]	Origin@	Plant infected /inoculated	First symptom appearance	Symptom severity[#]	Overall grade[*]
LA1777	*S. habrochaites*	IIHR, Bangalore	9/75 (12%)	12	+	HT
CLN2116B	*S. hirsutum*	AVRDC, Taiwan	28/75 (37.3%)	10	++	MT
TLBRH5	*S. habrochaites*	IIHR, Bangalore	29/75 (38.6%)	10	++	MT
TLBRH6	*S. habrochaites*	IIHR, Bangalore	29/75 (38.6%)	10	++	MT
EC520071	*S. peruvianum*	IIHR, Bangalore	27/75 (36%)	10	++	MT
IIHR2202	*S. habrochaites*	IIHR, Bangalore	45/75 (60%)	8	+++	S
H24	*S. habrochaites*	IIHR, Bangalore	39/75 (52%)	9	+++	S
FLA496-11-6-1-0	*S. chilense*	AVRDC, Taiwan	41/75 (54.6%)	8	+++	S
FLA478-6-3-1-11	*S. chilense*	AVRDC, Taiwan	42/75 (56%)	9	+++	S
FLA653-3-1-0	*S. chilense*	AVRDC, Taiwan	44/75 (58.6%)	8	+++	S
FLA456-4	*S. chilense*	AVRDC, Taiwan	41/75 (54.6%)	8	+++	S
CLN2026	*S. hirsutum*	AVRDC, Taiwan	38/75 (50.6%)	9	+++	S
CLN2498D	*S. hirsutum*	AVRDC, Taiwan	39/75 (52%)	9	+++	S
99S-C-39-20-11-24-17-0	NA	AVRDC, Taiwan	43/75 (57.3%)	8	+++	S
BL1172	NA	IIHR, Bangalore	45/75 (60%)	8	+++	S
EC20060	*S. hirsutum*	IIHR, Bangalore	68/75 (90.6%)	6	++++	HS
15SBSB	*S. lycopersicon*	IIHR, Bangalore	71/75 (94.6%)	6	++++	HS
EC520070	*S. peruvianum*	IIHR, Bangalore	67/75 (89.3%)	6	++++	HS
TY55	*S. chilense*	AVRDC,Taiwan	70/75 (93.3%)	6	++++	HS
TY52	*S. chilense*	AVRDC, Taiwan	71/75 (94.6%)	5	++++	HS

+, least severe; ++, moderately severe; +++, severe; ++++, highly severe

*T, tolerant (1–20%); MT, moderately tolerant (20.1–40%); S, susceptible (40.1–60%); HS, highly susceptible (60.1–100%)

@ IIHR, Indian Institute of Horticultural Research; AVRDC, Asian Vegetable Research and Development Center

[$] NA, Not available

Genetic analysis for ToLCNDV tolerance

An inter-specific hybrid was developed between S. *habrochaites* 'LA1777' (♂) and S. *lycopersicum* '15SBSB' (♀) in a reciprocal cross combination. LA1777 was used as a source of ToLCV resistance genes (tolerant parent), where as 15SBSB was used as susceptible parent. The inter-specific hybrid (15SBSB × LA1777) was crossed reciprocally with 15SBSB and a BC_1F_1 population was developed and used for inheritance study. A total of 135 plants of BC_1F_1 population were inoculated with ToLCNDV. Infectivity analysis revealed that out of 135 lines 16 lines were tolerant while remaining was susceptible to ToLCNDV (data not shown). Phenotypic observations were further validated by Southern and RCA analysis using viral coat protein DNA as probe. The result shows that, out of 135 lines, only 16 were tolerant (counting both tolerant/ moderately tolerant) while others were susceptible/highly susceptible to ToLCNDV (Table 2) and the segregation fit in a 1 (tolerant):7 (susceptible) ratio (at significant P=0.8-0.9; χ^2 = 0.0461) for the BC_1F_1 population, indicating that three dominant genes confer ToLCNDV resistance in LA1777 (Table 2).

Table 2. Segregation for *Tomato leaf curl New Delhi virus* tolerance in BC_1F_1 generation of cross between tolerant (S. *habrochaites* 'LA1777') and susceptible (S. *lycopersicum* '15SBSB') accessions based on RCA and Southern analysis.

Mapping population	Total No. of plants screened	No. of tolerant plants	No. of susceptible plants	Observed genetic ratio* (T:S)	*P*-value (0.005)
BC_1F_1 (15SBSB × LA1777)	135	16	119	1:7	0.8-0.9

* T, tolerant; S, susceptible

Screening of molecular markers and BSA

We performed PCR amplification of template genomic DNA from the parents of the BC_1F_1 mapping populations of LA1777 and 15SBSB with RAPD, ISSR, SSR, CAPS and RGA primers. Obvious polymorphism between the parents was observed with 214 of the total 707 primer pairs (Table 3). The tolerant and susceptible bulk was prepared by pooling the equal concentration and quantity of genomic DNA from five tolerant and five highly susceptible lines from BC_1F_1 population, respectively. The markers showing polymorphism between parents were used for bulk-segregant-analysis (BSA). The results of the total number different markers used and their percentage polymorphism in plants has been summarized in Table 3. Forty five ISSRs were surveyed on the two parental lines, *S. habrochaites* LA1777 and *S. lycopersicum* 15SBSB. Eleven markers of which showed parental polymorphism with approximate 24.4% polymorphic potential (Table 3). These polymorphic markers produced a total of 69 scorable fragments (ranged from 3 to 11 per primer) among the parental lines. Only one ISSR marker (UBC-815; 5' CTCTCTCTCTCTCTCTG 3') was able to differentiate tolerant and susceptible bulks and thus may be linked to ToLCNDV (Figure 1).

Table 3. Summary of different molecular markers used in the present study.

Markers*	Total number of makers	Number of polymorphic markers
CAPS	32	06 (18.75 %)
ISSR	45	11 (24.44 %)
RAPD	345	139 (40.29 %)
RGA	58	23 (39.65 %)
SSR	227	35 (15.41 %)

* CAPS, Cleaved Amplified Polymorphic Sequences; ISSR, Inter Simple Sequence Repeat; RAPD, Random Amplified Polymorphic DNA; RGAs, Resistance Gene Analogs; and SSR, Simple Sequence Repeat.

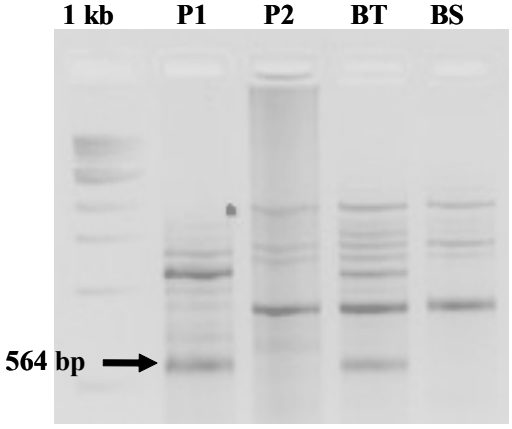

Figure 1. Bulk-Sergeant-Analysis of the ToLCNDV linked DNA marker (UBC-815). Arrow indicated the 564 bp allele amplified by marker UBC815 in P1 and BT. P1= LA1777 (Tolerant); P2= 15SBSB (Susceptible); BT= Bulk Tolerant; BS= Bulk Susceptible

Validation of linked marker for marker-assisted breeding

The identified marker obtained through BSA was further evaluated in 18 diverse tomato accessions (Table 1). The 564 bp allele was present in all the four tolerant accessions (LA1777, CLN2116B, TLBRH 5 and TLBRH 6) (Figure 2). To evaluate the potential of our identified ISSR marker for differentiation of accessions with tolerant and susceptible phenotypes the previously

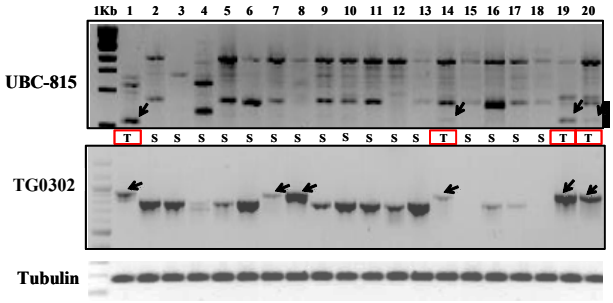

Figure 2. Validation of the ToLCNDV linked marker (UBC-815) in different tomato accessions. Tomato accessions were also validated with the known ty2 marker T0302. Tubulin gene used as a control. T= Tolerant; S= Susceptible; lane 1= LA1777 (T); lane 2= 15SBSB (S); lane 3=EC520071 (S); lane 4= EC520070 (S); lane 5= TY55 (S); lane 6= TY52 (S); lane 7= IIHR2202 (S); lane 8= H24 (S); lane 9= FLA496 (S); lane 10= FLA478 (S); lane 11= FLA653 (S); lane 12= FLA456 (S); lane 13= CLN2026 (S); lane 14= CLN2116B (T); lane 15= CLN2498D (S); lane 16= 99S-C (S); lane 17= BL1172 (S); lane 18= EC20060 (S); lane 19= TLBRH5 (T); & lane 20= TLBRH6 (T). Arrow indicates the 564 bp allele.

reported *Ty-2* linked SCAR marker T0302 (TG0302F-5'TGGCTCATCCTGAAGCTGATAGCGC 3'; T0302-5' AGTGTACATCCTTGCCATTGACT 3') was also validated in the same set of tomato accessions (Figure 2).

Discussion

Genetic analysis of 135 plants of BC_1F_1 population indicated that three dominant genes confer resistance to ToLCNDV in the accession *S. habrochaites* LA1777. In a study it was revealed that the resistance derived from *S. habrochaites* LA386 is controlled by more than one gene (Hassan *et al.,* 1984). Resistance derived from *S. hisutum* f. *glabratum* B-6013 has been reported to be conferred by *Ty-2* gene which was epistatically inherited (Banerjee and Kalloo 1987). An initial cross between resistant *S. habrochaites* LA1777 and LA386 was resulted in resistant and tolerant lines (Vidavsky and Czosnek 1998). The tolerance from these cross was conferred by a dominant major gene while resistance was controlled by two to three additive resistance genes. On the contrary, resistance derived from *S. chilense* LA1969 has been reported to be conferred by one major gene, *Ty-1*, with two minor modifier genes (Zamir *et al.,* 1994). Thus, results obtained here suggested that the inheritance of resistance derived from *S. habrochaites* LA1777 depends on the *S. lycopersicum* genetic background in which it was introgressed.

Breeding for TLCD resistance is very slow and difficult because of the complex genetics of the resistance (Pilowsky and Cohen 1990, Banerjee and Kalloo 1987, Lapidot *et al.,* 1997). Out of 45 ISSR markers, only 11 markers (24.4%) showed parental polymorphism (*S. habrochaites* LA1777 × *S. lycopersicum* 15SBSB). This result is in agreement with a study, where 23.58% polymorphic potential have been reported in a cross between *S. lycopersicum* XE 98-7 and *S. pimpinelifolium* LA2184 (Liu *et al.,* 2005). DNA polymorphism between *S. lycopersicum* and *S. habrochaites* is usually lower than between *S. lycopersicum* and *S. pennelli* (Miller and Tanksley 1990), which was later confirmed by using 98% RFLP polymorphic markers from a high-density linkage map of *S. lycopersicum* X *S. pennelli* (Zhang *et al.,* 2002).

The ISSR marker (UBC-815) identified in this study differentiated all the tolerant accessions. In conclusion, the polymerase chain reaction-based markers developed from the present study have great potential for identification of resistant/tolerant accessions of tomato and thus can be used for marker-assisted-selection for genetic improvement of tomato for ToLCNDV resistance.

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