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BRIEF COMMUNICATION

Marker assisted gene pyramiding for enhanced *Tomato leaf curl virus* disease resistance in tomato cultivars

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Abstract

The present research is aimed towards molecular marker assisted pyramiding *Tomato leaf curl virus* (ToLCV) disease resistance genes into two ToLCV susceptible tomato (*Solanum lycopersicum* L.) cvs. Pbc and H-86 (resistance genes recipient parents). Resistance gene donors were EC-538408 (*Solanum chilense)* and EC-520061 (*S. peruvianum)* in the case of cv. Pbc, and EC-520061 (*S. peruvianum*) and H-24 (*S. lycopersicum*) in the case of cv. H-86. A ToLCV resistance gene associated co-dominant simple sequence repeat (SSR) marker SSR-218 was used to discriminate between homozygotes and heterozygotes at the seedling stage prior to pollination, which enabled the rejection of non-target back crosses and pyramiding progenies of the crosses PbcxEC-520061 and H-86xEC-520061, whereas SSR-306 was used for the cross PbcxEC-538408. *Ty-2* gene cleaved amplified polymorphic sequences (CAPS) marker was used for the cross H-86xH-24. Out of 279 pyramiding progenies of the cross PbcxEC-538408/PbcxEC-520061, total 91 plants showed the presence of both resistance allele 1 and 2 along with both susceptibility alleles, and in 243 pyramiding progenies of the cross H-86xEC-520061/H-86xH-24, total 82 plants showed the presence of both resistance allele 1 and *Ty-2* along with both susceptible alleles. The pyramiding lines that carried both pyramided resistance genes were resistant to tomato leaf curl disease throughout its life cycle.

Additional key words: CAPS, molecular markers, *Solanum lycopersicum,* SSR.

Tomato (*Solanum lycopersicum* L., formerly *Lycopersicon esculentum* Mill.) is very important vegetable (Peralta *et al.* 2006). Unfortunately, numerous pathogens influence the cultivation of this crop. Among them, *Tomato leaf curl virus* disease (ToLCVD) is the most dominant (Moriones and Navas 2000). *Tomato leaf curl virus* (ToLCV) belongs to the genus *Begomovirus* of the family *Geminiviridae* and transmits to a host cell through its vector - whitefly (*Bemisia tabaci)* (Mehta *et al.* 1994). The 2.7 kb DNA genome of the virus encodes all the information required for viral DNA replication, transcription, and transmission across the plant cell (Pandey *et al.* 2009). When a plant is infected with the virus, leaves produce leaflets cupped downward and inward in a hook-like shape and become yellow and then become mis-shapen and smaller, showing interveinal and marginal chlorosis and upward curling the leaf border (Zhang *et al.* 2008). It is evident that ToLCV resistance sources are available in some of the wild tomato species (Scott 2007, Ji *et al.* 2007). Resistance genes *Ty-1* and *Ty-2* have been identified in *S. chilense* (Zamir *et al.* 1994) and *S. habrochaites f. glabratum* (Kalloo and

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Abbreviations: CAPS - cleaved amplified polymorphic sequence; DSI - disease severity index; MAS - marker assisted selection; SSR - simple sequence repeat; ToLCV - *Tomato leaf curl virus*.
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Banerjee 1990), whereas *Ty-3* and *Ty-4* have been identified in *S. chilense* (Ji *et al.* 2007, 2009). A resistance gene *Ty-5* was reported in *S. peruvianum*, and *Ty-6* has been identified in *S. chilense* (Hutton *et al.* 2012). Breeding and pyramiding ToLCVD resistance genes are considered to be the best approach to improve the resistance (Vidavski *et al.* 2008, Kottapalli *et al.* 2010, Jiang *et al.* 2012, Yang *et al*. 2013). Thus, in this study, marker assisted pyramiding was carried out with the aim of improving cultivars with enhanced and durable resistance against ToLCVD.

Tomato (*Solanum lycopersicum* L.) cultivars Pbc and H-86 (ToLCV resistance gene recipient parents), H-24, EC-538408, and EC-520061 (ToLCV resistance gene donor parents), and the corresponding crosses were cultivated under natural conditions at a research farm of the Indian Institute of Vegetable Research, Varanasi, Uttar Pradesh, India.

Genomic DNA was extracted from adult leaves of all the selected parents, BC_1F_1, BC_2F_2, and DC_1F_1 individual progenies of the crosses PbcxEC-538408, PbcxEC-520061, H-86xEC-520061, and H-86xH-24 following the modified protocol of Doyle and Doyle (1990). The quality of extracted DNA was measured by comparison of band intensity on ethidium bromide stained 0.8 % agarose gels. Quantity was measured with a *Dyna Quant 200* spectophotometer (Hoefer Pharmacia Biotech, San Francisco, CA, USA) at 260 and 280 nm. A total of 500 SSR markers (provided by *IDT*, CA, USA) belonging to different series *viz.* SSR, TG, T, CLEx, CLEr, *etc*., were used for the genomic DNA amplification and screening polymorphic markers based on the repetition and resolution of polymorphic band. Of these, 15 SSR markers (Table 1) were used for the background selection of recipient parents Pbc and H-86. Detailed information on SSR markers corresponding to twelve tomato chromosomes were obtained using a *Sol* genome data extractor (http://solgenomics.net).

Table 1. The description of SSR markers used for the background selection of recipient parents in pyramiding progenies. Chr - chromosome.

No.	Primer	Nucleotide sequence 5' - 3'	Repeat type	SGN ID No.	Chr	Allele	Band size
1	SSR-31	F: AAGACAGAAAGTGCACGTCAGA R: TGGATGAGAAGAGGGAATCCT	(TCTT)6	SGN-M704	3	3	103
2	SSR-51	F: CTACCCTGGTCTTGGTGGAA R: AAAGGATGCTCTAGCTTCTCCA	(ACAA)6	SGN-M724	1	3	148 (148/128)
3	SSR-65	F: GGCAGGAGATTGGTTGCTTA R: TTCCTCCTGTTTCATGCATTC	(AG)5 TG)7	SGN-M738	1	3	230
4	SSR-92	F: AAGAAGAAGGATCGATCGAAGA R: TCATGACCACGATACTACATGTTTC	(CT)11	SGN-M765	1	4	172
5	SSR-122	F: ACGGGATTGTACCCAATCAA R: AGGGTTTGAAGAGGAGGAGG	(AG)3 (AAG)7	SGN-M795	6	4	172
6	SSR-150	F: ATGCCTCGCTACCTCCTCTT R: AATCGTTCGTTCACAAACCC	(CTT)7	SGN-M823	1	3	215
7	SSR-188	F: TGCAGTGAGTCTCGATTTGC R: GGTCTCATTGCAGATAGGGC	(AT)11	SGN-M861	4	4	140 (148/140)
8	SSR-214	F: AAATTCCCAACACTTGCCAC R: CCCACCACTATCCAAACCC	(CTT)7	SGN-M887	4	3	221
9	SSR-218	F: GTGGTTATCCCAAGACCCAA R: CGCCAGTCTTCCTCTGACTT	(TCA)7	SGN-M891	10	3	127(143/127)
10	SSR-222	F: CTCATCTGGTGCTGCTGTT R: GACCCAGAAACAGGCATGAAA	(TCT)7	SGN-M895	1	4	1500 (800+700)
11	SSR-241	F: TCAACAGCATAGTGAGGAGG R: TCCTCGGTAATTGATCCACC	(AAT)13	SGN-M914	7	2	200 (185/200)
12	SSR-301	F: TTTCCACCTCAAACCACTCC R: CCCTTTGACCTGTGCC	(TTGGT)2 (TA)15	SGN-M974	10	4	181 (171/181)
13	SSR-304	F: TCCTCCGGTTGTTACTCCAC R: TTAGCACTTCCACCGATTCC	(CCA)7	SGN-M977	7	3	186
14	SSR-306	F: ACATGAGCCCAATGAACCTC R: AACCATTCCGCACGTACATA	(ATT)7	SGN-M979	4	3	258 (248/258)
15	SSR-308	F: TTTCCCTGTTT CAGCCTTTG R: GGCACGAGAAT TTAGCCACT	(TA)12	SGN-M981	1	3	293 (293/300)
16	SSR-350	F: GGAATAACCTCTAACTGCGGG R: CGATGCCTTCATTTGGACTT	(AT)13	SGN-M1023	6	4	267 (256/267)
17	TG-400	F: TCCAAATCCACCACCTATCC R: AGCATTGCTCCCTGCTAAAG	NA	NA	11	3	404 (404/300)

PCR amplification was carried out in a 0.025 cm^3 reaction mixture which contained 50 ng template DNA, a 10× reaction buffer (10 mM Tris HCl, pH 8.3, and 50 mM KCl), 2.0 mM $MgCl_2$, 1.3 U Taq DNA polymerase, 100 µM dNTPs, 0.2 µM primers. The amplification was performed in a *Thermo* (Pittsburg, USA) PCR thermal cycler programmed as follows: 1 cycle of initial denaturation at 94 °C for 1 min, 40 cycles each of denaturation at 94 °C for 1 min; the annealing step was performed at an optimum temperature for each primer, from 0.8 to 1.2 °C below its original average annealing temperature, elongation at 72 °C for 1 min, and final elongation at 72 °C for 10 min. Amplified DNA bands were resolved through electrophoresis at 60 V for 120 min using a 1× TAE buffer in a 2.2 % (m/v) agarose gel containing 0.005 cm^3 of ethidium bromide, and photographed under UV radiation with an *Alpha InnoTech* (Santa Clara, CA, USA) gel documentation system.

For ELISA study, samples were collected 45 d after the inoculation from all the individual pyramiding progenies and measured according to Clark and Adams (1977). The colour reaction was measured as absorbance at 405 nm on an ELISA plate reader (*Multiskan EX*, *Themo Labsystems OY*, Vantaa, Finland).

To analyze specific amplified DNA fragments from resistance gene donor parents EC-538408 (derived from *S. chilense*), EC-520061 (*S. hirsutum*), and H-24 (*S. lycopersicum*) in the background of recipient parents Pbc and H-86 (*S. lycopersicum*), the F_1 plants from the crosses (PbcxEC-538408, PbcxEC-520061, H-86xEC-520061, and H-86xH-24) were backcrossed to Pbc and H-86. BC_1F_1 populations were developed by crossing healthy true tested F_1 plants from the crosses PbcxEC-538408, PbcxEC-520061, H-86xEC-520061, and H-86xH-24 with the recipient female parents Pbc and H-86. After crossing, these were kept for setting fruits, BC_1F_1 seeds were grown in pots under above mentioned conditions, and 25 to 30-d-old seedlings were transplanted to the field for subsequent growth, phenotypic evaluations, and the generation of BC_2F_1 population. Then 10-14 BC_1F_1 progenies carrying the desired genes (resistance gene 1, resistance gene 2, and *Ty-2* gene) were screened with the SSR-306 marker sequence (forward primer 5'-ACATGAGCCCAATG AACCTC-3' and reverse primer 5'-AACCATTCC GCACGTACATA-3') in the cross PbcxEC-538408, with the SSR-218 marker sequence (F: 5'-GTGGTTATC CCAAGACCCAA-3' and R: 5'-CGCCAGTCTTCCTC TGACTT-3'), in the crosses H-86xEC-520061 and PbcxEC-520061, and through the *Ty-2* gene CAPS marker in the cross H-86xH-24. BC_2F_1 populations were developed by crossing resistant BC_1F_1 plants of crosses PbcxEC-538408, PbcxEC-520061, H-86xEC-520061, and H-86xH-24 carrying resistance gene 1, resistance gene 2, or *Ty-2* gene (F: 5'-CTTCAGAATTCCTGT TTTAGTCAGTTGAACC-3' and R: 5'-ATGTCACAT TTGTTGCTTGGACCATCC-3') with the recipient parents Pbc and H-86. After crossing, plants were kept for setting fruits, BC_2F_1 seeds were grown in pots, and 25 to 30-d-old seedlings were transplanted to the field for subsequent growth, phenotypic evaluations, and the generation of double cross F_1 (DCF_1) population.

After reaching the BC_2F_1 generation through marker aided backcrossing for individual resistance genes, in the next step, two BC_2F_1 lines carrying different resistant genes were crossed to produce the double cross F_1 (DCF_1) {(PbcxEC-538408)/(PbcxEC-520061)}, {(H-86xEC-520061)/(H-86xH-24)} for pyramiding two resistance genes. The pyramiding progenies DCF_1 carrying two ToLCV resistance genes were selected with the help of markers, and the selected plants were selfed to produce DCF_2 in the background of Pbc and H-86. The markers of particular interest were those ones that link to disease resistance genes. A *Ty-2* CAPS marker and two SSR markers were used effectively for the marker assisted selection (MAS) of the resistance genes. To study the ToLCVD resistance and susceptibility in all the backcrosses and pyramiding lines, the severity of development of yellowing and curling symptoms were studied in terms of disease severity index (DSI) as described by Lapidot and Friedmann (2002); 1 = slight yellowing of leaflet margins on apical leaves; 2 = some yellowing and minor curling leaflet ends; 3 = a wide range of leaf yellowing, curling and cupping, with some reduction in size, yet plants continue to develop; 4 = very severe plant stunting and yellowing, pronounced leaf cupping and curling.

ToLCV resistance gene associated markers SSR-218 and SSR-306 were used to screen the genuine BC_1F_1 and BC_2F_1 progenies. Among all backcross progenies, a total of 106 individuals were BC_1, where the highest 16 (69.5 %) out of 23 BC_1F_1 progenies were heterozygous in the cross H-86xH-24, whereas the lowest 13 (59 %) out of 22 BC_1F_1 progenies were heterozygous in the cross PbcxEC-538408. In the cross PbcxEC-520061 out of 23 progenies genotyped, 15 showed the resistance allele (175 bp) as well as the susceptibility allele (140 bp), whereas 8 progenies showed only the susceptibility allele (140 bp) with the marker SSR-218. In the cross PbcxEC-538408, out of 22 progenies, 13 showed the resistance allele (254 bp) as well as susceptibility allele (284 bp), whereas 9 progenies showed only susceptibility allele (284 bp) with the marker SSR-306. Likewise in the cross H-86xEC-520061, out of 23 progenies genotyped, 14 showed the resistance allele (175 bp) as well as susceptibility allele (140 bp), whereas 9 progenies showed only the susceptibility parent gene allele (140 bp) with the marker SSR-218.

Similarly, 90 individuals were genotyped in BC_2, where the highest 14 (60.86 %) out of 23 individuals were heterozygous in the cross H-86xH-24, whereas the lowest 11 (47.8 %) out of 23 were heterozygous in the cross H-86xEC-520061. The cross PbcxEC-520061, out of

22 progenies genotyped, 13 were heterozygous carrying the respective allele (175 and 140 bp), whereas 9 progenies carried only the susceptible parent gene allele of 140 bp indicating homozygosity, as assayed with the marker SSR-218. In the cross PbcxEC-538408, out of 22 progenies genotyped, 12 carried the resistance allele (254 bp) as well as susceptibility allele (284 bp), whereas 10 progenies carried only the susceptibility allele (284 bp). Similarly in the cross H-86xEC-520061, out of 23 progenies, 11 carried the resistance allele (175 bp) as well as susceptibility allele (140 bp), whereas 12 progenies carried only the susceptible parent gene allele (140 bp). In the cross H-86xH-24, out of 23 progenies genotyped, 16 carried the resistance allele (200 bp) as well the as susceptibility allele (350 bp), whereas 7 progenies carried only the susceptible parent (recurrent parent H-86) allele (350bp) as assayed with the *Ty-2* gene CAPS marker.

The co-dominant nature of the polymorphism exhibited by SSR-218 and SSR-306 enabled discrimination between homozygotes and heterozygotes in the seedling stage prior to pollination, which aided in the rejection of non target BC plants. It resulted in saving labour and material resources, thus our finding is in favour of the objective of background selection to recover rapidly a maximum proportion of the recurrent parent genome at non target loci through markers that are distributed evenly throughout the genome (Young and Tanksley 1989, Vischer *et al.* 1996). In the BC_1 and BC_2 populations, for each cross, it was found that the banding patterns of each backcross progenies were segregated in the expected segregation ratio of 1:1 (resistance alleles : susceptibility alleles). Similar segregation ratios have been observed in various interspecific crosses (Vallejos and Tanksley 1983, Bonierbale *et al.* 1988, Paterson *et al.* 1991) and backcross breeding has been used for the incorporation of ToLCV resistance genes into tomato cultivars (Frary *et al.* 2004, Elazbieta and Marzena 2010).

The pyramiding progenies of crosses (PbcxEC-538408/PbcxEC-520061 and H-86xEC-520061/H-86xH-24) were classified into different groups; group 1: plants carrying only resistance allele 1, group 2: plants carrying only resistance allele 2, group 3: plants carrying both resistance alleles 1 and 2, and group 4: plants without any resistance allele. In the present investigation, out of 279 pyramiding progenies of the cross PbcxEC-538408/PbcxEC-520061, 91 plants carried both resistance allele 1 (from the exotic tomato accession EC-538408) and resistance allele 2 (from an exotic tomato accession EC-520061) along with both susceptible alleles, 68 plants carried only resistance allele 1 and both susceptible alleles, 53 plants carried only resistance allele 2 and both susceptible alleles, whereas 67 plants carried only susceptible alleles from the ToLCV resistance gene donor parents. Out of 243 pyramiding progenies of the cross H-86xEC-520061/H-86xH-24, 82 plants carried both resistance allele 1 (from an exotic tomato accession EC-538408 and resistance allele *Ty-2* (from H-24) along with both susceptible alleles, 51 plants carried only resistance allele 2 and both susceptible alleles, 63 plants carried only *Ty-2* and both resistance gene alleles and 47 plants progenies did not show any resistance allele from any of donor parents EC-520061 and H-24.

Table 2 Detection of the presence of ToLCV using ELISA technique. Group 1 - plants carrying only resistance allele 1, group 2 - plants carrying only resistance allele 2, group 3 - plants with two resistance alleles, group 4 - plants without any resistance allele.

Crosses	Groups	Number of progenies	ELISA reading
PbcxEC-538408/	1	68	0.592
PbcxEC-520061	2	53	0.773
	3	91	0.153
	4	67	1.319
H-86xEC-520061/	1	51	0.865
H-86xH-24	2	63	0.690
	3	82	0.210
	4	47	1.507
Susceptible control		10	2.144

The plants were grown from fungicide treated seeds in the research farm of the Indian Institute of Vegetable Research, Varanasi, U.P., India; for subsequent growth and phenotypic evaluations during years 2008, 2009, and 2010 when the virus inoculum pressure was highest. The severity of ToLCV in the recurrent parents Pbc and H-86 were high (DSI 3.6 ± 0.4 and 2.9 ± 0.3) before pyramiding ToLCV resistance genes. The parent EC-520061 showed the highest resistance (DSI 1.2 ± 0.1) throughout all growth stages and during the entire season which started in August and ended in next May. EC-538408 was also highly resistant for the most part of its life cycle (after five months at the fruit ripening stage it showed mild leaf curling) (DSI 1.4 ± 0.2). The cultivar H-24 carrying *Ty-2* gene showed leaf curling two months after transplanting and it was also considered tolerant to the ToLCV Varanasi strain. Both BC_1 and BC_2 populations showed a large variation in ToLCV severity. The backcross progenies carrying resistance gene 1 from EC-538408 or resistance gene 2 from EC-520061 were highly resistant to the ToLCV disease, whereas the plants carrying *Ty-2* allele showed the ToLCV symptoms at the later stages of plant growth and were indistinguishable from susceptible checks, though the disease development was very fast in the plants devoid of any resistance gene. Thus, unlike the situation in the southern states of India, *Ty-2* alone was not effective against the ToLCV strain found in the Varanasi region. One progeny of PbcxEC-538408 and two progenies of H-86xEC-520061 were highly resistant with all symptomless plants within each

population. Intermediate disease severity was found in many progenies of PbcxEC-538408, PbcxEC-52006, and H-86xEC-520061.

The ToLCV disease symptom severity index of all the backcrosses and pyramiding lines were observed visually and scored weekly for four weeks, after which DSI was calculated. The ToLCV disease symtom resistant lines had considerably less DSI and less or no symtoms as compared to the susceptible lines which showed both severe symptoms and high DSI. Further, the lines that had resistance gene 1 (derived from EC-538408) incorporated showed resistance to ToLCV symtoms with less DSI (1.5 ± 0.1) for the most part of the season and the lines that had resistance gene 2 (derived from EC-520061) incorporated showed more resistance to ToLCV symtoms with less DSI (1.3 ± 0.2) throughout the season at all crop growth stages. Up to the fruiting stage (four months from the date of transplanting), there was no observable difference in the resistance among the lines carrying resistance gene 1 or 2. Similarly, the lines that carried both resistance gene 1 and 2 were resistant throughout the life cycle. The effect of pyramiding was not immediate because one of the resistance genes alone was able to provide a very high degree of resistance. However, it is a fact that pathogens are unable to break resistance if it is provided by more than one resistance gene. The lines that carried only *Ty-2* gene became susceptible to ToLCV symtoms with high DSI (2.8 ± 0.2) before flowering, whereas the lines with resistance gene 1 or *Ty-2* + resistance gene 1 were resistant to ToLCV symtoms with less DSI (1.5 ± 0.4). These finding support earlier studies having demonstrated that pyramiding can enhance the resistance in crop plants including tomato (Huang *et al.* 1997, Hittalmani *et al.* 2000, Vidavski *et al.* 2008).

The ELISA absorbance reading 2.144 indicated a high amount of the virus present in the susceptible control, whereas its minimum (0.153 and 0.210) in progenies carrying two resistance alleles in the pyramiding crosses PbcxEC-538408/PbcxEC-520061 and H-86xEC-520061/H-86xH-24. Interestingly, it was observed reading 1.319 and 1.507 for progenies which did not carry any resistance allele.It was higher in comparison with the group carrying only one resistance allele (Table 2). The resistant plants accumulated considerably less virions than the susceptible individuals. Values dramatically increased in the susceptible individuals which showed both severe disease symptoms and high readings.

The development of cultivars resistant to ToLCVd is the most efficient way to combat the disease. The strategy of pyramiding resistance genes through marker assisted selection is a valuable method due to an increased resistance and its durability.

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