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# VALIDATION OF MOLECULAR MARKERS LINKED TO ToLCV RESISTANCE IN TOMATO VARIETIES / HYBRIDS / LINES

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## ABSTRACT

Tomato (*Solanum lycopersicum* L.) is one of the most important and extensively grown vegetables around the world. Successful cultivation of tomato crop has been hindered due to attack by numerous pests and devastating diseases. Chiefly of these limiting factors, the tomato leaf curl disease caused by Tomato Leaf curl Virus (ToLCV) is a destructive disease of tomato in many parts of India and world. The use of molecular markers linked to genes for resistance is a tool, which can be used efficiently in plant breeding through marker assisted selection (MAS). Three molecular markers *Ty1*, *Ty2* and *Ty3* linked to ToLCV resistance were validated with fourteen ToLCV resistant entries and Pusa Ruby as a susceptible check, after screening against ToLCV resistance in the green house using white flies. Among these, two advanced breeding lines IIHR-2822 and IIHR-2823 showed the presence of the all three genes *TY1*, *Ty2* and *Ty3* for ToLCV resistance, the wild accession *S. habrochaites* LA 1777 (IIHR-2101) showed the presence of two genes *Ty2* and *Ty3*, Abhinava showed the presence of *Ty1* gene and Hisar Anmol (H-24), Vyabhav, Arka Ananya, Lakshmi, NS-501 showed the presence of only *Ty2* gene. The varieties Nandhi and Sankranthi; hybrids Shakthiman and US-618 and the advanced breeding line IIHR-2611 (TV 55) did not show any presence of the *Ty1*, *Ty2* and *Ty3* resistant genes.

**KEYWORDS:** Tomato leaf curl virus, ToLCV linked resistant markers, Ty1, Ty2, Ty3, MAS

## INTRODUCTION

Tomato (*Solanum lycopersicum* L) is one of the most important and extensively grown vegetables around the world next to potato and onion. 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 rich source of Vitamin 'A', Vitamin 'C', minerals and organic acids. To meet this requirement, numbers of high yielding varieties / hybrids with improved yield and fruit quality have been released for commercial cultivation. It is grown in an area of 0.88 m ha with the production of 18.73 million tonnes in India and in Karnataka it is cultivated in an area of 0.61 lakh ha with an estimated production of 2.07 million tonnes (NHB, 2014).

Tomato has the chromosome number 2n= 24, self pollinated, and native to South America. Successful cultivation of tomato has been hindered, due to attack by numerous pests and devastating diseases. Chiefly of these limiting factors, the tomato leaf curl disease caused by *Tomato Leaf curl Virus* (ToLCV) is a destructive disease of tomato in many parts of India (Muniyappa and Saikia, 1983). *Tomato leaf curl disease* (ToLCD) was, first reported in India by Vasudeva and Samraj (1948). The infected plants showed the following symptoms: stunted growth, leaves and internodes are greatly

reduced in size, severe curling, twisting and rolling of the leaves accompanied by dark green outgrowth or enations of the vein on the under surface of the leaflets (Smith, 1935). The disease is caused by a begomovirus, transmitted naturally by whitefly vector (*Bemisia tabaci* Genn.). 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 its virulence. This has posed serious problem for the breeders to take up any successful breeding programme to develop varieties / hybrids with stable resistance to ToLCV. Wild tomato species have been screened for their response to the virus and a number of ToLCV resistant accessions have been identified in wild species such as *Solanum chilense, S. habrochaites, S. peruvianum, S. pimpinellifolium* and *S. cheesmaniae*. Thus, breeding programs have been based on the transfer of resistance genes from accessions of wild origin to the cultivated tomato.

The use of molecular markers linked to genes for resistance is a tool, which can be used efficiently in plant breeding for the indirect selection of quantitative resistance and for accelerated transfer of resistance from different sources into a single cultivar by gene pyramiding through marker assisted selection (MAS), compared to traditional methods of screening germplasm for disease resistance and screening of the segregating populations against the disease which is laborious and time consuming. Hence, the present study was carried out for the validation of the reported markers with genes (*Ty1, Ty2* and *Ty3*) linked to ToLCV resistance in the commercial varieties and hybrids of tomato.

## MATERIALS AND METHODS

A total of fourteen ToLCV resistant entries were used for screening against ToLCV. These resistant entries included, four ToLCV resistant varieties (Hisar Anmol, Vyabhav, Nandhi and Sankranthi), six ToLCV resistant commercial hybrids (Abhinava, Arka Ananya, Lakshmi, NS-501, Shakthiman and US-618); three advanced breeding lines (IIHR-2611, IIHR-2822 and IIHR-2823) and a ToLCV resistant wild accession *Solanum habrochaites* LA 1777 (IIHR-2101). Tomato variety Pusa Ruby was used as susceptible check.

Seeds of all the fourteen ToLCV resistant entries along with susceptible check Pusa Ruby were sown in the 98 cavities seedling trays. After the emergence of the true leaf (10 days), the trays were kept in the screen house and mass inoculation was done with help of viruliferous whiteflies (*Bemisia tabaci* Genn.). The seedling trays were placed near ToLCV culture plants for transmission of virus in the screen house for the infection. Seedlings were inspected at regular intervals for ToLCV incidence. When all the susceptible check Pusa Ruby seedlings showed ToLCV symptoms, disease scoring was done of these seedlings for resistance and expressed in percentage of ToLCV infection and leaf samples were collected for DNA extraction.

DNA was extracted using CTAB method from fresh tomato leaves collected from the 14 resistant entries after screening along with Pusa Ruby and the integrity and purity of the DNA was checked using agarose gel electrophoresis. 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 *Ty1* Marker

The PCR reaction was carried out in a total volume of 25 µl and was standardized using 10x Incomplete buffer, 25 mM $MgCl_2$, 5 µM of each forward and reverse primer, 1 mM dNTPs, 3U/ µl 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$^{o}$c for 3 min, 20 cycles of 94$^{o}$C for 10 s, 55$^{o}$C for 30 s and 72$^{o}$C for 70 s, 10 cycles of 94$^{o}$C for 10 s, 53$^{o}$C for 30 s and 72$^{o}$C for 70 s, Followed by final extension step of 10 min at 72$^{o}$C and Store at 4$^{o}$C.

**PCR Reaction Mixture and Conditions for *Ty2* Marker**

The PCR reaction was carried out in a total volume of 25 µl and standardized using 10x Incomplete buffer, 25 mM $MgCl_2$, 5 µM of each forward and reverse primer, 1 mM dNTPs, 3U/ µl 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$^{o}$C for 5 min, 35 cycles of 94$^{o}$C for 30 sec, 55$^{o}$C for 1 min and 72$^{o}$C for 2 min, Followed by final extension of 72$^{o}$C for 8 min and Store at 4$^{o}$C.

**PCR Reaction Mixture and Conditions for *Ty3* and *Ty3a* Marker**

The PCR reaction was carried out in a total volume of 25 µl and standardized using 10x Incomplete buffer, 25 mM $MgCl_2$, 5 µM of each forward and reverse primer, 1 mM dNTPs, 3U/ µl 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$^{o}$C for 4 min, 35 cycles of 94$^{o}$C for 30 sec, 53$^{o}$C for 1 min and 72$^{o}$C for 1 min, Followed by final extension of 72$^{o}$C for 10 min and Store at 4$^{o}$C.

## RESULTS AND DISCUSSIONS

After 15 to 18 days of seedling emergence, the susceptible check Pusa Ruby seedlings expressed typical symptoms of ToLCV while screening with mass inoculation by viruliferous white flies in the screenhouse. None of the resistant entries expressed ToLCV symptoms. Leaf samples were collected of the resistant entries along with the susceptible check Pusa Ruby and DNA extraction was done using CTAB method (Doyle and Doyle, 1990). The variation in DNA concentration was due to the varying concentration of interfering constituents like carbohydrates, proteins, phenols, etc in different genotypes. Further, the DNA was diluted to 20 ng after quantification and PCR was carried out using the identified molecular markers linked to ToLCV resistance in tomato *viz.*, *Ty1*, *Ty2* and *Ty3* (Table 1).

The tomato varieties and hybrids upon amplification with *JB-1* primers, showed a band size of 900 bp before restriction (Plate 1) and upon restriction with *TaqI* showed a susceptible band size of 450 bp and resistant band size of 500 bp (Plate 1), confirming the absence and presence of the *Ty1* gene respectively. The Abhinava hybrid, and advanced breeding lines IIHR-2822 and IIHR-2823 showed heterozygous bands of size 500 bp and 450 bp which confers the presence of the *Ty1* gene whereas, all the other resistant varieties / hybrids showed a susceptible band of size 450 bp with absence of the gene. While screening for *Ty2* marker Hisar Anmol, Arka Ananya, a wild accession of *S. habrochaites* LA 1777 (IIHR-2101) and advanced breeding lines IIHR-2822, IIHR-2823 showed resistant band of size 600 bp confirming the presence of the *Ty2* gene for ToLCV resistance; Vyabhav, Lakshmi and NS-501 showed heterozygous bands of size 600 bp and 450 bp with the presence of *Ty2* gene whereas, all others showed a susceptible band size of 450 bp in which the *Ty2* gene was absent (Plate 4). *Ty3* specific primers FLUW-25 were screened for the fourteen resistant entries, among

which the ToLCV resistant wild accession *S. habrochaites* LA1777 (IIHR-2101) showed a resistant band of 600 bp and the advanced breeding lines IIHR-2822, IIHR-2823 also showed resistant bands of size 640 bp. Both the band size confirms the presence of *Ty3* gene where as, others showed a susceptible band size of 480 bp which confirms the absence of *Ty3* gene (Plate 2). *Ty3a* marker was validated with P6-25 primers and none of the entries showed the presence of *Ty3a* gene with the resistant band size of 630 bp, instead all showed the susceptible band size of 320 bp with absence of the *Ty3a* fragment (Plate 3).

The presence of the gene corresponds to the lines or hybrid derived from *S. chilense* accession line LA 1932 whereas, absence of gene indicates the lines derived from *S. lycopersicum* cultivated species with the *Ty1* marker (De castro *et al*., 2007). The resistant band for *Ty2* marker, indicates the presence of the *Ty2* gene for resistance to ToLCV corresponds to the lines/ varieties/ hybrids derived from the *S. habrochaites* H-24 line and the absence of the *ty2* gene corresponds to the lines/ varieties/ hybrids derived from the cultivated species *S. chilense* accession LA 2279 (Brenda *et al*., 2007). The presence of *Ty3* gene for ToLCV resistance corresponds to the advanced breeding lines derived from the *S. chilense* LA 2279 and absence of *ty3* gene corresponding to the lines derived from cultivated species *S. lycopersicum* (Melinda *et al.,* 2006). When FLUW-25 primers were used with the begomovirus resistant lines derived from LA 1932 *S. chilense*, no fragment was produced. Therefore, a new set of primers (P6-25) were designed for *Ty3a* marker, which amplified different size fragments from *S. lycopersicum*, LA 2279 derived lines and LA 1932 derived lines (Jensen *et al*., 2007). None of the resistant entries showed the presence of the *Ty3a* allele correspond, to the lines derived from the *S. lycopersicum* species.

The varieties Nandhi and Sankranthi; hybrids Shakthiman and US-618 and the advance breeding line IIHR-2611 (TV 55), did not show any presence of the *Ty1, Ty2* and *Ty3* resistant genes when validated with the *Ty1, Ty2* and *Ty3* resistant marker linked to ToLCV resistance. The resistance in these resistant entries may be due to the presence of other resistant genes linked for ToLCV and hence there is need to identify these markers.

Validation of three molecular markers linked to ToLCV resistance in the resistant tomato varieties/hybrids/advanced breeding lines/wild accession used in the present study, revealed the presence of *Ty1*, *Ty2* and *Ty3* resistance 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. Thus, with the development of molecular markers (PCR based) tightly linked to the resistant genes, and their identification help the plant breeders efficiently to incorporate these resistant genes into elite tomato genotypes there by accelerating the breeding of resistant cultivars of tomato. Further more available markers linked to ToLCV resistance have to be used for the development of resistant tomato cultivars.

## CONCLUSIONS

With the development of molecular markers (PCR based) tightly linked to To LCV resistant genes, and their validation help the plant breeders efficiently to incorporate these resistant genes into elite tomato genotypes, thus accelerating the breeding of resistant cultivars. 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 moreoptions in providing tomato growers with durable resistance to the be gomoviruses and their production.

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## APPENDICES

**Table 1: Details of the Molecular Markers Linked to ToLCV Resistance in Tomato used in the Study**

| Name of the Marker | Primer Name | Type of Marker | Sequence of the Primer | Expected Amplicon Size | | Reference |
|---|---|---|---|---|---|---|
| | | | | Resistant | Susceptible | |
| Ty1 | JB-1 | CAPS | F 5'AACCATTATCCGGTTCACTC 3' R 5'TTTCCATTCCTTGTTTCTCTG 3' | 900 bp (BR) 500 bp (AR) | 450 bp (AR) | De Castro *et al*., 2007 |
| Ty2 | TG030 2F/TY2 R1 | SCAR | F 5'TGGCTCATCCTGAAGCTGATAGCGC 3' R 5'TGAT (T/G)TGATGTTCTC (T/A)TCTCT (C/A)GCCTG 3' | 600 bp | 450 bp | Brenda *et al*., 2007 |
| Ty3 | FLUW 25 | SCAR | F 5'CAAGTGTGCATATACTTCATA (T/G)TCACC 3' R 5'CCATATATAACCTCTGTTTCTATTTCG AC 3' | 640 bp or 600 bp or 450 bp | 480 bp | Melinda *et al*., 2007 |
| Ty3a | P6-25 | SCAR | F 5' F 5'GGTAGTGGAAATGATGCTGCTC 3' R 5'GCTCTGCCTATTGTCCCATATATAACC 3' | 630 bp | 320 bp | Jensen *et al*., 2007 |

**AR- After Restriction, BR- Before Restriction, F-Forward, R-Reverse**

**Table 2: Identification of Resistant *Ty1*, *Ty2*, *Ty3* and *Ty3a* genes using Markers Linked to ToLCV**

**(+/- Gene Present in Heterozygous, R- Resistant Plant, S- Susceptible Plant, + Gene Present, - Gene Absent)**

| Sl No. | Name of the Varieties/Hybrids | Screenhouse Screening | *Ty1* | *Ty2* | *Ty3* | *Ty3a* |
|---|---|---|---|---|---|---|
| 1. | Hisar Anmol (H-24) | R | - | + | - | - |
| 2. | Vyabhav | R | - | +/- | - | - |
| 3. | Nandhi | R | - | - | - | - |
| 4. | Sankranthi | R | - | - | - | - |
| 5. | Pusa Ruby (Susceptible Check) | S | - | - | - | - |
| 6. | Abhinava | R | +/- | - | - | - |
| 7. | Arka Ananya | R | - | + | - | - |
| 8. | Lakshmi | R | - | +/- | - | - |
| 9. | NS-501 | R | - | +/- | - | - |
| 10. | Shakthiman | R | - | - | - | - |
| 11. | US-618 | R | - | - | - | - |
| 12. | IIHR- 2101 (LA 1777) | R | - | + | + | - |
| 13. | IIHR- 2611 (TV 55) | R | - | - | - | - |
| 14. | IIHR- 2822 | R | +/- | + | + | - |
| 15. | IIHR- 2823 | R | +/- | + | + | - |

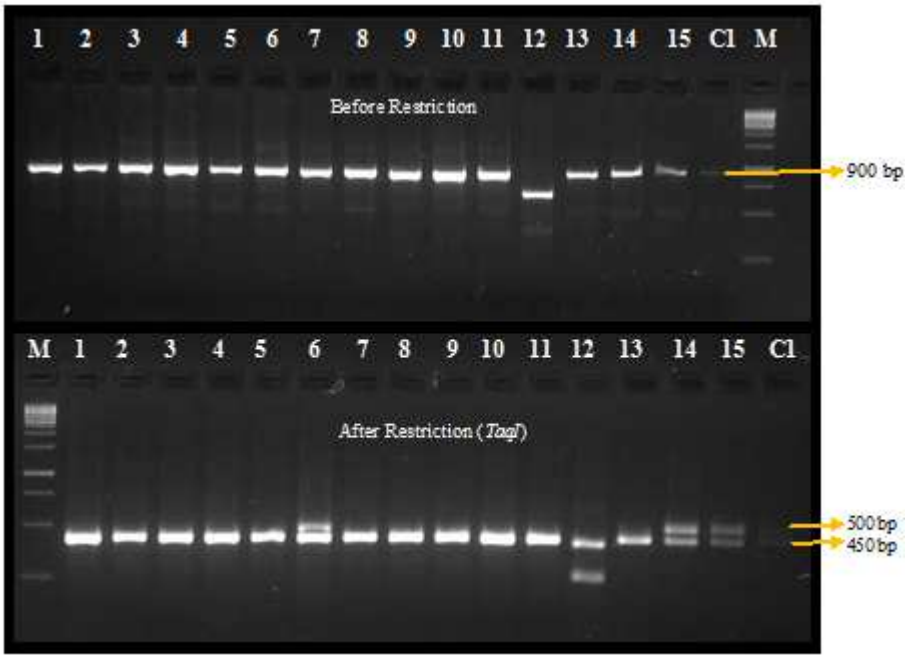


**Plate 1: Gel Picture Showing *Ty1* Resistant Marker Amplification**

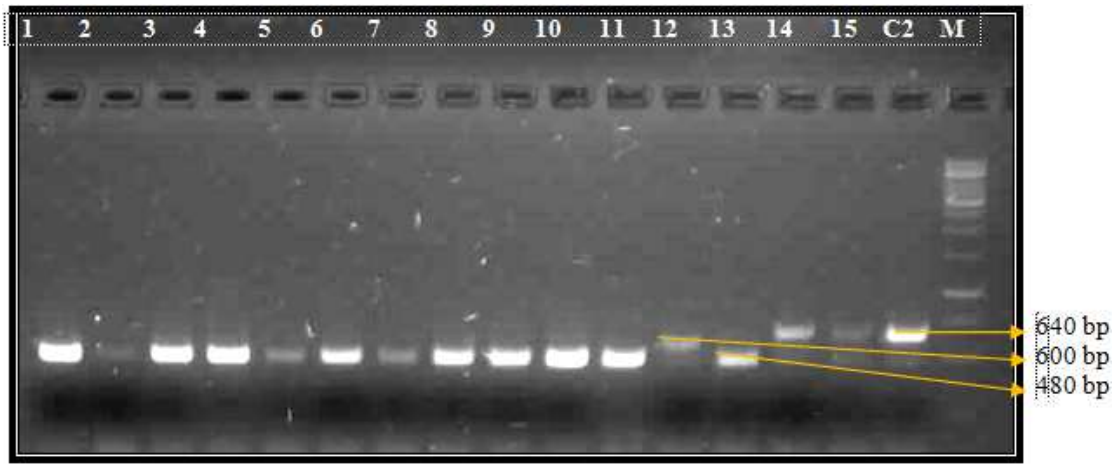


**Plate 2: Gel Picture Showing *Ty3* Resistant Marker Amplification**

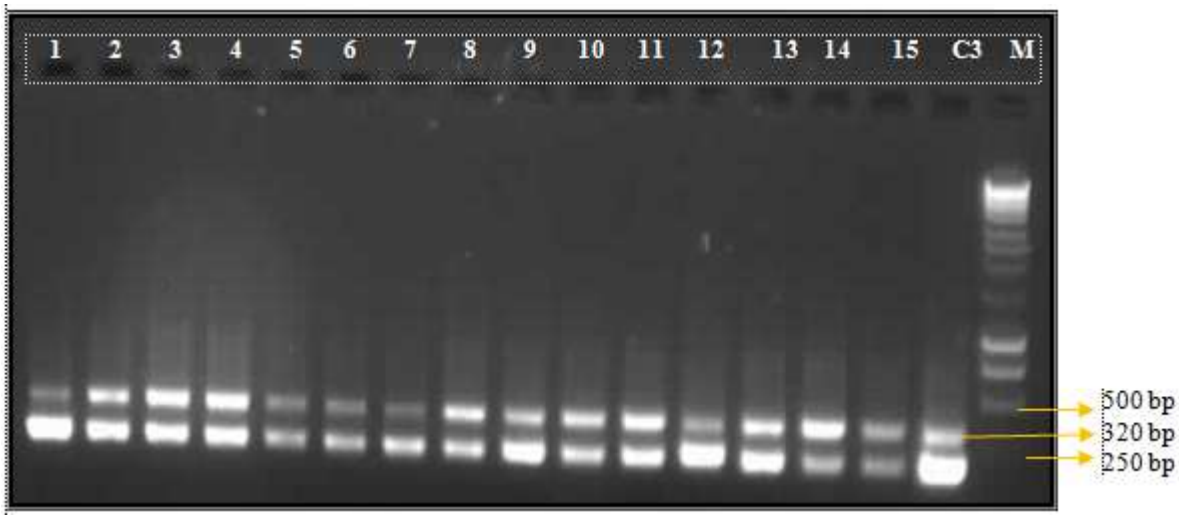


**Plate 3: Gel Picture Showing *Ty3a* Resistant Marker Amplification**

Lanes 1-15: H 24 (Hisar Anmol), Vyabhav, Nandhi, Sankranthi, Pusa Ruby, Abhinava, Arka Ananya, Lakshmi, NS-501, Shakthiman, US-618, IIHR 2101, IIHR 2611, IIHR 2822, IIHR 2823, C1- Check (LA 1969), C2 - Check (LA 2279), C3 - Check (LA 1932), Marker (1 kb)

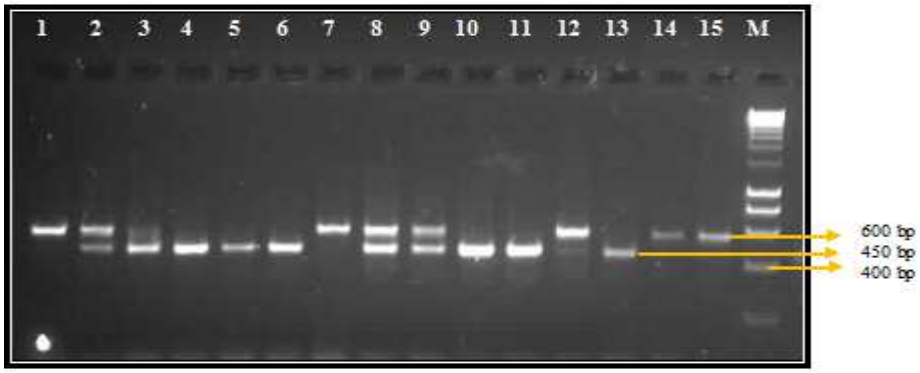


**Plate 4: Gel Picture Showing *Ty2* Resistant Marker Amplification**

Lanes 1-15: H 24 (Hisar Anmol) (Check), Vyabhav, Nandhi, Sankranthi, Pusa Ruby, Abhinava, Arka Ananya, Lakshmi, NS-501, Shakthiman, US-618, IIHR 2101, IIHR 2611, IIHR 2822, IIHR 2823, Marker (200 bp)