Validation of SSR Markers for Imparting Disease Resistance in Tomato (Solanum lycopersicum L.)

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A B S T R A C T

The simple sequence repeat (SSR) or microsatellite marker is currently the most preferred molecular marker because of its reproducibility and codominant nature. The aim of this study was to validate SSR markers for bacterial wilt (BW) and tomato leaf curl virus disease (ToLCV) in tomato. DNA isolated from the parents Mukthi and IIHR-2195 was used to validate five SSR primers already reported for BW and ToLCV. One primer SSR20 which showed good polymorphism and reproducibility among parents were selected for further validation in F3 and F4 population. SSR20 was validated on resistant F4, their corresponding F3 parental lines, along with susceptible checks. The SSR20 segregated with the trait in the F3 and F4 resistant plants and was also found expressed in few susceptible checks. SSR20 identified in the study could be utilized for marker assisted selection with respect to bacterial wilt in tomato.

Keywords
Simple Sequence repeats (SSR's), Bacterial wilt, Tomato leaf curl virus (ToLCV)

Introduction

Tomato (Solanum lycopersicum L.) is one of the world’s most important vegetable crop and has been the subject of genetic study for more than a century. It has offered insights into genetics, breeding and evolution. It belongs to the family Solanaceae and diversified first in Peru, Mexico where it was domesticated from its ancestor, Solanum lycopersicum cerasiforme (Cox, 2000). It then spread to all the important agroecologies of tropical, subtropical and temperate regions. The productivity of tomato in India is very less compared to world scenario. There are many constraints for less productivity and quality. The production and quality of tomato fruits are considerably affected by plant disease at different stages of crop growth and perishable nature of fruit respectively. Over two hundred diseases are listed worldwide (Gry, 1994). Among these, bacterial wilt disease is a major limiting factor in tropical, subtropical and humid regions of the world (Yabuuchi et al.,
1995). *Ralstonia solanacearum*, the causal agent of bacterial wilt, is one of the most devastating plant pathogenic bacteria (Mansfield et al., 2012) with a large host range encompassing more than 200 plant species which include major agricultural crops such as tomato, potato and banana (Hayward, 1991; Elphinstone, 2005).

Leaf curl caused by the Tomato Leaf Curl virus (ToLCV), a heterogenous complex of whitefly transmitted geminivirus is another serious production constraint of tomato worldwide, particularly in the Indian subcontinent. The effect of the disease is near total loss of crops. Each year ToLCV infection causes millions of dollar damage to tomato crops all over the world. Despite the efforts taken up so far, tomato leaf curl virus disease and bacterial wilt (BW) still continues to be the major limiting factors in tomato cultivation. The leaf curl virus infects the crop in all locations while bacterial wilt is more severe in warm humid tropics. Acidic soils, humid climate and high temperature favour bacterial wilt incidence in Kerala and it affects the crop at all stages of growth resulting in total crop loss. Leaf curl virus incidence is also gaining importance in the state recently and hence it is the need of the hour to develop varieties with combined resistance. Conventional breeding has helped to develop location specific varieties and molecular breeding have identified several Resistant Gene Analogues and QTLs mapped on different chromosomes. Considering the importance of bacterial wilt in Kerala, Kerala Agricultural University has developed varieties with relatively good resistance to Bacterial wilt (eg- Mukthi), but are susceptible to ToLCV and fruit qualities are not superior. Genotypes resistant to different strains of ToLCV have been developed at Indian Institute of Horticultural Research (eg- IIHR-2195) and this project is an attempt to incorporate combined resistance to BW and ToLCV through molecular breeding. The markers that will be validated will be of great use in marker assisted selection. An ideal genotype with ToLCV resistance in bacterial wilt resistance background and having desirable horticultural traits is targeted in the programme.

**Materials and Methods**

Bacterial wilt resistant variety Mukthi, released from Kerala Agricultural University and ToLCV resistant genotype IIHR-2195 identified at Indian Institute of Horticulture Research, Bangalore, were raised in pots during March-June, 2013, for screening the primers (SSR) already reported for disease resistance in tomato.

Five bacterial wilt resistant genotypes viz., Anagha, Sakthi, Mukthi, LE 1-2 and LE 626 were crossed with seven Tomato leaf curl virus (ToLCV) resistant genotypes viz., IIHR 2195, IIHR 2196, H 24, H 86, Hawaii 7998, LE 474 and LE 640 in a line x tester fashion in an earlier work by the research group (Yadav, 2011). The thirty five F \(_1\) hybrids of the cross Mukthi X IIHR 2195 along with their parents were grown in a wilt sick field to study their reaction to bacterial wilt and ToLCV during August-November, 2010 (Yadav, 2011). The F \(_2\)’s of thirty five crosses were grown in bacterial wilt sick field to screen for bacterial wilt and ToLCV resistance during February-May, 2011 (Yadav, 2011). Among the F \(_2\) segregants, 30 segregants were found promising and resistant to both ToLCV and bacterial wilt (Yadav, 2011). F \(_3\) population was raised from the seeds obtained from five F \(_2\) plants which showed combined resistance (Dheemanth et al., 2017). F \(_4\) population was raised from the seeds obtained from 22 F \(_3\) plants which showed combined resistance and 35 plants were found tolerant to both the diseases (Dheemanth et al., 2017). In the present study, selected SSR markers validated
in parental population were further applied on resistance and susceptible plants in F3 and F4 population.

**Screening and analysis of SSR primers**

DNA was extracted using CTAB procedure developed by Rogers and Bendich (1994). Five SSR primers already reported in tomato are listed in Table 1 were screened with parents (Mukthi and IIHR-2195) and those primers which gave polymorphism in parents were selected for screening F3 and F4 population by PCR for SSR analysis. The amplified products were run on two per cent agarose gel using 1X TAE buffer stained with ethidium bromide along with molecular weight marker (100bp ladder). The profile was visualized under UV (312 nm) transilluminator and documented. The documented SSR profiles were carefully examined for amplification of DNA.

**Results and Discussion**

Five SSR primers were screened using the DNA isolated from Mukthi and IIHR-2195 to select the primers showing good amplification and polymorphism among the parents. The number of bands obtained using the SSR primers ranged from 1 to 2 (Table 2). The amplification pattern obtained for SSR primers is shown in Plate 1. Among the five SSR primers only one (SSR 20) gave polymorphism among the parents Mukthi and IIHR-2195. The primer SSR 20 gave two distinct bands for IIHR-2195 out of which shared one with the variety Mukthi, thus the band of size 180 bp was found polymorphic among the two parents. The other primers gave monomorphism among the parents so they were not selected for validation in F3 and F4 plants. Different sources of resistance and linkage of the markers with the QTL may be the reason for not obtaining polymorphism to characterize Mukthi and IIHR-2195 with all the reported markers in the present study.

The selected SSR marker was further tested on F3 and F4 population for confirming their segregation pattern. The bacterial wilt specific primer SSR 20 which showed polymorphism among the parents Mukthi and IIHR-2195 was evaluated on F4 progenies with combined resistance for bacterial wilt and ToLCV along with its F3 parent and susceptible F3 lines. These F4 lines, their F3 parent; when analyzed indicated monomorphism to wilt resistant parent Mukthi representing 180bp band (Table 3).

The bacterial wilt specific band was however also found present in some of the susceptible F3 progenies evaluated. The selected F4 plants derived from 5 F2 lines were validated against the primer SSR20 along with their corresponding F3 parents and few susceptible lines.

In all the 5 sets of resistant plants the specific band for wilt resistance (180 bp) was amplified. However the susceptible once gave different amplification patterns. Some of them gave heterozygous banding pattern as expected (Plate 3b, 4b, 6b). Few other susceptible once gave banding pattern similar to resistant once (Plate 2a, 3b, 4b, 6b) and others did not amplify at all (Plate 2b, 3a, 4a, 5, 6a). This can be expected in a segregating population for a trait controlled by recessive genes and multiple alleles. Nazeem et al., (2010) reported that polymorphic band in resistant genotypes and several SNP and other PCR-based markers associated with BW resistance genes on tomato chromosomes 6 and12.
**Table.1** List of SSR primers screened with tomato samples

| Sl No | Name of Primers | Sequence                                      | Source                  |
|-------|-----------------|-----------------------------------------------|-------------------------|
| 1     | LEaat 007       | F 5’ CAA CAG CAT AGT GGA GGA GG 3’           | (He et al., 2003)       |
|       |                 | R 5’ TAC ATT TCT CTC TCT CCC ATG AG 3’       |                         |
| 2     | LEat 006        | F 5’ CAT AAT CAC AAG CTT CTT TCG CCA 3’      |                         |
|       |                 | R 5’ CAT ATC CGC TCG TTT CGT TAT GTA AT 3’   |                         |
| 3     | LEaat 002       | F 5’ GCG AAG AAG ATG AGT CTA GAG CAT AG 3’   |                         |
|       |                 | R 5’ CTC TCT CCC ATG AGT TCT CCT CTT 3’      |                         |
| 4     | SSR 20          | F 5’ GAG GAC GAC AAC AAC AAC GA 3’           | Sol Genome             |
|       |                 | R 5’ GAC ATG CCA CTT AGA TCC ACC A 3’        | Project                |
| 5     | SSR 306         | F 5’ ACA TGA GCC CAA TGA ACC TC 3’           |                         |
|       |                 | R 5’ AAC CAT TCC GCA CGT ACA TA 3’           |                         |

**Table.2** Number of bands and amplification patterns of SSR primers in parental genotypes Mukthi and IIHR-2195

| Sl.No | SSR Primers | Number of amplicons observed | Amplification pattern |
|-------|-------------|------------------------------|-----------------------|
|       |             | Number | Specificity | Mukthi | IIHR-2195 |                         |
|       |             |        |             |        |           | Monomorphic             |
| 1.    | LEaat 007   | BW     | ToLCV       | 1      | 1         |                         |
| 2.    | LEaat 002   | BW     | ToLCV       | 1      | 1         |                         |
| 3.    | SSR 306     | Nil    | Nil         | 2      | 2         | Monomorphic             |
| 4.    | LEaat 006   | BW     | ToLCV       | 1      | 1         | Monomorphic             |
| 5.    | SSR 20      | BW     | Nil         | 1      | 2         | Polymorphic             |

**Table.3** Segregation pattern of SSR20 marker in F₃ and F₄ population

| S. No. | Marker type | Name  | F₄ line | Number of plants with combined resistance | Marker segregations |
|--------|-------------|-------|---------|-------------------------------------------|---------------------|
| 1.     | SSR         | SSR20 | 54-31   | 4                                         | 4/4 | 1/1 |
| 2.     |             |       | 38-50   | 5                                         | 5/5 | 1/1 |
| 3.     |             |       | 54-67   | 4                                         | 4/4 | 1/1 |
| 4.     |             |       | 38-49   | 3                                         | 3/3 | 1/1 |
| 5.     |             |       | 54-57   | 3                                         | 3/3 | 1/1 |
Plate.1 Screening of SSR primers with the parents Mukthi and IIHR-2195

M and 14- Marker (100bp), 1- LEaat 007 with Mukthi, 2- LEaat 007 with IIHR-2195, 3- LEaat 002 with Mukthi, 4- LEaat 002 with IIHR-2195, 5- SSR 306 with Mukthi, 6- SSR 306 with IIHR-2195, 7 - LEaat 006 with Mukthi, 8- LEaat 006 with IIHR-2195, 9- SSR 20 with Mukthi, 10- SSR 20 with IIHR-2195.

Plate.2a Validation of BW specific marker SSR 20 on F₄ segregants with combined resistance along with the F₃ parent and F₃ susceptible ones

L- Ladder (100bp), 1- Mukthi, 2- IIHR-2195, 3- F₃ Parent (F₂-54-31), 4- F₄ resistant (F₃-54-31-19), 5- F₄ resistant (F₃-54-31-25), 6- F₄ resistant (F₃-54-31-33), 7- Susceptible (F₂-47-6), 8- Susceptible (F₂-47-14).

Plate.2b Validation of BW specific marker SSR 20 on F₄ segregants with combined resistance along with the F₃ parent and F₃ susceptible ones

L- Ladder (100bp), 1- Mukthi, 2- IIHR-2195, 3- F₃ Parent (F₂-54-31), 4- F₄ resistant (F₃-54-31-19), 5- F₄ resistant (F₃-54-31-25), 6- F₄ resistant (F₃-54-31-33), 7- Susceptible (F₂-38-1), 8- Susceptible (F₂-38-3).
Plate 3a Validation of SSR 20 in F₃ and F₄ (38-50 line) for bacterial wilt resistance in tomato

L- Ladder (100bp), 1- Mukthi, 2- IIHR-2195, 3- F₃ Parent (F₂-38-50), 4- F₄ resistant (F₃-38-50-18), 5- F₄ resistant (F₃-38-50-26), 6- F₄ resistant (F₃-38-50-31), 7- F₄ resistant (F₃-38-50-35), 8- F₄ resistant (F₃-38-50-39), 9- Susceptible (F₂-38-1), 10- Susceptible (F₂-38-3).

Plate 3b Validation of SSR 20 in F₃ and F₄ (38-50 line) for bacterial wilt resistance in tomato

L- Ladder (100bp), 1- Mukthi, 2- IIHR-2195, 3- F₃ Parent (F₂-38-50), 4- F₄ resistant (F₃-38-50-18), 5- F₄ resistant (F₃-38-50-26), 6- F₄ resistant (F₃-38-50-31), 7- F₄ resistant (F₃-38-50-35), 8- F₄ resistant (F₃-38-50-39), 9- Susceptible (F₂-38-66), 10- Susceptible (F₂-41-5), 11- Susceptible (F₂-41-74).

Plate 4a Validation of SSR 20 in F₃ and F₄ (54-67 line) for bacterial wilt resistance in tomato

L- Ladder (100bp), 1- Mukthi, 2- IIHR-2195, 3- F₃ Parent (F₂-54-67), 4- F₄ resistant (F₃-54-67-18), 5- F₄ resistant (F₃-54-67-22), 6- F₄ resistant (F₃-54-67-23), 7- F₄ resistant (F₃-54-67-28), 8- Susceptible (F₂-38-1), 9- Susceptible (F₂-38-3).
Plate 4b Validation of SSR 20 in F₃ and F₄ (54-67 line) for bacterial wilt resistance in tomato

L- Ladder (100bp), 1- Mukthi, 2- IIHR-2195, 3- F₃ Parent (F₂-54-67), 4- F₄ resistant (F₃-54-67-18), 6- F₄ resistant (F₃-54-67-22), 7- F₄ resistant (F₃-54-67-23), 8- F₄ resistant (F₃-54-67-28), 9- Susceptible (F₂-38-66), 10- Susceptible (F₂-41-5).

Plate 5 Validation of SSR 20 in F₃ and F₄ (38-49 line) for bacterial wilt resistance in tomato

L- Ladder (100bp), 1- Mukthi, 2- IIHR-2195, 3- F₃ Parent (F₂-38-49), 4- F₄ resistant (F₃-38-49-2), 5- F₄ resistant (F₃-38-49-13), 6- F₄ resistant (F₃-38-49-16), 7- Susceptible (F₂-41-3), 8- Susceptible (F₂-41-4).

Plate 6a Validation of SSR 20 in F₃ and F₄ (54-57 line) for bacterial wilt resistance in tomato

L- Ladder (100bp), 1- Mukthi, 2- IIHR-2195, 3- F₃ Parent (F₂-54-57), 4- F₄ resistant (F₃-54-57-1), 5- F₄ resistant (F₃-54-57-5), 6- F₄ resistant (F₃-54-57-21), 7- Susceptible (F₂-41-3), 8- Susceptible (F₂-41-4).
Plate 6b Validation of SSR 20 in F3 and F4 (54-57 line) for bacterial wilt resistance in tomato

Different types of gene actions have been reported for bacterial wilt in tomato. Tikoo et al., (1983) have reported the presence of two independent genes for wilt resistance. The resistance was reported to be governed by multiple recessive genes in CRA 66 Sel A from Hawaii and another by single dominant gene in 663-12-3 from Taiwan. Sreelathakumari (1983) reported a complimentary and hypostatic type of digenic recessive gene system for wilt resistance in tomato. BWR-1 a pure line selection with a dominant gene for bacterial wilt resistance was developed from AVRDC accession L33 (VC 8-1-2-1) (Tikko et al., 1986). Anand et al., (1992) reported dominant gene action in the F1S of BWR-1, BWR-5, 1661, 15 SB and 1836 and incomplete dominance in the F1S of 1881 and Sonali for resistance to bacterial wilt.

In most cases resistance has been reported to be polygenic (Danesh et al., 1994; Thoquet et al., 1996; Hanson et al., 1998; Mangin et al., 1999) although in a few cases the presence of major resistance genes has been suggested. In particular, a single dominant resistance gene was reported in the genotype Hawaii 7998 (Scott et al., 1988) and Hawaii 7996 (Grimault et al., 1995). Traditional breeding for BW resistance has proven difficult for various reasons, including variation in pathogen populations, environmental effects on disease expression and association of resistance with undesirable horticultural characteristics such as small fruit size (Scott et al., 2005; Yang and Francis, 2007).

Thus, the use of molecular markers to assist separating BW resistance from undesirable horticultural characteristics, and to pyramid resistance genes from multiple sources, has been advocated (Yang and Francis, 2007). SSR20 identified in the study could be utilized for marker assisted selection in tomato.

The markers found to segregate along with the trait could be recommended for marker assisted selection in tomato. To increase the utility of MAS in tomato breeding, it is imperative that additional efforts are made to identify allele specific markers and validate reported markers, which could be used across breeding populations. In some cases it may be necessary to fine map the gene(s) of interest and identify markers based on gene sequences or closely flanking sequences.
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