Validation of Molecular Markers for Tagging the Combined Resistance for Bacterial Wilt and Tomato Leaf Curl Virus Diseases in Tomato

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Abstract

Molecular markers have been used for identification and mapping of genes and QTLs for numerous agriculturally important traits in tomato including resistance / tolerance to biotic and abiotic stresses and fruit and flower-related characteristics. Tomato leaf curl virus (ToLCV) disease caused by begomovirus and the bacterial wilt, caused by *Ralstonia solanacearum* are rated as the most serious production constraints in tomato. Though the breeding for disease resistance is an important objective in tomato improvement, the extent to which markers have been utilized in such programs has not been clearly determined. The utility of molecular markers for use in tomato breeding programs is limited as most of the markers reported are not validated across tomato genotypes or are not polymorphic within tomato breeding populations. In this study, the validity of available markers specific for bacterial wilt and ToLCV resistance traits in tomato was examined by testing them with an F_3 population produced by crossing two resistant genotypes - Sakthi (BW resistant) and IIHR 2196 (ToLCV resistant). Most of the primers needed PCR optimization for successful amplification and some were not informative in the genotypes studied. Out of the eight markers examined, two were specific for resistance to bacterial wilt ($TSCAR_{AAG/CAT}$) and ToLCV (Ualty 16). Most of the available markers need to be further refined or examined for trait association and presence of polymorphism in the breeding lines and populations. However, with recent advances in tomato genome sequencing, it has become increasingly possible to develop more informative markers to accelerate the use of MAS and tagging the genes of interest in tomato breeding.

Keywords: Bacterial wilt; Disease resistance; Marker Assisted Selection; Molecular breeding; ToLCV; *Solanum lycopersicum*

The cultivated tomato (*Solanum lycopersicum* L.) is the second most commonly consumed vegetable crop (after potato) and unquestionably, the very popular garden vegetable in the world. There exists large number of varieties of tomato sold worldwide than any other vegetable crop. Most commercial cultivars of tomato are the results of conventional plant breeding programs, where trait evaluation and phenotypic selection under field or greenhouse conditions are the routine procedures. However, with the advent of molecular markers and genetic maps, there has been an increased interest in the use of marker technology to facilitate tomato crop improvement.

The main limiting factor for the cultivation of this crop in the hot and humid tropics is the incidence of bacterial wilt caused by *Ralstonia solanacearum*. Symptoms of the disease include rapid and complete wilting of grown up plants. Tomato leaf curl virus (ToLCV) is another serious problem which is caused by a gemini virus transmitted by the whitefly *Bemisia tabaci* (Anbinder et al., 2009). The infected plant exhibit curling, puckering, reduction in leaflet

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size, severe stunting and reduced in fruit set. Resistance breeding taken up in Kerala Agricultural University has so far developed four bacterial wilt resistant varieties viz., Sakthi, Mukthi, Anagha and Manulakshmi. However, these varieties are susceptible to ToLCV, necessitating the development of varieties with combined resistance. Tomato is one among the first crop species for which genetic markers were suggested as indirect selection criteria for breeding purposes and for which molecular markers and maps were developed (Tanksley, 1983; Tanksley et al., 1992; Foolad, 2007). Over the past 30 years, several molecular maps of tomato have been developed; including the high-density linkage map of tomato based on *S. Lycopersicum* and *S. pennellii* (Pillen et al., 1996; Fulton et al., 2002). Furthermore, molecular markers associated with genes or QTLs underlying many agriculturally important traits in tomato, including disease and insect resistance, abiotic stress tolerance, and flower and fruit-related characteristics, have been identified and mapped. Such developments have provided opportunities for selection of economically important traits, based on the genotype of the associated markers instead of the trait itself, a process known as marker-assisted selection (MAS).

Markers are considered useful if they detect association with the trait of interest across breeding lines and populations. In this study, the utility and reproducibility of the reported markers were analysed for BW and ToLCV resistance in tomato, using the F_3 population of the parents with definite resistance to the diseases.

Materials and methods

Plant material

Two tomato genotypes Sakthi (BW resistant) and IIHR 2196 (ToLCV resistant), and the F_3 population (200 nos.) of a cross between Sakthi and IIHR 2196, were used to examine the utility of the available molecular markers for bacterial wilt and ToLCV disease resistance traits in tomato. The parents have been checked under lab conditions with high dose of inoculum and confirmed for their resistance/ susceptibility. The genotypes were chosen based on their field reaction for disease resistance; however, the homozygosity of resistance at genic level was not expected in these lines since they were in the F_3 stage only. All plants have been grown in bacterial wilt sick field, during the peak summer months when the virulence of the viral vectors will be maximum. Regarding the bacterial wilt disease, the field is referred as sick since the soil is proven to contain more than enough inoculum of *Ralstonia solanacearum* that is required to initiate bacterial wilt symptoms in tomato (10^7 cfu g^{-1} of soil). At KAU, the particular field is regularly used to screen the tomato genotypes for the levels of wilt resistance. With respect to ToLCV, the presence of infected plants and optimum population of whitefly vectors was confirmed in and around the field, as specified by the pathologist. The peak summer which is the optimum weather for both these diseases was also chosen for the field screening experiments. The identified resistant and susceptible plants were subsequently used for DNA extraction and marker analysis.

DNA extraction and marker analysis

Genomic DNA was extracted from young leaves of one month old plants using the standard CTAB protocol (Rogers and Bendich, 1994). DNA concentration was measured using a NanoDrop ND-1000 and the concentration of the working sample was adjusted to 30 ng/µl. Primer sequence information for markers associated with the bacterial wilt and ToLCV disease resistance genes were obtained from published literature (Table 1). Specific SCAR (Sequence Characterised Amplified Region) markers which are separately reported for tagging the resistance for BW and ToLCV in tomato were selected and employed in the study. Out of the eight SCAR markers evaluated, $TSCAR_{AAG/CAT}$ and $TSCAR_{AAT/CAT}$ were specific to BW and the remaining 6 for ToLCV. All the SCAR primers were

Table 1. List of SCAR (Sequence Characterised Amplified Region) primers used for screening the parents (Sakthi and IIHR 2196)

Sl No	Name of Primers		Sequence	Marker type BW	Marker type ToLCV	Reference
1	$TSCAR_{AAT/CGA}$	F	5‘ TAG ATG GAA TCC AAT ATC AGG 3‘	√		Miao et al. (2009)
		R	5’ AAC CAC AGT GAA GGA ATA TAC A 3’			
2	$TSCAR_{AAG/CAT}$	F	5’ AGA AGG TCA CGG CGA GA 3’	√		
		R	5’ TGA GTC CTG AGT AAC TGG 3’			
3	Ualty3a	F	5’GAC CTT CAA AAT GAT CAG ATA 3’		√	Gonzalez-cabezuela et al. (2012)
		R	5’ TGG ACC CTT TTT ACC CTA AGC 3’			
4	Ualty3b	F	5’ CTC CAC AGC TTC AAT GCA AA 3’		√	
		R	5’ CGT GAA TAC CTT GAT TCT TGA 3’			
5	Ualty 5	F	5’ TAG GAA ATG TTG AAC TAT TGT GTT 3‘		√	
		R	5’ TCA TGC GAT GAA GAG GTC TAT G 3‘			
6	Ualty 6	F	5’ TGT TGT GAT TGT TAT TGT CAA C 3‘		√	
		R	5’ CTG GCA AGC GTG TAA CTC AC 3’			
7	Ualty 11	F	5’ TTA ATT CTA GGG ATT TGG CAG T 3’		√	
		R	5’ CCC AAG CCA TCA TGA GAT TC 3’			
8	Ualty 16	F	5’ GCA CAA AAA TGC TTT TGG ACA 3’		√	
		R	5’ TTC CGA ATT AAC AGA GTC TCC AC 3’			

ordered from Sigma Technologies (Banglore, India) and the PCR conditions as given in the corresponding reports were followed. Briefly, PCR was conducted in 20.0 µl volume consisting of 4.4 µl master mix (2.0 µl dNTPs, 0.4 µl Taq polymerase, 2.0 µl reaction buffer A), 1.0 µl DNA (30-35 ng/ µl), 0.75 µl each of forward and reverse primers and 13.1 µl of nuclease free water. After PCR amplification, PCR products have been separated on 2 per cent agarose gel and the bands were visualized with ethidium bromide staining. Fragment sizes were determined using a 100 bp DNA ladder.

Results and Discussion

Numerous sources of genetic resistance to BW have been identified and the genetics of resistance have been extensively studied (Hanson et al., 1998; Scott et al., 2005). 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).

Among the eight markers used in the present study, two markers, $TSCAR_{AAG/CAT}$ and Ualty 16 gave polymorphic bands (~500bp) in bacterial wilt and ToLCV resistant genotypes, respectively. In the F_3 population, the $TSCAR_{AAG/CAT}$ showed 113 polymorphic amplicons indicating bacterial wilt resistance (Fig. 1A and B) and Ualty 16 showed 52 polymorphic amplicons with respect to ToLCV resistance. In field reaction, under sufficiently high levels of pathogen and highly congenial environmental conditions, 104 plants were resistant

Table 2. The marker segregation scored against disease reaction in F_3 population

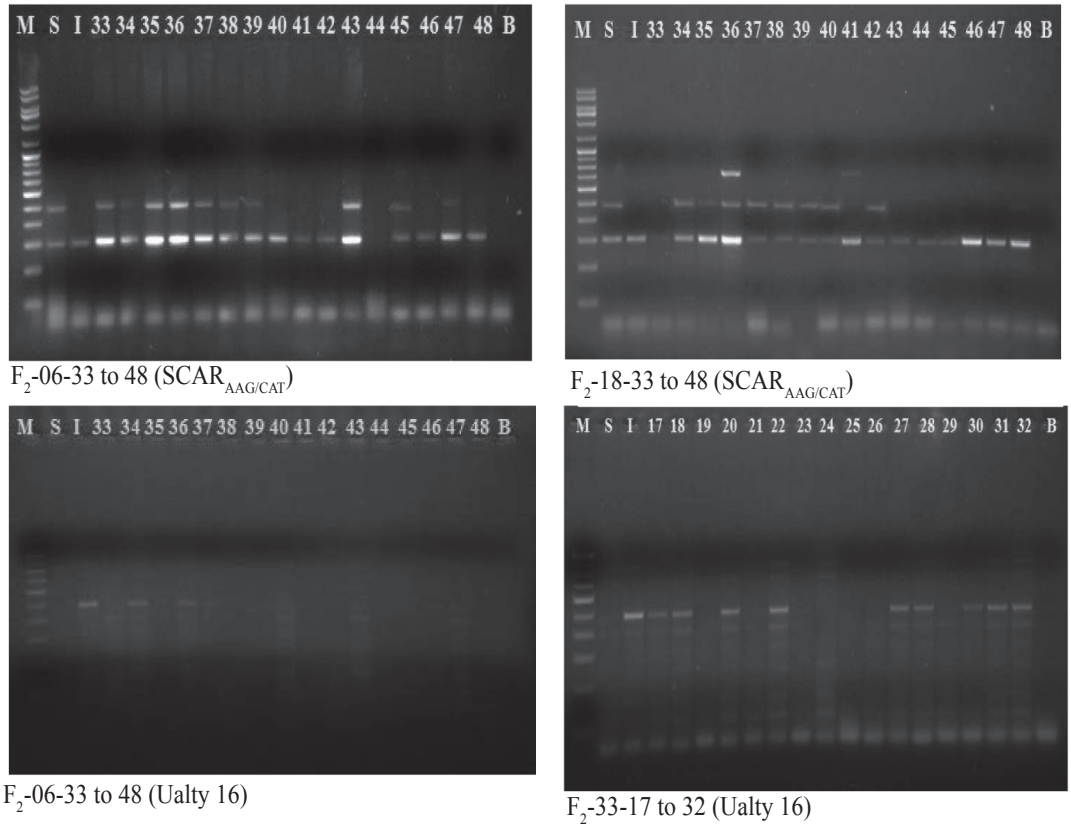
Sl. No.	Marker system	Primers	No. of plants analysed through marker segregation	In Field		Polymorphism of markers for disease reaction				Field reaction			
						BW		ToLCV		BW		ToLCV	
				BW	ToLCV	R	S	R	S	R	S	R	S
01	SCAR	TSCAR $_{AAG/CAT}$	200	200	104	113	87	-	-	104	96	12	92
02	SCAR	Ualty 16	200	200	104	-	-	52	148	104	96	12	92

R – Resistant, S – Susceptible

to BW while only 12 plants showed combined resistance to bacterial wilt and ToLCV and these were included among the ones identified by the marker system (Table 2). Marker studies indicated ToLCV resistance in 52 plants. However in field screening, all these 52 have not survived due to wilt incidence that is why only 12 plants with combined resistance were mentioned in text. It was difficult to score ToLCV resistance alone in field reaction since many of the resistant ones would not have survived BW at early stages. It was difficult to score ToLCV resistance alone in field reaction since many of the resistant ones would not have survived BW at early stages. Twelve F_3 lines with combined resistance to both BW and ToLCV were screened out in the study. Thus it could be inferred that among the 52 plants with ToLCV resistance identified by the marker system, only twelve are having combined resistance and these were screened out in field reaction too. Both the traits - BW and ToLCV are reported to be polygenic in nature and the source of resistance may differ in different parents. Further, the F_3 population on which this study has been performed, was forwarded from the selected F_2 plants which had shown combined resistance for both these diseases. Thus the number of plants with combined resistance to both these diseases was sufficiently high in the F_3 population. Screening of BW resistance with the marker system was fairly good. Since the marker could identify 113 progenies with resistance as against 104 resistant ones in field reaction. The difference of nine plants in field reaction can be contributed to wilting of plants due to physical injuries or transplanting shock. All the other six markers evaluated in the study have failed to give polymorphism in accordance with the actually observed resistance since sources of resistance for both these diseases, reported

M-Marker (100-3Kb), 1- Empty, 2- TSCAR $_{AAT/CGA}$ with Sakthi, 3- TSCAR $_{AAT/CGA}$ with IIHR 2196, 4-TSCAR $_{AAG/CAT}$ with Sakthi, 5-TSCAR $_{AAG/CAT}$ with IIHR 2196, 6-Ualty3a with Sakthi, 7-Ualty3a with IIHR 2196, 8-Ualty3b with Sakthi, 9-Ualty3b with IIHR 2196, 10-Ualty 5 with Sakthi, 11-Ualty 5 with IIHR 2196, 12-Ualty 6 with Sakthi, 13-Ualty 6 with IIHR 2196, 14-Ualty 11 with Sakthi, 15-Ualty 11 with IIHR 2196, 16-Ualty 16 with Sakthi, 17-Ualty 16 with IIHR 2196, 18 and 19-Empty, 20-Blank

Figure 1 A. Screening of tomato lines using the reported primers



F_2-06-33 to 48 ($SCAR_{AAG/CAT}$)

F_2-18-33 to 48 ($SCAR_{AAG/CAT}$)

F_2-06-33 to 48 (Ualty 16)

F_2-33-17 to 32 (Ualty 16)

M- Marker (1 Kb), S- Sakthi, I- IIHR 2196, B- Blank

B. Amplification pattern of F_3 population with primers $SCAR_{AAG/CAT}$ & Ualty 16

Figure 1B**.** Screening of primers with parental DNA and amplification of selected primers on F_3

elsewhere in the world are highly diverse and the failed markers were designed for the resistant genes other than those involved in the present crossing programme. The objective of the present study was to identify the suitable marker that shows segregation along with the resistance in the available sources, facilitating a marker assisted selection for the combined resistance. The final confirmation of field level resistance has to be done in advance generation like F_6 or $F_{7,}$ with virulent pathogen.

In a similar study, molecular markers associated with BW resistance in a highly-resistant cultivar (T51A) were reported (Miao et al., 2009). The BW resistance in T51A has been reported to be conferred by two complementary, co-dominant genes, located on tomato chromosome 6. No amplification has been observed in their susceptible lines. In another study, 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 and 12.

Several genes conferring resistance to TYLCV (Tomato Yellow Leaf Curl Virus) and ToLCV, mainly *Ty-1* and *Ty-3* genes, have been introgressed to cultivated tomato (*Solanum lycopersicum*) from the wild relative, *Solanum chilense* (Gonzalez-Cabezuelo et al., 2012). Scott (2007) has reported that the disease response of the resistant cultivars often varies from location to location, and it has been difficult to develop resistant cultivars with horticultural characteristics similar to those of susceptible ones. Four resistance loci, *Ty-1, Ty-2,*

Ty-3 and *Ty-4*, have been identified and mapped to tomato chromosomes 6, 11, 6 and 3, respectively (Zamir et al., 1994; Ji et al., 2007; 2009). When we analysed both the marker for combined resistance (BW and ToLCV) in F_3 segregants, 12 plants were identified for both the resistance in the field and the markers identified were also found to segregate along with the trait. $TSCAR_{AAG/CAT}$ showed good correlation with respect to bacterial wilt and Ualty 16 was good enough for ToLCV.

In general, resistance breeding for various bacterial and viral diseases in tomato, including bacterial canker, bacterial spot, bacterial wilt, ToMoV (Tomato Mosaic Virus), ToSWV (Tomato Spotted Wilt Virus), and ToLCV has been challenging. While some resistance genes have been identified and associated molecular markers reported, MAS is far from a routine application for improving combined disease resistance in tomato. It is either because of the complexity of pathogen or complexity of the genetic resistance.

Molecular markers associated with genes or QTLs underlying many simple and complex traits in tomato have been identified. Theoretically, the marker information should be useful for improving such traits via marker-assisted breeding. However, not all markers reported in the literature are readily applicable in tomato breeding programs, even for simple traits. Often additional efforts are needed to refine the markers or to identify and develop new polymorphic markers with greater utility and reproducibility in specific breeding populations. In this study, the utility of 8 markers previously reported for the resistance to bacterial wilt and ToLCV diseases of tomato has been examined and 2 markers, $TSCAR_{AAG/CAT}$ and Ualty 16, were found useful. Sequencing of transcript or cDNA is not envisaged in the study since it pertains to the validation of already reported markers. Genotypes having $TSCAR_{AAG/CAT}$ and Ualty 16 specific amplicons have been reported to be resistance for BW and ToLCV respectively (Nazeem et al., 2010). The failure of rest of the markers could be due to various reasons, including absence of genomic sequences complementary to the primers used, breakdown of linkages between markers and genes, and differences in PCR conditions. To increase the utility of MAS in tomato breeding, it is imperative that additional efforts are made to identify allele-specific 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. With recent advances in tomato sequencing, it is becoming increasingly possible to develop such markers to accelerate the use of MAS in tomato breeding.

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