Validation of markers linked to *Ty-2* gene imparting resistance to ToLCV disease in tomato (*Solanum lycopersicum* L.)

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Abstract

ToLCV disease caused by geminivirus transmitted through whitefly (Bamisia tabaci Gennadius) causes 90-100% yield loss in tomato. Molecular markers reported to be linked with resistance to ToLCV could accelerate the identification of resistant lines as they are independent of the environmental effect. However, before they can be utilized for marker assisted selection (MAS), validation in an independent population is required. The present investigation was carried out to validate two SCAR markers (TG0302 and P1-16) and one SSR marker (TES0344) linked to Ty-2 gene resistance to ToLCV disease of tomato. The susceptible varieties GPBT-08 and DMT-2 were crossed with resistant lines CLN2768A and CLN2777H respectively and F_1,s were selfed to get F_2. Two F_2 populations were screened for response to ToLCV under field conditions.

Al the three makers used in the study showed polymorphism between resistant and susceptible parents. Single marker analysis was performed to ascertain the relationship between the marker and the trait and showed significant R^2 value of all the three markers with resistance to ToLCV disease for the F_2 populations of the crosses GPBT-08 × CLN2768A and DMT-2 × CLN2777H. Kruskal - Wallis ANOVA test also indicated the significant H value for all the three markers in both the populations. These markers which clearly differentiated between resistant and susceptible lines of tomato can be efficiently used in MAS for developing resistant cultivars.

Keywords: Tomato, ToLCV, *Ty-2* gene, Single marker analysis, Kruskal – Wallis ANOVA.

Introduction

Tomato (*Lycopersicon esculentum* L.) is one of the most important solanaceous vegetable crops and widely grown in a variety of climatic conditions and is popular for its high nutritive value, taste and versatile uses. Tomato originated in South America in the general area of Peru and Ecuador was first domesticated in Mexico. It belongs to the family Solanaceae with chromosome number of 2n=24 and is a self-pollinated, annual crop[17]. It has become an important commercial crop so far as the area, production, industrial values and its contribution to human nutrition are concerned and for its high nutritive value, taste and versatile uses.

The production and quality of tomato fruits are considerably affected by array of insect pests infesting at different stages of crop growth. Of these, tomato leaf curl disease (ToLCV) has been a global constraint to tomato (*Solanum lycopersicum*) production since the 1980s. ToLCV has a wide host range that includes tomato (*S. lycopersicum*), sweet pepper (*Capsicum annuum*), chili pepper (*C. chinense*), tobacco (*Nicotiana tabacum*), common bean (*Phaseolus vulgaris*), petunia (*Petunia × hybrida*) and lisianthus (*Eustoma grandiflora*)[6].

Till now, ToLCV is still one of the most devastating diseases of tomato. Infected susceptible tomato plants show symptoms that include yellowing, curling and cupping of leaves, severe stunting and abortion of flowers and fruits, all of which can lead to yield reduction of up to 100%[1]. ToLCV can be caused by genus *Begomovirus* of the *Geminiviridae* family.

The *Ty-2* tomato yellow leaf curl resistance gene is derived from *S. habrochaites* and has been successfully used in breeding programs to provide resistance against monopartite *Tomato leaf curl virus* which is highly prevalent in southern India. The *Ty-2* locus has recently been fine-mapped to a 300 Mb region of chromosome 11, allowing sourcing of new, tightly linked markers for this locus.[13,31]

However, breeding for resistance has been slow due to ineffectiveness of selection methods[8]. Selection of resistant lines has been done by screening tomato genotypes in several environments, 'hot spot' sites by use of disease phenotyping to identify resistance sources in order to develop new varieties with improved resistance[7]. Phenotypic markers such as hairiness of leaves, toughness of leaf veins, thickness of leaf lamina, length of hair and angle of insertion were reported to be associated with sucking insect resistance used as an aid for selection for resistance to ToLCV in tomato.[18-20]

The problem associated with phenotypic evaluation of genotypes / lines is that it requires large sample sizes for screening, it is laborious and time consuming, relies greatly on repeated ratings in diverse environments confounded by environmental factors and incurs high experimental errors[9]. Nonetheless, molecular markers linked to ToLCV resistance genes can provide an alternative approach to overcome these

drawbacks and accelerate identification and development of resistant genotypes.[5,16]

Duveiller and Sharma[7] and Sharma et al[26] reported that use of molecular markers linked to *Ty-2* gene resistance to ToLCV disease in combination with field selection could increase the efficiency and speed of improving resistance in tomato lines. This is because the use of molecular markers saves time, reduces experimental errors and is always reliable and accurate as they are not confounded by environmental factors[5].

Validation of molecular markers with another population is required before utilizing in marker assisted breeding to determine their effectiveness[3]. Validation establishes the value of a molecular marker reported to be linked to a particular trait in an independent population with varying genetic background.[16,27] P1-16 and TES0344 molecular markers for their association with resistance to ToLCV disease for probable use in marker-assisted breeding.

Material and Methods

The present scientific investigation was carried out at Botany Garden, Department of Genetics and Plant Breeding, University of Agricultural Sciences, Dharwad. Two high yielding tomato varieties GPBT-08 and DMT-2 released by University of Agricultural Sciences, Dharwad and two lines CLN2768A and CLN2777H obtained from AVRDC, Taiwan were used for the presnt study.

Two F_1 hybrids were developed in Kharif by crossing GPBT-08 (susceptible to ToLCV) × CLN2768A (Resistant to ToLCV carrying *Ty-2* gene) and DMT-02 (susceptible to ToLCV) × CLN2777H (Resistant carrying *Ty-2* gene). The F_1's were raised during rabi. True F_1's were selfed to obtain two F_2 populations. The two F_2 population of both the crosses were screened for ToLCV during summer season along with F_1 and parents.

Molecular analysis

Genomic DNA extraction: Genomic DNA was isolated from young leaves from individual plants by following CTAB method described by Bernatzky and Tanksley[4] with certain modifications. The quality and concentration of DNA were assessed by using gel electrophoresis (0.8 % agarose) with known concentrations of uncut lambda DNA. 1-2 µl of RNase (10 mg/ml) were added, incubated incubate at 37^{o}C for 40-45 minutes and stored at -20^{o}C.

To amplify regions of genomic DNA, polymerase chain reaction (PCR) was performed in a 20 µl volume reaction mixture containing 1 µl of them DNA (50 ng/µl), 0.8 µl deoxynucleoside 5′-triphosphates (dNTPs) (2.5 mM each), 1 µl magnesium chloride ($MgCl_2$) (25 mM), 2 µl buffer (10×), 0.2 µl Taq polymerase (3 U/µl), 13.4 µl PCR grade water, 0.24 µl dye (10 µM), 0.06 µl forward primer and 0.3 reverse primer. The amplification reactions were performed in a heated lid thermal cycler programmed at 95°C for 51 min for one cycle followed by 30 cycles of denaturing at 94°C for 2 min, primer annealing at 58 °C for 1 min and an extension of one cycle of 72°C for 2 min, followed by a final extension at 72°C for 10 min. Amplification products were separated in 3 per cent agarose gel visualized using documentation system. The three co-dominant markers, TG0302[22], P1-16[30] and TES0344[29] previously reported to be linked to *Ty-2* gene resistance to ToLCV disease were used for validation.

Phenotypic evaluation: Phenotypic screening of two F_2 populations and its parents against resistance to ToLCV disease was done during summer in Botany garden, University of Agricultural Sciences, Dharwad ($15^0$31′N, 75^0 07′ 678 m a.s.l). Seeds were sown in the 98 cavities seedling trays. In separate glass house pure culture of whiteflies (*Bemisia tabaci* Genn.) were reared on large scale on brinjal and cotton plants.

These whiteflies were allowed to feed on ToLCV infected tomato cultivar Pusa Ruby for acquisition of ToLCV virus for 24 hours for the mass production of viruliferous flies. 10 days old seedling trays were kept in the screen house and mass inoculation was done with help of viruliferous whiteflies. Twenty five days old seedlings were transplanted into the experimental plots during summer. Plants were examined visually for disease symptoms at 30, 60 and 90 days after transplanting and observations recorded and per cent disease incidence (PDI) was calculated.

The disease severity score was based on Saari and Prescott's 0-4 scale for assessing foliar disease where zero = No symptoms, 1 = Light yellowing along the leaf margins and mild vein clearing, 2 = Yellowing of leaves and slight curling, growth, flowering and yield not greatly affected, 3 = Pronounced leaf curling, yellowing, stunting and reduced fruiting and 4 = Very severe curling, puckering, stunting and reduction in leaf size and no fruit formation.

The genotypes were classified on a 5-point scale using the resistance criterion proposed by Muniyappa et al[23]. Lines with 0 per cent incidence were considered as resistant (R), upto 25 per cent incidence considered as moderately resistant (MR), 26-50 per cent incidence considered as tolerant (T), 51-75 per cent incidence considered as susceptible (S) and >75 per cent incidence considered as highly susceptible (HS).

Data analysis: Single marker analysis (SMA) using regression analysis in Rstudio was done to determine the association between marker genotype data and field ToLCV disease resistance values. The band amplified from each marker data was scored as either 0 to indicate absence of the marker or 1 to indicate presence of the marker for regression analysis.[10,21] Significance of the regression coefficient suggests that there is a relationship between the marker and the trait[2]. The SMA was conducted following the linear model below:

Y = a + bX + error

where Y = trait value, a = constant, b = slope or regression coefficient of X and X = molecular marker.

Kruskal-Wallis test, a non-parametric method was also used to identify markers potentially associated with ToLCV disease resistance in two F_2 populations. The test statistic *Hc*, that has approximately a χ^2 distribution on k-1 degrees of freedom, was used to test its significance and to estimate the *P* value. To reduce the type I error, the following formula was used to calculated and compare to critical values of F for degrees of freedom of V_1 = k-1 and V_2 = N-k-1[32].

F= (N-k) *Hc*/ (k-1) (N-1-*Hc*)

Results and Discussion

Association of molecular markers to ToLCV disease resistance by single marker analysis (SMA): Marker TG0302 amplified PCR product of 900 base pair (bp) in the resistant parents CLN2768A and CLN2777H. The 900 bp fragment was also present in moderately resistant and tolerant F_2 progenies from these parental lines which were absent in the susceptible parental lines.

Susceptible parental lines GPBT-08 and DMT-2 and some F_2 progenies showed a 800 bp fragment upon amplification with marker TG0302. The 800 bp amplicon was present in all susceptible genotypes and not in the resistant and moderately resistant genotypes (Figure 1, 4 and 7)[22]. The similarity observed in the amplified fragments in the resistant parental and the F_2 progenies, resistant and moderately resistant ones, is one of the indications that the marker was associated with resistance.[11,15]. Upon screening with marker P1-16, a 600 bp fragment was observed in the susceptible parent, GPBT-08 and DMT-2.

This amplicon only appeared in the susceptible parents and in some F_2 progenies. For the same marker P1-16 amplified a 300 bp fragment in the resistant parent CLN2768A and CLN2777H and in moderately resistant and tolerant F_2 progenies (Figure 2, 5 and 7)[14].

Marker TES0344 amplified an identical fragment size of 205 bp in the resistant parental genotype CLN2768A and CLN2777H and resistant F_2 progenies derived from them. Susceptible parental lines GPBT-08 and DMT-2 and resistant F_2 progenies showed a 184 bp fragment (Figure 3, 6 and 8).

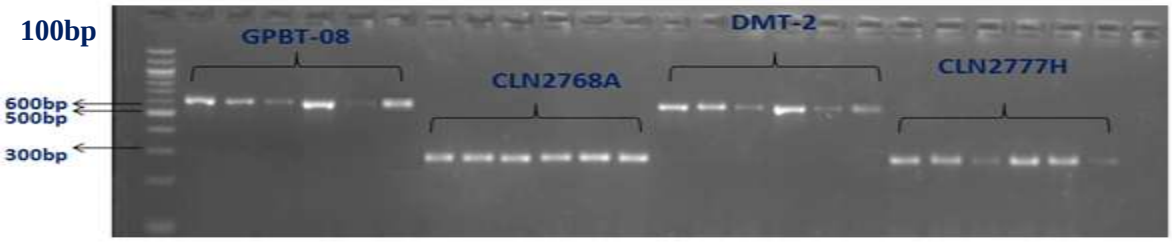

Figure 1: Parental polymorphism between GPBT-08 and CLN2768A and between DMT-2 and CLN2777H using SCAR marker P1-16

Figure 2: Parental polymorphism between GPBT-08 and CLN2768A and between DMT-2 and CLN2777H using SCAR marker TG0302

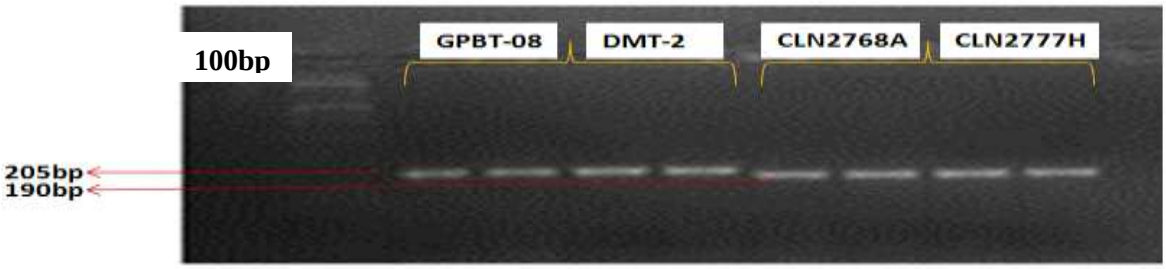

Figure 3: Parental polymorphism between GPBT-08 and CLN2768A and between DMT-2 and CLN2777H using SSR marker TES0344

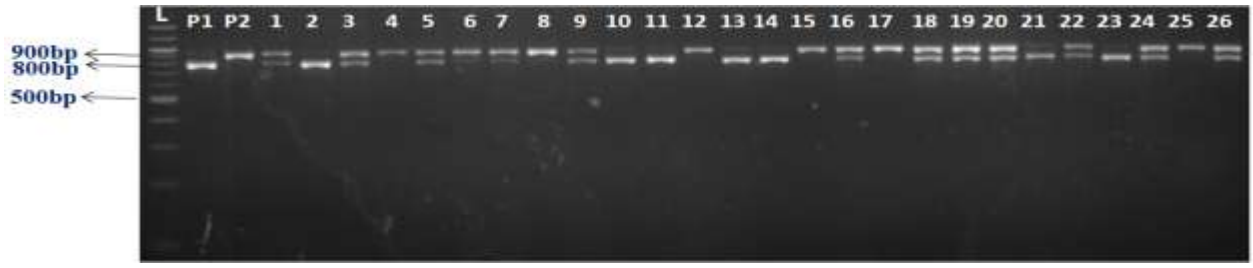

Figure 4: Amplification profile of F_2 with SCAR marker TG0302
[L: 100bp ladder, P1: GPBT-08. P2: CLN2768A, 1 to 26: samples of F_2]

Figure 5: Amplification profile of F_2 with SCAR marker P1-16
[L: 100bp ladder, P1: GPBT-08. P2: CLN2768A, 1 to 26: samples of F_2]

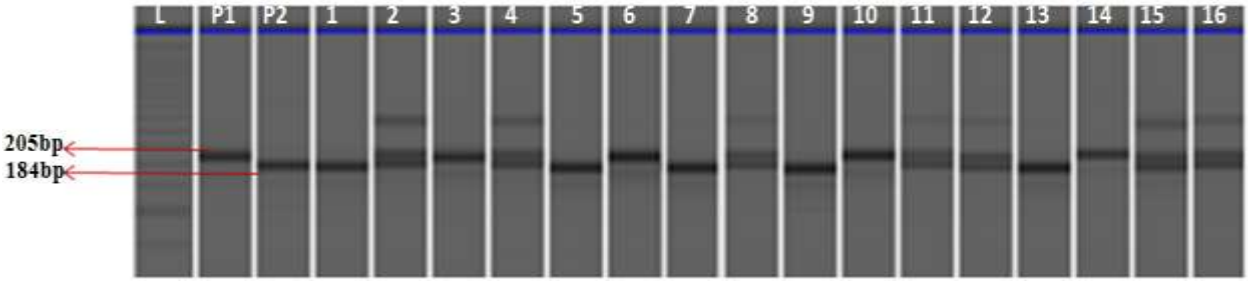

Figure 6: Amplification profile of F_2 with SSR marker TES034
[L: 100bp ladder, P1: GPBT-08. P2: CLN2768A, 1 to 16: samples of F_2]

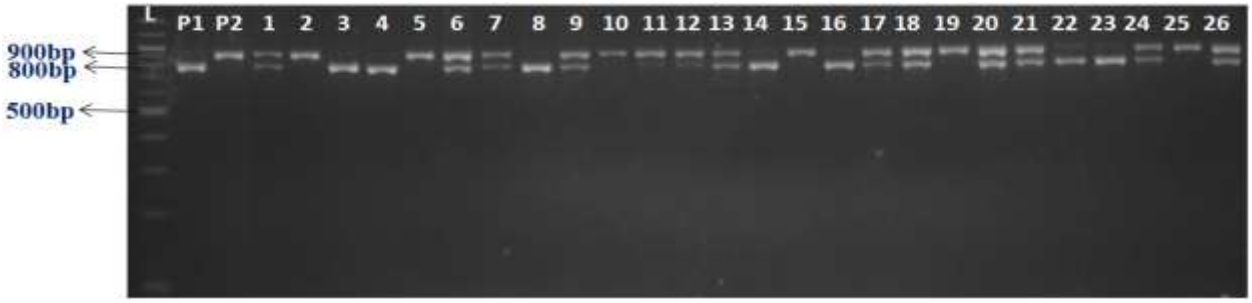

Figure 7: Amplification profile of F_2 with SCAR marker TG0302
[L: 100bp ladder, P1: DMT-2. P2: CLN2777H, 1 to 26: samples of F_2]

Figure 8: Amplification profile of F_2 with SCAR marker P1-16
[L: 100bp ladder, P1: DMT-2. P2: CLN2777H, 1 to 27: samples of F_2]

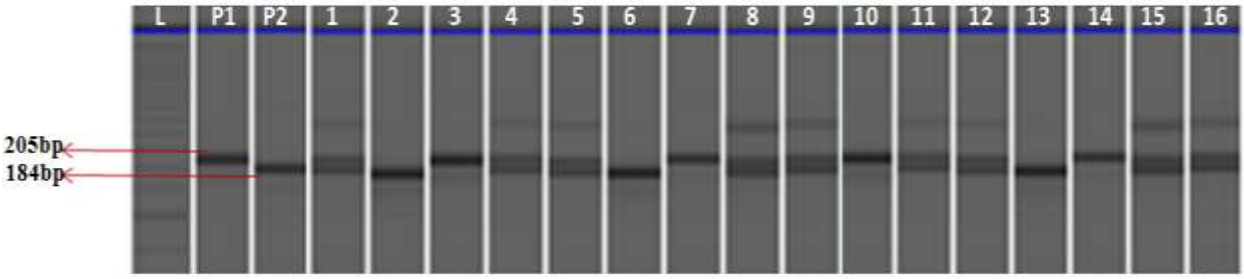

Figure 9: Amplification profile of F_2 with SSR primer TES0344
[L: 100bp ladder, P1: DMT-2. P2: CLN2777H, 1 to 16: samples of F_2]

Table 1
List of markers used for validation in tomato

S.N.	Marker name	Type of marker	Forward (F) and Reverse (R)	Amplicon size (bp)
1	TG0302	SCAR[11]	**F:** TGGCTCATCCTGAAGCTGATAGCGC **R:** AGTGTACATCCTTGCCATTGACT	900
2	P1-16	SCAR[30]	**F:** CACACATATCCTCTATCCTATTAGCTG **R:** CGGAGCTGAATTGTATAAACACG	300
3	TES0344	SSR[29]	**F:** GCCTTTTCCCACTTATATTCCTCTC **R:** ACACATACGACGTTCCGTCA	205

Table 2
Association of molecular markers with ToLCV disease reaction (30, 60 and 90 DAT) by single marker analysis and Kruskal-Wallis ANOVA F_2 population of the cross GPBT-08 × CLN2768A

Marker	Tests		TG0302	P1-16	TES0344
ToLCV-30	SMA	F *(P)*	58.29 (1.15E-21)	66.13 (6.63E-24)	51.41 (1.09E-19)
		R^2	30.79*	33.55*	28.34*
	KWA	H *(P)*	51.39* (2.23E-18)	54.34* (5.88E-20)	46.27* (5.65E-17)
		F	81.29*	88.56*	74.83*
ToLCV-60	SMA	F *(P)*	14.67 (1.83E-06)	31.27 (6.16E-13)	25.75 (6.19E-11)
		R^2	18.65*	19.27*	16.43*
	KWA	H *(P)*	26.59* (1.87E-10)	25.96* (3.84E-10)	29.94* (8.95E-12)
		F	44.80*	43.36*	50.88*
ToLCV-90	SMA	F *(P)*	16.84 (1.32E-07)	18.11 (4.3E-08)	15.71 (3.58E-07)
		R^2	11.39*	12.15*	10.71*
	KWA	H *(P)*	18.59* (2.96E-7)	17.75* (7.24E-7)	19.52* (1.09E-7)
		F	30.06*	28.28*	32.07*

*****: Significant at 5% level of probability; SMA: Single marker analysis; KWA: Kruskal- wallis ANOVA; H: Statistic test value (chi-square value); P: Probability value; ToLCV-30: ToLCV score at 30 DAT; ToLCV-60: ToLCV score at 60 DAT; ToLCV-90: ToLCV score at 90 DAT

SMA showed a significant association ($P < 0{\cdot}01$) of the markers and ToLCV resistance for all the three markers. In the cross GPBT-08 × CLN2768A P1-16 recorded the highest R^2 value of 33.55%, 19.27% and 12.15% at 30, 60 and 90 DAT respectively followed by TG0302 (30.79%, 18.65% and 11.39% at 30, 60 and 90 DAT respectively) and TES0344 (28.34%, 16.43% and 10.71% at 30, 60 and 90 DAT respectively). Kruskal-Wallis ANOVA test was conducted for all the three markers. Marker P1-16 recorded highest H value of 54.34, 25.96 and 17.75 at 30, 60 and 90 DAT respectively followed by the marker TG0302 (51.39, 26.59 and 18.59 at 30, 60 and 90 DAT respectively) and TES0344 (46.27, 29.94 and 19.52 at 30, 60 and 90 DAT respectively) (Table 2).

Table 3
Association of molecular markers with ToLCV disease reaction (30, 60 and 90 DAT) by single marker analysis and Kruskal-Wallis ANOVA F_2 population of the cross DMT-2 × CLN2777H

Marker	Tests		TG0302	P1-16	TES0344
ToLCV-30	SMA	F *(P)*	50.05 (9.81E-19)	52.81 (1.47E-19)	48.47 (2.95E-18)
		R^2	30.60*	31.75*	29.93*
	KWA	H *(P)*	47.99* (1.19E-16)	46.56 (1.62E-16)	41.29 (1.31E-15)
		F	73.34*	72.72*	68.53*
ToLCV-60	SMA	F *(P)*	35.06 (5.37E-14)	18.62 (9.11E-08)	31.06 (1.19E-12)
		R^2	23.62*	24.46*	21.49*
	KWA	H *(P)*	34.05* (1.83E-12)	21.09 (7.95E-8)	31.74 (2.07E-11)
		F	54.05*	32.69*	49.20*
ToLCV-90	SMA	F *(P)*	16.37 (2.3E-07)	16.99 (1.33E-07)	16.29 (2.43E-07)
		R^2	12.61*	13.02*	12.56*
	KWA	H *(P)*	18.34* (5.38E-7)	18.78* (3.36E-7)	18.26* (5.71E-7)
		F	28.87*	29.81*	28.75*

*: Significant at 5% level of probability; SMA: Single marker analysis; KWA: Kruskal- wallis ANOVA; H: Statistic test value (chi-square value); P: Probability value; ToLCV-30: ToLCV score at 30 DAT; ToLCV-60: ToLCV score at 60 DAT; ToLCV-90: ToLCV score at 90 DAT.

In F_2 population of the cross DMT-2 × CLN2777H observed phenotypic variance for resistance to ToLCV disease with the marker P1-16 recording the highest significant R^2 value of 31.75%, 24.46% and 13.02% at 30, 60 and 90 DAT respectively followed by TG0302 (30.60%, 23.62% and 12.61% at 30, 60 and 90 DAT respectively) and TES0344 (29.93%, 21.49% and 12.56% at 30, 60 and 90 DAT respectively).

Kruskal-Wallis ANOVA test was conducted for all the three markers. Marker P1-16 recorded highest significant H value of 46.56, 21.09 and 18.78 at 30, 60 and 90 DAT respectively followed by the marker TG0302 (47.99, 34.05 and 18.34 at 30, 60 and 90 DAT respectively) and TES0344 (41.29, 31.74 and 18.26 at 30, 60 and 90 DAT respectively) (Table 3).

Similar findings were reported by Vithoba[28] with phenotypic variance 28.1 per cent and 26.71 per cent for the marker TG0302 in crosses Pusa Ruby× CLN2768A and Pusa Ruby× CLN2777H and Pooja et al[24] observed phenotypic variance of 11.67% for the marker MB-SSR238 for powdery mildew resistance. This finding indicates significant association between the markers and the phenotypic[12] and also indicates usefulness as a tool for identifying resistant lines in early breeding generations.

This shows that the all the three markers have a high potential for use in MAS for resistance to ToLCV disease.

Conclusion

The markers TG0302, P1-16 and TES0344 previously reported to be linked with resistance to *Ty-2* gene were validated in the present study.

Since the R^2 values are significant for all the three markers, these markers can be used in MAS in increasing the efficiency for identification of resistant lines in the seedling stage even in the absence of the disease epiphytotic conditions.

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