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qPCR analysis of *Ty-2* and *Ty-3* gene pyramided lines of tomato for resistance to tomato leaf curl New Delhi virus (ToLCNDV)

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ABSTRACT

The present study was carried out at the ICAR-IARI, New-Delhi, India during 2017-18 to analyze the tomato genotypes having *Ty-2* and *Ty-3* genes incorporated through molecular breeding for resistance against the tomato leaf curl New Delhi virus (ToLCNDV).ToLCNDV is most predominant virus and causes huge economic loss in tomato, chilli, many cucurbits and cotton. Presently six genes *Ty-1, Ty-2, Ty-3, Ty-4, ty-5* and *Ty-6* are being utilized to address the tomato leaf curl disease (ToLCD). The tomato genotypes under study were grown in the field, along with susceptible and resistant checks. Genotyping was done to know the status of *Ty-2* and *Ty-3* genes in all the tomato samples.The tomato lines showing phenotypic resistance against ToLCNDV in the field were grown under controlled conditions in phytotron and agroinoculated with ToLCNDV genome. ToLCNDV agroinoculated tomato lines were checked for ToLCNDV specific *AC4* transcript by qPCR assay.ToLCNDV specific *AC4* transcript level was 4 log fold high in the susceptible check (Pusa Ruby) as compared to donor line (EC814916). However, *AC4* transcript level was 2.1-2.8 log fold lower in the tomato line 218 (P. Ruby ×EC814916) as compared to susceptible var. Pusa Ruby. The study shows the importance of *Ty-3* gene for imparting resistance against ToLCD disease in tomato.

Keywords: *AC4*, Tomato, Tomato leaf curl disease (ToLCD), Tomato leaf curl New Delhi virus (ToLCNDV), qPCR

Tomato (*Solanum lycopersicum*) is the world's most cultivated vegetable crop after potato. However, tomato is prone to viral infections and its production is affected by many viral diseases.*Tomato leaf curl virus* (ToLCV) is one of the most devastating viruses of tomato in India. Tomato leaf curl disease (ToLCD) caused by ToLCV is the major constraint in improving tomato production in India, infecting elite cultivars (Sahu *et al.* 2010).

ToLCV belongs to begomovirus, transmitted by whitefly *Bemisia tabaci*. Symptoms of ToLCV include yellowing, vein clearing, crinkling and upward or downward curling of leaves which results in significant yield loss (Chakraborty S 2009). In India,ToLCV viruses carrying both monopartite and bipartite genome are reported (http://www.ncbi.nlm.nih.gov). ToLCV strains affecting tomato are bipartite in Northern India (Padidam *et al.* 1995) and monopartite in Southern India.

The cultivated tomato species have no resistance for ToLCV, so wild species were screened to identify and introgress resistance loci into *S. lycopersicum* (Vidavski 2007). So far, six resistance genes i.e. *Ty-1* to *Ty-6* have been known for ToLCD. *Ty-1* and *Ty-3* are derived from *S. chilense,* shown to be allelic and code for RNA-dependent RNA polymerase (Verlaan *et al.* 2013). *Ty-4* (Ji *et al.* 2009) and *Ty-6* (Hutton and Scott 2013) are also originated from *S. chilense* accessions. *Ty-2* is derived from *S. habrochaites* (Yang *et al.* 2014) and *ty-5* from *S. peruvianum* (Hutton *et al.* 2012). The exact mechanism of action of these genes are not known, beside this single gene is not capable of providing resistance in tropical climate like India.

ToLCNDV has a broad host range including plants of Solanaceae like tomato, chili pepper and potato (Chakraborty 2009, Usharani *et al.* 2004, Pratap *et al.* 1995) and plants of Cucurbitaceae, such as luffa, melon, pumpkin and cucumber (Sohrab *et al.* 2003, Ito *et al.* 2008). Efforts have been made to control ToLCV infection through introgression of resistance genes (*Ty-1* to *Ty-6*) from wild cultivars of tomato. In this study we have investigated *Ty-2* and *Ty-3* gene pyramided tomato lines for resistance against

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ToLCNDV using qPCR assay along with genotyping for *Ty-2* and *Ty-3* genes.

MATERIALS AND METHODS

Plant material: The *Ty-2* and *Ty-3* genes pyramided tomato lines (F_2 population), Pusa Ruby (susceptible var.), EC814916 (resistant donor for ToLCNDV) used in this study is listed in (Table 1). All these tomato plants were grown and maintained at the research field of the Division of Vegetable Science, ICAR-IARI, New Delhi, India. Tomato line 218-2 (Pusa Ruby × EC814916) was also grown along with Pusa Ruby and EC814916 at ±25°C in the National Phytotron Facility, IARI.

DNA isolation and Genotyping: Young leaves were taken from each tomato line (Table 1) for genomic DNA isolation using the C-TAB method (Murray MG and Thomson 1980). Primers used for verification of *Ty-2* and *Ty-3* genes are mentioned in Table 2. PCR reaction was carried using 1 unit of *Taq* DNA polymerase (G- Biosciences, USA), 50-100 ng of genomic DNA as template, 10 mM each dNTP mix,0.1 µM of each forward and reverse primer and 10X PCR buffer in 25 µl reaction volume under standard conditions. PCR cycle programme used to amplify *Ty-2* and *Ty-3* genes involve cycle of 95°C for 3 min followed by 35 cycles of 94°C for 60 sec, 53°C for 30 sec and 72°C for 1 min and final extension at 72°C for 10 min. Amplified products were visualized on 1.5% (w/v) agarose gel stained with ethidium bromide dye (Sigma Aldrich Chemical Pvt. Ltd, Bengaluru, India).

Agroinoculation of tomato genotypes with ToLCNDV: The tomato line (218-2) along with susceptible and resistant check as per listed in (Table 1) were grown at ±25°C with 16 h light/8 h dark photoperiod in the National Phytotron, IARI, New Delhi. For artificial injection of ToLCNDV virus into the tomato seedlings, *Agrobacterium tumefaciens* strain EHA105 harbouring ToLCNDV viral genome A, B and beta was used. Three days prior to agroinfilteration the *Agrobacterium tumefaciens* strain was incubated in Yeast Extract Peptone (YEP) media (10 ml) with appropriate antibiotics at 28°C in a shaker (180 rpm). Two days later, 1 ml of this primary culture was inoculated in a 1L flask containing 250 ml of YEP medium containing antibiotics and incubated again at 28ºC with shaking (180 rpm) for 16-18 h. The *Agrobacterium* culture was centrifuged at 6000 rpm for 10 min and the cell pellet was resuspended in MS medium (Murashige and Skoog 1962).The Syringe infiltration procedure was done to inject *Agrobacterium* cells into the tomato seedlings (25-30 day old). The entire inoculation procedure was repeated after one week.

RNA isolation and cDNA synthesis: Leaf samples for RNA isolation were collected from ToLCNDV agroinoculated plants grown in phytotron after 40-45 days of agroinoculation. Spectrum plant total RNA isolation kit (Sigma, USA) was used to isolate total RNA from leaf samples using manufacturer's protocol. On column DNaseI treatment (Sigma, USA) was given to remove DNA contamination from RNA samples. cDNA was synthesized using Applied biosystem cDNA synthesis kit from Random hexamers using 1 µg of RNA according to the manufacturer's protocol. Nanodrop spectrophotometer (Thermo Scientific, USA) was used to check concentration of cDNA samples. The concentration of cDNA samples was adjusted to 50 ng/µL.

qRT PCR analysis: qRT–PCR was done on Real–time PCR system (Roche Light Cycler- 96) using SYBR® Green PCR Master Mix (Roche). *AC4* and *elongation factor 1* (*EF1*) (reference gene) specific primer sequences are listed in Table 2. *AC4* and *EF1* primers were synthesized from Sigma Aldrich Chemical Pvt. Ltd, Bengaluru, India. The *AC4* primers were used for ToLCNDV titre estimation.*EF1* transcript levels were used to normalize variations among samples and relative expression was calculated using $-\Delta\Delta C_T$ method (Livak and Schmittgen 2001).

Table 1 List of genotypes taken for study, *Ty-2* and *Ty-3* gene status and details of the cross

Genotype	*(Ty-2)*	*(Ty-3)*	Details of cross
EC814916	Hm (R)	Hm (R)	(Ty-2/Ty-2,Ty-3/Ty-3)
Pusa Ruby	Hm (S)	Hm (S)	(ty-2/ty-2, ty-3/ty-3)
232-1	Hm(S)	Ht	P1 (ty-2/ty-2, ty-3/ty-3) × EC814916 (F2)
232-2	Ht	Ht	
232-3	Hm (R)	Ht	
224-6	Hm (R)	Hm (S)	P2 (Ty-2/Ty-2, ty-3/ty-3) × EC814916 (F2)
224-8	Hm (R)	Hm (R)	
213-4	Ht	Ht	P3 (ty-2/ty-2, ty-3/ty-3) ×EC814916 (F2)
213-7	Ht	Hm(R)	
218-2	Ht	Hm (R)	P. Ruby X EC814916 (F2)
218-5	Ht	Hm (S)	

Table 2 List of primers used in this study

Primer Name	Primer sequence (5´-3´)	Reference
T0302 FP	TGGCTCATCCTGAAGCTGATAGCGC	Yang *et al.* (2014)
T0302 RP	AGTGTACATCCTTGCCATTGACT	
TY3 SCAR1 FP	GCTCAGCATCACCTGAGACA	Dong *et al.* (2016)
TY3 SCAR1 RP	TGCAGGAACAGAATGATAGAAA	
AC4 FP	CTAGAACGTCTCCGTCTTTGTCGATGT	
AC4 RP	GGGTCTCCGCATATCCATGTTCTCA	
EF1 FP	GATTGACAGACGTTCTGGTAAGGA	
EF1 RP	ACCGGCATCACCATTCTTCA	

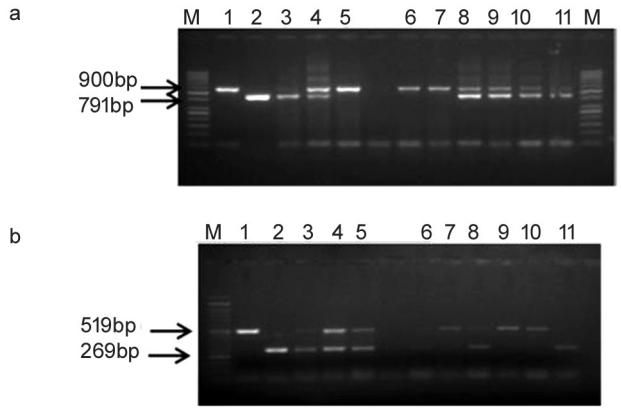

Fig 1 Gel picture depicting Ty-2 and Ty-3 specific resistant and susceptible alleles.(a) PCR amplicon of 900 bp and 791 bp specific for Ty-2 resistant and susceptible allele, respectively.(b)PCR amplicon of 519 bp and 269 bp specific for Ty-3 resistant and susceptible allele respectively. L-50 bp. Lane: 1 (Donor line EC814916), Lane: 2 (Susceptible var. Pusa Ruby), Lane 3-12 (3, 4, 5 –P1x EC814916; 6, 7 – P2x EC814916; 8,9 –P3 X 814916;10,11- Pusa Ruby × EC814916).

RESULTS AND DISCUSSION

Genotyping for Ty-2 and Ty-3 genes: The F_2 population (Table 1) was developed by crossing between indigenous tomato varieties susceptible for ToLCV and *Ty-2* and *Ty-3* genes carryingline EC814916 (ToLCV resistant donor line). DNA was isolated from the young leaves to identify the *Ty-2* and *Ty-3* gene specific resistant and susceptible allele in the tomato lines listed in Table 1. *Ty-2* gene specific TA302 marker identified by Yang *et al.* 2014 was used to genotype tomato lines for *Ty-2*. A PCR amplicon of 900 bp specific to resistant allele was found in donor line EC814916 and the homozygous plants (for resistant allele) of F_2 population (Fig 1) and an amplicon of 791 bp specific to susceptible allele was found in susceptible var. Pusa Ruby and the homozygous plants (for susceptible allele) of F_2 population (Fig 1). Both resistant and susceptible allele (900 bp/791 bp) specific to TA302 marker was found in heterozygous progenies derived from F_2 (Fig 1). Similarly all the above mentioned tomato lines (Table 1) were used to genotype for *Ty-3* gene using *Ty-3* SCAR1marker (Dong *et al.* 2016). An amplification of 519 bp for *Ty-3* resistant allele was observed in ToLCV resistant line EC814916 and F_2 homozygous plants for resistant allele (Fig 1). PCR product of 269 bp specific for *ty-3* loci was found in Pusa Ruby (susceptible var. for ToLCV) and the homozygous plants (for susceptible allele) of F_2 population (Fig 1). In heterozygotes, both alleles (*Ty-3/ty-3*) specific to *Ty-3* locus were observed (Fig 1).

Phenotypic analysis for ToLCNDV symptoms: Phenotypic analysis of tomato lines grown in field (Table 1) showed that *Ty-2* and *Ty-3* genes pyramided tomato lines are resistant against ToLCNDV. We found severe symptoms of ToLCV like yellowing, vein clearing, crinkling and upward or downward curling of leaves as reported by Chakraborty S (2009), in our susceptible variety Pusa Ruby. However, the leaves of tomato plants (F_2 population, Table 1) containing *Ty-2* and *Ty-3* resistant alleles were found devoid of ToLCV specific severe symptoms.

Agroinoculation of ToLCNDV into selected tomato lines: To test the resistance level of *Ty-2* and *Ty-3* gene pyramided line 218 (Pusa Ruby × EC814916) it was grown at ±25°C in the National Phytotron Facility, IARI along with Pusa Ruby and EC814916. Syringe infiltration of ToLCNDV viral genome was done in the all the above mentioned tomato seedlings at 25-30 day old stage. The agroinoculation procedure was repeated after one week to increase the efficiency of infection. ToLCNDV specific symptoms like leaf yellowing and curling first started to appear in susceptible var. Pusa Ruby after (25-30 days) of first agroinoculation (Fig 2a). Later on 40-45 days after agroinoculation plants of Pusa Ruby developed clear symptoms specific to the virus (Fig 2a). However, in the plants of line 218 (Pusa Ruby × EC814916) mild chlorosis was found (Fig 2a) and in EC814916 (donor for *Ty-3*) we could not find phenotypic symptoms specific to the ToLCNDV virus after 40-45 days of agroinoculation

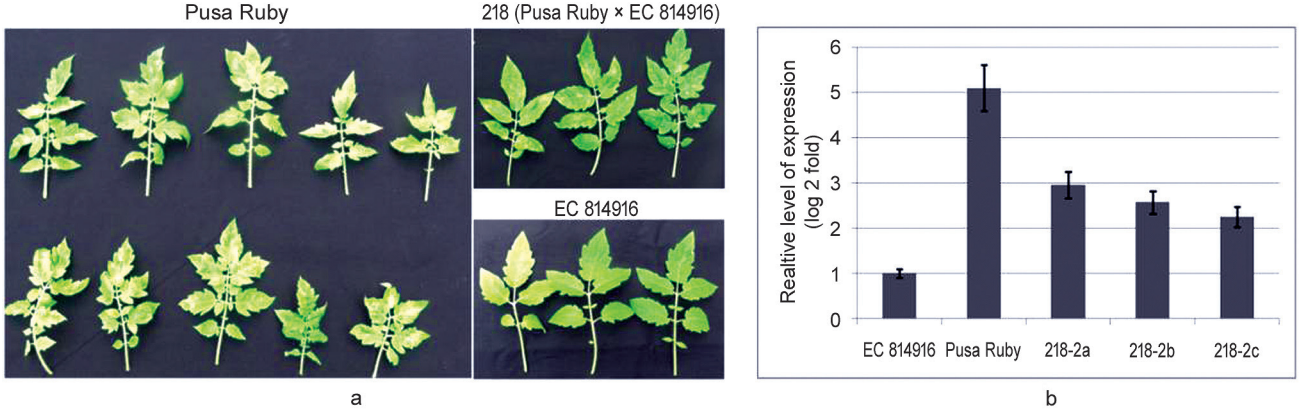

Fig 2(a) Photograph of leaves of tomato (Pusa Ruby, Pusa Ruby × EC814916 and EC814916) agroinoculated with ToLCNDV. (b) qPCR estimation of AC4 transcripts in tomato leaves. AC4 expression in EC814916 is taken as calibrator. Error bar indicates the S.D. of individual plants each with three technical replicates.

(Fig 2a).

qPCR analysis: Leaf samples were collected from the ToLCNDV agroinoculated tomato plants (40-45 days) after first agroinoculation. Total RNA was isolated from the leaf and cDNA was synthesized. qRT PCR was performed to analyze the ToLCNDV specific *AC4* gene transcript levels. *EF1* was used as a reference gene for qPCR analysis. Results of qPCR are shown in Fig 2b. *AC4* transcript level in the resistance check (R.C) was taken as calibrator. *AC4* transcript level was 4 log fold high in the susceptible check (Pusa Ruby) as compared to donor line (EC814916). However, *AC4* transcript level was 2.1-2.8 log fold lower in the tomato line 218 (P. Ruby × EC814916) as compared to susceptible var. Pusa Ruby. Genotyping assay with *Ty-3* gene specific marker *Ty-3* SCAR-1 showed that line 218 contains *Ty-3* gene in homozygous condition. The decrease in virus specific transcript in the line 218 (P. Ruby × EC814916) might be due presence of *Ty-3* gene. The study shows the importance of *Ty-3* gene for imparting resistance against ToLCNDV disease.

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