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TOLERANT TOMATO (*SOLANUM LYCOPERSICUM)* DEVELOPMENT FOR LEAF CURL VIRUS USING T-*REP* GENE

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ABSTRACT
Tomatos highly tolerant to tomato leaf curl virus disease (ToLCVD) were developed by genetic engineering using truncated replicase (T-rep) gene. The gene construct developed by scientists at IARI using conserved sequences of ToLCV New Delhi virus was cloned in pCambia 2301 plant expression vector. A binary vector carrying the antisense T-rep gene (Truncated sequence, 479 bp) along with the NPTII gene and GUS gene cloned and transformed in Agrobacterium LBA 4404 strain. Transgenic tomato plants were developed using Agrobacterium mediated genetic transformation system. High level of tolerance and inheritability of the transgene was observed following challenge inoculation with the tomato leaf curl virus disease using whitefly (Bemisia tabaci). The mechanism of resistance appears RNA-mediated inhibition of ToLCV disease.

Keywords: Agrobacterium, Tomato, Transformation, whitefly, TOLCV etc.

INTRODUCTION
ToLCV is one of the most devastating viral diseases of cultivated tomato (*Solanum lycopersicon)* in tropical and subtropical regions of the world. Affected plants produce either no fruit or a few small fruits, causing more than 90% yield loss in severe cases. In India alone five distinct geminiviruses have been reported to cause the disease. The disease causes severe leaf curling, cupping of leaf lamina and overall stunting of growth in tomato. Several laboratories, worldwide have tried to introduce resistance to ToLCV by transforming tomato with viral derived resistance. Nevertheless, only partial resistance to the virus has been obtained.

The present study was undertaken for developing trait stable transgenic resistance with broader coverage against *Tomato Leaf Curl Virus Disease* (ToLCVD) using RNAi based transgenic approach. Here efforts have been made to develop transgenic tomato using T-*rep* gene and evaluation of the second generation plants was carried out for ToLCV resistance by challenge inoculation using whitefly vector.

MATERIAL AND METHODS
Here we describe the strategy for cross inhibition of ToLCV replication by siRNAs targeted to various conserved regions of the AC1 gene. The multiple siRNAs have been used to target the AC1 gene, including a small overlapping AC4 gene essential for pathogenicity and having silencing suppression activity.

For this study the gene construct T-*rep* was obtained from Indian agriculture Research Institute, New Delhi. Transgenic tomato development and evaluation was planned using highly ToLCVD susceptible breeding line obtained from Bejo Sheetal Seeds Pvt. Ltd. Tomato seeds of breeding line TomD4 were obtained in sufficient quantity to use for developing transgenic tomato using *Agrobacterium* mediated genetic transformation system. Further this line was used as susceptible control during all experimental stages of transgenic tomato evaluation.

TRANSGENIC TOMATO DEVELOPMENT
Transgenic tomato plants were developed using *Agrobacterium* mediated genetic transformation containing gene construct; pCAMBIA2301 with T-*rep* antisense gene along with NPTII gene as selectable marker gene (for antibiotic kanamycin as plant selection marker) & GUS gene as scorable marker gene was used for transformation of Tomato (var. TomD4). Nine promising transgenic tomato lines were selected based on PCR confirmations and natural tolerance of the transgenic plants in controlled condition in T_0 generation. T_1 generation progenies were developed from these lines, total genomic DNA was isolated by CTAB method with some modifications originally described by (Sambrook *et al*. 1989). 2-3 juvenile leaves were taken from primary transgenic plants Total DNA was dissolved in 30 ul of TE (pH 8.0), 2ul DNA was used to confirm the DNA quality using agarose gel electrophoresis

PCR analysis was performed using gene specific primers for NPTII and GUS gene amplification and for T-*rep* gene gene cassette specific primers were designed. PCR reactions were set up using approximately 80-100 ng template DNA in total 25 ul PCR reaction volume. Non transgenic plant DNA control, PCR reaction mix without DNA, plasmid DNA as positive control were used as controls with the test PCR samples. Reaction mix was containing 10x buffer, 1U Taq DNA polymerase (Invitrogen), 10mM DNTP's 25mM MgCl2.

PCR was performed using the program of initial denaturation at 94^0C for 5 min. then 35 cycles of denaturation at 94 ^{0}C for 1 min, annealing 57 ^{0}C for T-*rep*, 60^0C for NPTII and 63^0C for GUS gene and extension at 72^0C

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for 1min. Final extension was given at 72^0C for 7 min. using Biorad C-1000 thermal cycler. PCR product with 3 µl of bromophenol blue dye was then electrophoresed in 1% agarose gel along with 1Kb DNA ladder (Invitrogen molecular weight marker). Electrophoresis was done at 50 V for 1 hour using 1x TAE (pH 8.0). After separation, the DNA bands were visualised and documented using gel documentation system (Alpha digi-doc, USA). PCR positive plants for all the three genes were further selected for ToLCV resistance evaluation. ToCLV resistance evaluation was investigated by challenge inoculation of disease using whitefly mediated disease transmission.

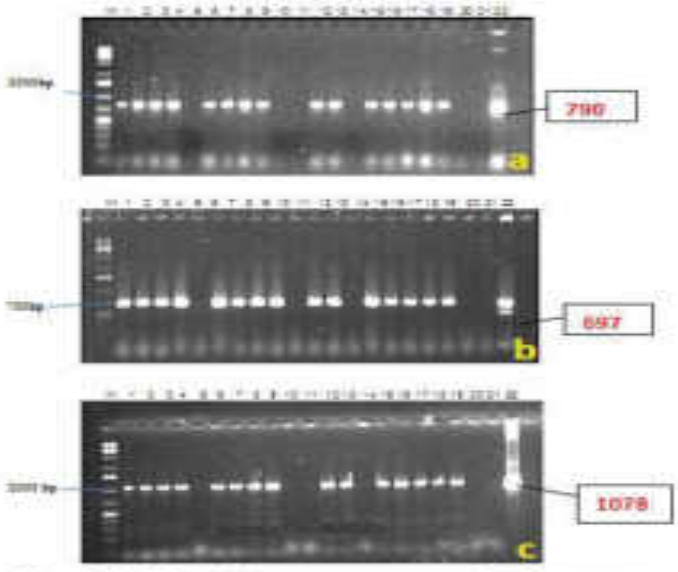
CHALLENGE INOCULATION SCREENING FOR TOLCV EVALUATION:

The tomato leaf curl virus (ToLCV) inoculum used during the present investigation was obtained from a tomato plant showing typical leaf curl symptoms, from Jalna region & it was confirmed by PCR. The viruliferous whiteflies (*Bemisia tabaci*) from the infected plants were collected and maintained on susceptible tomato host plant in insect proof net. These whiteflies were released on test tomato population. The test tomato population trail was planted in insect proof net in controlled condition. Transgenic test tomato population was selected based on PCR confirmed for T-*rep* gene containing transgenic plants along with non transgenic control plants. The ToLCV disease transmission was confirmed by PCR & morphological symptoms were observed after three weeks of transmission. The diseased susceptible plants were maintained for investigation studies throughout the experimentation period.

RESULTS AND DISCUSSION

Generation advancement of primary transgenic tomato plants

Transgenic tomato T_1 generation population was established by germinating 40 seeds of nine promising transgenic tomato lines in portrays grown in controlled condition polyhouse. After emergence of primary leaves Total DNA was isolated from primary transgenic tomato plants of T_1 generation by following CTAB method, 20 progeny samples from each primary transgenic event were subjected to PCR analysis using GUS gene, NPTII gene and T-*rep* gene cassette specific primers. The PCR analysis of all three genes for one primary transgenic event is shown in (Figure 1.) showing 3:1 segregation ratio which is following standard Mendelian ratio for single dominant gene.



All the nine primary transgenic events samples were analyzed by PCR and segregation ratio were recorded. Most of the primary putative events were nearly following Mendelian segregation ratio except two putative events which was near to 1:1 ratio, this could be due to small sample size.

Evaluation of ToLCV tolerance by challenge inoculation of ToLCV acquired whiteflies

The selected PCR positive progeny from these putative events was then transplanted in controlled condition polyhouse for artificial challenge inoculation of ToLCV disease using viruliferous whiteflies (*Bemisia tabaci*) carrying ToLCV disease as vector. These nine putative transgenic lines were exposed to these whiteflies at

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seedling level for 24 hrs of infection transmission process. Symptoms were clearly observed on nontransgenic susceptible plants after 3-4 weeks. The transgenic lines ToVR 5,6,27,28,41,44,53,59 & 63 were exhibited varied degree of ToLCV tolerance. Each line was then analyzed for percent resistance based on the plants showed susceptibility in few plants and tolerance. The percent of complete tolerance was varied from 50% to 73.33%. The susceptible and tolerance symptoms are shown in (Figure 2:)

Figure 2: Screening ToLCV tolerance by challenge inoculation of whiteflies
a Susceptible Non transgenic plant showing typical ToLCV symptoms
b Transgenic ToLCV tolerance plant showing high tolerance to ToLCV disease

Out of tested nine lines five line exhibited low to moderate level of tolerance and the percent tolerance of the line was in the range of 40-60% where as control susceptible plant was showing only 20% of the plants tolerance to ToLCV. Four lines were showing high tolerance to ToLCV disease the tolerance plant percent was ranging from 60-73.33% These lines were also showing normal healthy fruits and till maturity high level of tolerance to the ToLCV disease. These results of ToLCV resistance at par with the earlier reported transgenic tomato resistance using full length *rep* gene (Praveen-at-al., 2005). The resistance to leaf curl disease demonstrated in this study, with the antisense rep gene construct, indicates RNA-mediated resistance

CONCLUSION

Various studies have focused on using partial, entire, sense, antisense or mutated begomoviruses rep gene (Noris et al., 1996; Bendahmane and Gronenborn, 1997; Yang et al., 2004). The original rationale of antisense RNA technology (Gizant and Weintraub, 1984), leading to gene silencing, presents an effective defense mechanism against viruses (reviewed by Wassengger 2002). Homology - dependent gene silencing using antisense approach for development of resistance was also demonstrated for development of Tomato yellow leaf curl virus resistance in tomato (Yang et al., 2004), Cotton leaf curl disease (Asad et al., 2003), and Bean golden mosaic virus (Aragao et al., 1998). Thus an antisense rep gene of ToLCV would in principle block the viral rep gene expression either by preventing translation or through homology dependent degradation of target viral RNA (Praveen et al., 2005). Here in this we demonstrated the development of highly tolerant line of transgenic tomato using truncated *rep* gene which would be significantly inhibiting the ToLCV disease for various isolates of India and the sequence of antisense truncated rep gene is similar to various Indian isolates. Hence these lines can be further used for identifying the homozygous lines in T_2 generation and after evaluation superior line can be selected for further use in tomato breeding program.

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