



ISSN: 0976-3031

Available Online at <http://www.recentscientific.com>

CODEN: IJRSFP (USA)

International Journal of Recent Scientific Research
Vol. 9, Issue, 3(I), pp. 25262-25265, March, 2018

**International Journal of
Recent Scientific
Research**

DOI: 10.24327/IJRSR

Research Article

TRANSGENIC APPROACHES FOR DEVELOPMENT OF HIGHLY TOLERANT TOMATO FOR LEAF CURL VIRUS (TOLCV)

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DOI: <http://dx.doi.org/10.24327/ijrsr.2018.0903.1827>

ARTICLE INFO

Article History:

Received 15th December, 2017

Received in revised form 25th
January, 2018

Accepted 23rd February, 2018

Published online 28th March, 2018

ABSTRACT

Transgenic approaches provides solutions to the problems, which cannot be solved by conventional breeding methods. Tomato leaf curl virus disease is very important disease of Tomato particularly in winter and summer season. Highly tolerant Tomato were developed using T-rep gene construct. High level of tolerance for TOLCV and inheritability of the transgene were observed following challenge inoculation with the viruliferous White- flies. The mechanism of resistance appears RNA-mediated, since the plants carried the untranslatable antisense rep gene.

Key Words:

Tomato, Transformation, whitefly,
TOLCV etc.

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INTRODUCTION

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

The present study was undertaken for development of stable resistance with broader coverage against *Tomato Leaf Curl Virus Disease* (ToLCVD) using RNAi based transgenic approach. 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 Dr. Shelly Praveen, Advanced Center for Plant Viruses, Indian agriculture Research Institute, New Delhi. Transgenic Tomato

development and evaluation was planned using ToLCVD susceptible breeding line obtained from Bejo Sheetal Seeds Pvt. Ltd. Jalna. Seeds of breeding line TomD4 were obtained in sufficient quantity for development of transgenic Tomato using *Agrobacterium* mediated genetic transformation system. Further this line was used as susceptible control during all experimental stages of transgenic Tomato evaluation.

Transformation in Tomato

Agrobacterium cells containing 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) using protocol of McCormick (1991) with some modifications. Single colony *Agrobacterium tumefaciens* LBA4404 transformed with pCAMBIA2301 construct was grown in Luria broth liquid medium with Rifampicillin (25 µg/ml) and kanamycin (50 µg/ml) at 28°C for 18 hrs. With constant agitation at 180 rpm. 25ml of well grown culture was centrifuged at 4000 rpm at 4°C for 5 min. pellet was then re-suspended in 25 ml of Luria broth medium without any antibiotic, with acetosyringone at the concentration of 200 µM. Culture was again incubated at 28°C for 18 hrs. With constant agitation at 180 rpm. Till the OD

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reaches to ~ 0.8 (at 600 nm). Finally before infection to the leaf explants the culture was diluted to 1:1 ratio using Murashige & Skoog liquid medium with 1% sucrose pH 5.8 before autoclaving.

Healthy seeds were sterilized with 0.1% HgCl_2 for 2 minutes, followed by sterile distilled water wash for 6-7 times and placed on germination medium (1/2 MS medium) after blot drying on sterilized blotting paper. Seedlings were developed in 16 hr photoperiod at $25 \pm 2^\circ\text{C}$ for one week after initial incubation in dark for 2-3 days. Cotyledonary leaves were excised from 10 days old seedlings and explants were prepared by cutting all the sides of cotyledonary leaves. Placed the prepared leaf explants of size $\sim 0.5\text{ cm}^2$ in adaxial position on the pre-culture medium (MS salts and B5 vitamins, 3% Sucrose, 1mg/l Zeatin, 0.1mg/l IAA, phytoagar 0.25 %, pH 5.8 adjusted before autoclaving). Cultures were incubated at $25 \pm 2^\circ\text{C}$ in 16 hr photoperiod for two days before infection.

Infection was carried out in sterile glass beaker, two days pre-cultured leaf explants were removed for tissue culture medium safely without keeping any traces of phytoagar before infection. 50 leaf explants at a time were infected with the *Agrobacterium* culture prepared as described above. Cultures were left for 20 minutes for Agroinfection in dark with gentle shaking. Excess *Agrobacterium* culture was removed. These co-cultivated explants were blot dried on sterile filter paper and then placed on co-cultivation medium (MS salts and B5 vitamins, 3% Sucrose, 1mg/l Zeatin, 0.1mg/l IAA, phytoagar 0.25 %, pH 5.8 adjusted before autoclaving). Incubated the cultures in dark for 2 days at 25°C .

The cotyledonary leaves were then transferred to (MS salts and B5 vitamins, 3% Sucrose, 1mg/l Zeatin, 0.1mg/l IAA, phytoagar 0.25 %, pH 5.8 adjusted before autoclaving) 80mg/l kanamycin, 300mg/l cephotaxime was added to autoclaved medium and poured in sterilized glass plate. The cultures were incubated at 25°C under 16 hr photoperiod for 3 to 4 weeks, with sub culturing at every 15-20 days. Young shoots were then transferred to MS medium containing 3% sucrose, 0.1 mg/l Zeatin, 0.1 mg/l IAA and kanamycin at 50 mg/l for shoot elongation. The shoots so obtained were transferred to MS medium with 0.05 mg/ml of IBA for rooting. Cultures were incubated for 2 to 3 weeks and rooted plants were transferred to pots containing peat and hardened. Hardened plants were shifted gradually to green house for further establishment and analysis. The observations on plant regeneration after co-cultivation were recorded. The seedlings were transplanted and maintained in greenhouse for further analysis along with a set of control plants.

Screening

PCR confirmed Tomato leaf curl virus (ToLCV) inoculum was used during the present investigation was obtained from a Tomato plant showing typical leaf curl symptoms. The whiteflies were released on inoculum for 12 hours and viruliferous whiteflies from these plants were collected and were released on confirmed T-rep gene containing plants (Transgenic) and control plants for 24 hours and then were killed by spraying insecticide. The ToLCV disease transmission was confirmed by PCR & morphological symptoms were observed after three weeks of transmission.

The diseased plants were maintained for investigation studies throughout the experimentation period.

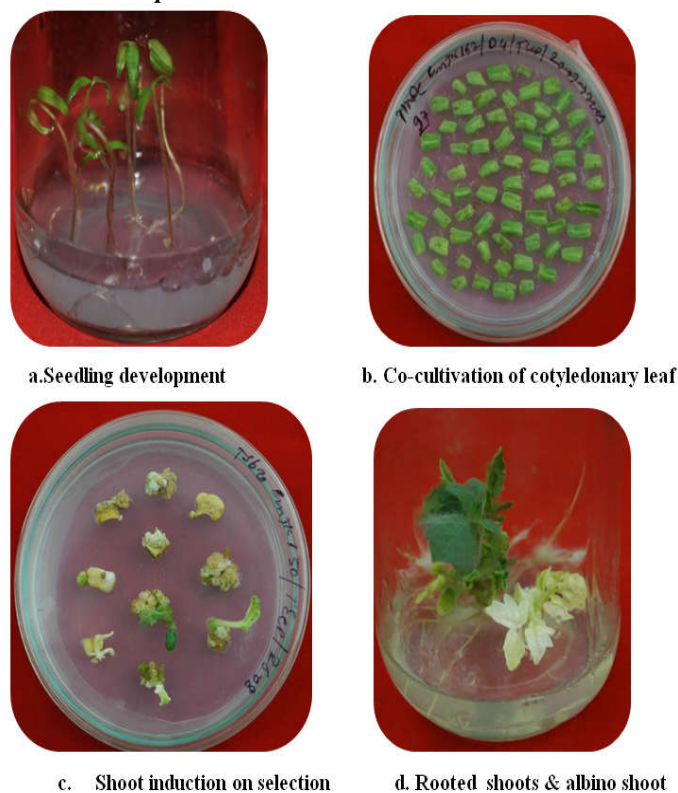
RESULTS AND DISCUSSION

Table Transgenic Tomato development summary

Experiment	No. of explants infected	Percent of explants responded on selection	Percent of callus induced shoots	No. of plantlets developed
Set 1	156	48	28	11
Set 2	151	42	33	8
Set 3	145	46	40	13
Set 4	147	40	35	17
Set 5	155	51	35	10
Set 6	150	39	32	7

Regenerated shoots from callus were harvested & transferred to selection free elongation medium (MS salts with B5 vitamins, 3% Sucrose and 300mg/l cephotaxime. pH 5.8). Well developed shoots transferred to half-strength MS medium with 300mg/l Cephotaxime pH 5.8 for rooting. After 15 days root initiation started & seventy three rooted plantlets transferred to primary hardening and kept in controlled condition green house with high humidity ($< 90\%$). Well established plantlets were then transferred to soil in contained polyhouse as per DBT norms. Plantlets were grown till maturity and seeds were collected from self pollinated fruits. Leaf sample from these primary transformants were collected for histochemical GUS assay, genomic DNA isolation for PCR analysis to confirm the gene integration prior to maturity.

Figure: Steps during Agrobacterium mediated transgenic Tomato development





e. Plantlets ready to harden



f. Hardening of plant



Figure:4.8 Evaluation of ToLCV resistance by challenge inoculation
 a. Non transgenic tomato plant showing typical leaf curl symptoms
 b. Transgenic tomato plant showing resistance to ToLCV disease
 c. Non transgenic apical shoot showing typical leaf curl symptoms
 d. Transgenic apical shoot remains healthy after challenge inoculation



Histochemical GUS assay

Gene construct T-*rep* used in this study to develop transgenic Tomato was cloned in pCAMBIA 2301 which has GUS reporter gene along with selection marker nptII gene. During transformation experiments kanamycin antibiotic was used as selectable marker and GUS as reporter gene to check the gene

expression at different stages of transformation in laboratory. To confirm the expression of inserted gene in primary transgenic Tomato plant was carried out using the leaf pieces of the well established plants from contained polyhouse. Among the plants tested thirty plants were showing prominent GUS expression, control check was used from non transgenic leaf tissue from the Tomato seedling which was positive control in transformation experiments. These GUS expressing plants were further used to confirm the gene integration by polymerase chain reaction (PCR).

PCR Analysis of Primary Transgenic Tomato

Total DNA was isolated from primary transgenic Tomato plants of T₀ generation grown in controlled polyhouse by following CTAB method with some modifications originally described by (Sambrook *et al.* 1989). 2-3 juvenile leaves were taken from thirty primary transgenic plants which were showing GUS expression by histochemical assay along with the one non transgenic control plant. 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.

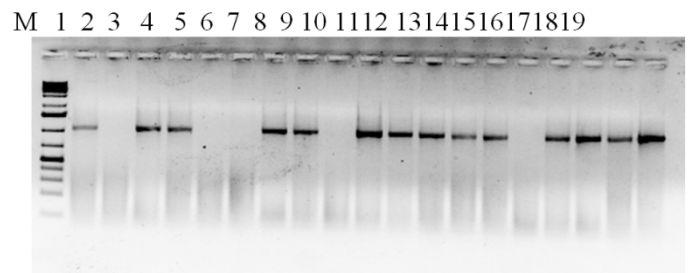
Primers were designed and get synthesized from Sigma, USA:

1. T-*rep* gene, gene of interest (whole cassette)
 Forward : 5' CATCAAGATCTGTGGAGAGAGC 3'
 Reverse: 5' CGTCGATTGGGTATCGTCTA 3'
2. NPTII gene, selectable marker (within gene)
 Forward: 5' CAATCGGCTGCTCTGATGCCG 3'
 Reverse: 5' AGGCGATAGAAGGCGATGCGC 3'
3. GUS gene, reporter gene (within gene)
 Forward : 5' CAACGAACTGAACTGGCA 3'
 Reverse : 5' TTTTGTACACGCGCTATCAG 3'

PCR was performed 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. The primers were used individually for each gene detection. PCR was performed as per the program specified in Table: using Biorad C-1000 thermal cycler.

After PCR amplification samples were subjected to agarose gel electrophoresis for separation of amplified fragments. The results are summarized in Table:

Fig: PCR amplification of T-*rep* gene cassette in T₀ plants
 M: Marker
 Lane: 1-19 test plant DNA sample



CONCLUSION

Tomato was successfully transformed & regenerated using cotyledon explants cultured on MS salts and B5 vitamins, 3% Sucrose, 1mg/l Zeatin, 0.1mg/l IAA, phytoagar 0.25 %, pH 5.8 and this has been proved by GUS histochemical assay hence this protocol can be utilized for transferring gene of interest to Tomato system. The integration of T-rep gene was confirmed by PCR and the challenged inoculation of transgenic plants showed very high level of tolerance hence such high expression showing events can be used in breeding programmes after bio-safety studies.

Acknowledgement

We are very much thankful to Dr. N.S. Kunchge, Director Bejsheetal Research Pvt. Ltd .Jalna for his support.

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