Research Note :

Development of gene construct of conserved *rep* gene from tomato leaf curl virus from natural host plant for resistance against tomato leaf curl virus

JITENDRA KUMAR PAL AND MAJOR SINGH

International Journal of Plant Protection, **Vol. 4 No. 2 (October, 2011) : 411-414**

See end of the article for authors' affiliations

Correspondence to :
JITENDRA KUMAR PAL
National Research Centre on Plant Biotechnology, Pusa Campus, NEW DELHI (INDIA)
Email : palijitendracish @gmail.com

Pal, Jitendra Kumar and Singh, Major (2011). Development of gene construct of conserved *rep* gene from tomato leaf curl virus from natural host plant for resistance against tomato leaf curl virus. *Internat. J. Plant Protec.,* **4**(2): 411-414.

Key words : Tomato leaf curl virus, Rep gene

Received : *May, 2011*
Accepted : *August, 2011*

The tomato is grown worldwide for its edible fruits, with thousands of cultivars having been selected with varying fruit types. India ranks 4th in tomato production in the world. It produces about 10.2 million tones tomato annually from about half a million hectares (FAOSTAT, Crop statistics, 2008). Tomatoes are now eaten freely throughout the world, and their consumption is believed to benefit the heart among other things. They contain lycopene, one of the most powerful natural antioxidants. Tomato consumption has been associated with decreased risk of breast cancer, head and neck cancers and might be strongly protective against neurodegenerative diseases. (Zhang *et al*., 2009). There are over 10 important viruses which naturally infect tomato and some of these viruses have a large number of distinct strains. The most destructive viruses affecting tomato are Tomato leaf curl virus (ToLCV) causing tomato leaf curl disease (ToLCD), whose occurrence in India has been known since 1948 (Varma and Malathi, 2003). Efforts to develop tomato varieties resistant to ToLCV by traditional breeding have not been successful, as natural sources of resistance are not available. The virus is transmitted by whiteflies (*Bemisia tabaci)* that are attracted to young leaves and growing tips. The virus is not transmitted mechanically nor via seed. The concept of pathogen-derived resistance was introduced in plant virology based on the report that transgenic tobacco expressing tobacco mosaic virus (TMV) coat protein showed a resistance to TMV infection. This type of resistance generally referred to as coat protein-mediated resistance, has been described for many virus/host systems, but is restricted to the virus closely related to the expression of functional or altered introduced viral replicase gene (Powell-Abel *et al*., 1986). Viral gene suppression involves a coordinated series of sub cellular events that ultimately lead to the post-transcriptional silencing of gene expression. This phenomenon has been termed as co-suppression or post-transcriptional gene silencing, which operates through homology-dependent gene silencing. Various models have been proposed to demonstrate the mechanism of virus resistance and PTGS of endogenous gene in transgenic plants, containing sense or antisense transgenes. The Cauliflower Mosaic Virus (CaMV) 35S Promoter is the most well-known and widely used promoters in genetics research. One of the reasons is that it was one of the earliest promoters discovered, and it is also a *constitutive* promoter, meaning that it is always "on" - it makes the gene it is linked up to express constantly (Baulcombe 1996).

Five leaf curl infected samples of tomato plants were collected from Indian Institute of Vegetable Research Varanasi, U.P. Out of those five samples two were found positive through electron microscopy. The DNA was isolated from one positive plant's leaf sample by DNeasy Plant Maxi Kit (QIAGEN Ltd.). Three sets of PCR primers were tried to get the sequence from the *rep* gene out of which primer, Forward Primer 'catcaagatctgt-ggagagagc' Reverse Primer 'tagacgagacc-caatcgacg' are able to amplify *rep* gene. and sequence confirmed by sequencing. The condition was set for PCR as initial denuration 94°C for 5 min, 94°C for 30 sec, 60°C for 40

sec, 72° for 30 sec repeat 30 times and final extension 72°C for 10 min. The T-rep was initially cloned in pGEM –T Easy vector (Promega, Madison, USA) followed by *Not 1* cleavage which added a nonviral sequence of 20 nucleotides ending with an in-frame stop codon downstream of the T-Rep sequence, after ligation vector was inserted in to E. coli strain DH5á. In next step *Not 1* fragment was further subcloned into the corresponding restriction sites of pUC118 containing a plant expression cassette, thus introducing the T-Rep gene between the CaMV 35S promoter and a nopaline synthase terminator (Fig. 1).

Presence of T-rep with CaMV 35S promoter and a nopaline synthase terminator was determined by PCR, using CaMV 35S promoter specific primer 'attgcgataaagggaaggcc' and nopaline synthase terminator specific primer 'cctgcaggtaccactggatt while PCR cycle was set as as initial denuration 94°C for 3 min, 94°C for 30 sec, 58°C for 40 sec, 72° for 30 sec repeat 30 times and final extension 72°C for 5 min and further the entire expression cassette containing the T-Rep gene was cleaved by *Pst1* and inserted into a *Pst1* sites in the polylinker region of the binary vector pCambia 2301 at and transformed to E. coli strain DH5á. The binary vector pCambia 2301 carrying conserved rep gene sequence. was mobilized into *A. tumefaciens* disarmed strain LBA 4404 by freeze–thaw method (Hofgen and Willmitzer, 1988). Transformant was selected on Luria Broth (LB)-agar (Casein enzymic hydrolysate10 mg/l, Yeast extract 5 mg/l, Sodium chloride 5 mg/l), LB agar plate was solidified by 1% w/v agar, containing kanamycin acid sulphate(potency 650 lg/mg) 50 mg/l and streptomycin sulphate (potency 950 lg/mg) 200 mg/l.

Presence of entire expression cassette containing

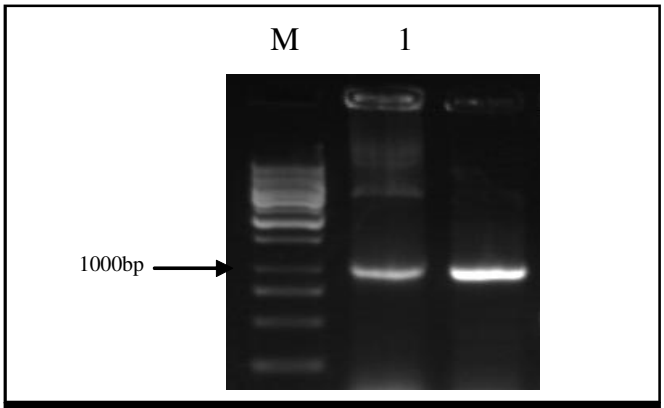

Fig. 2 : Release of T-rep gene from pCambia2301, using *Pst I* ; M is DNA marker(1kb); Lane. 1 and 2- T-rep release

the T-Rep gene with 35S promoter and 35S terminator (~ 1000bp) was confirmed by restriction with *Pst I*. An Amplicon of ~ 450 bp of conserved *rep* region was obtained through PCR after insertion in *Agrobacterium tumefaciens* which confirmed the presence of pCambia 2301 binary vector with *rep* gene in *Agrobacterium tumefaciens* (Fig. 3). The sequence of nucleotides of conserved rep region was determined by sequencing of rep gene. The sequence of rep gene has been submitted to Gene Bank, with accession number HM214580. Most of the strategies for genetically engineered resistance to begomoviruses have involved replication-associated protein. Various studies have focused on using partial, entire, sense, antisense or mutated begomoviruses *rep* gene. The original rationale of antisense RNA technology leading to gene silencing, presents an effective defense mechanism against viruses, (Yang *et al*., 2004). Genes that encode complete or partial replicase proteins can confer near immunity to infection that is generally, but

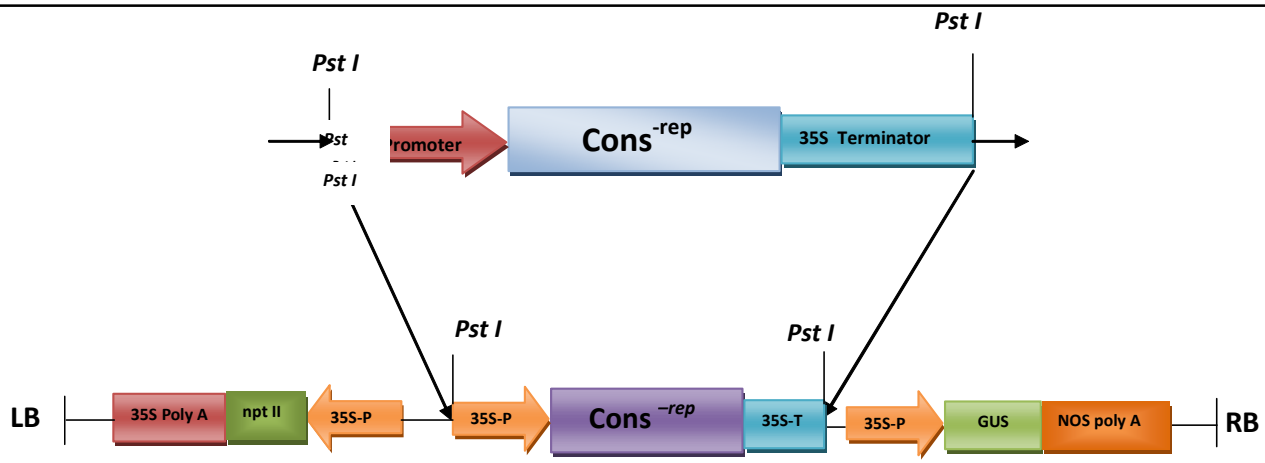

Fig. 1 : Diagram demonstrating the development of tomato leaf curl antisense construct having conserved truncated replicase gene

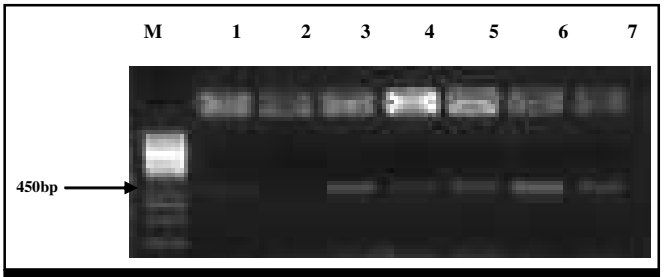

Fig. 3 : PCR amplification of T rep from construct in *Agrobacterium tumefaciens* LBA4404; PCR amplicon of 450bp *conserved rep* gene specific fragment (marked with an arrow) using *rep* gene specific primer, M is DNA marker lane (GeneRuler™ 100 bp DNA Ladder, Fermentas).lane, 1-7 randomly selected colonies after mobilization of vector carrying conserved *rep* gene in *Agrobacterium tumefaciens*

not always, limited to the virus strain from which the gene sequence was obtained. Replicase-mediated resistance (Rep-MR) to TMV was first described in transgenic plants that contain a sequence encoding a 54 kDa fragment of replicase, although the protein fragment was not detected. Sijen *et al*. (1996), although suggested that certain examples of Rep-MR are RNA- rather than protein-mediated,other examples require an open reading frame and, apparently, production of protein. A truncated mutant of replicase derived from a cucumber mosaic virus (CMV) subgroup I virus conferred high levels of resistance in tobacco plants to all subgroup I CMV strains, but not to subgroup II strains or other viruses. In Rep-MR against PVY and AlMV, mutant but not wild-type replicase conferred resistance to infection; a similar approach provided resistance to tomato yellow leaf. Extreme virus resistance conferred by the virus-derived transgene has been shown in number of cases to be mediated by a mechanism of PTGS (Post-transcriptional gene silencing (Baulcombe, 1996). There have been a number of models proposed for induction and operation of PTGS involved with antisense, co-suppression and virus resistance. One of the model proposed that gene silencing and viral immunity involves the rapid degradation of dsRNA molecules formed with sense and antisense genes. Viral sense and antisense homologous transgene construct confer different degree of recovery from viral infection (Baulcombe, 1996). By analogy with other studies, the ToLCV recovery has been observed, mediated by sequence-specific degradation of existing ToLCV genome in the plants. The corner stone of our model is gene silencing, induced by formation of duplex RNA with sense (viral origin) and antisense (transgene)), resulted in recovery of virus-infected plants.

The production of dsRNA (double stranded RNA) template is the key to produce sequence-specific degradation of viral genome and helps in recovery. The dsRNA is formed by hybridization of complementary antisense transgenes RNA with viral sense gene RNA. If the antisense transgene is derived from the viral sequence then this will provide sequence-specific cleavage of viral genome thus preventing viral infection. The present work predicts that ds RNA formed by the antisense RNA and the already existing target viral sense RNA will induce the gene silencing mechanism and thus recovering the plant from the virus infection. Similarly, gene silencing mechanism with simultaneous expression of both polarities has been found to be effective than the either polarity alone. This was explained to occur due to the hypothetical threshold level of dsRNA needed to induce the system and leads to the accumulation of siRNAs. However, our study predicts that PTGS should be readily induced by using simple antisense construct, as the target viral sense RNA is already present in the virus-infected plant for the duplex formation and siRNAs production. Irrespective of the mechanism, delivery of RNA with the potential to form duplexes is required for gene silencing and virus suppression in transgenic plants. Studies are in progress to determine the spectrum and mechanism of resistance.

Acknowledgements:

The authors greatfully thank to Dr. Shelly Praveen for their assistance during work.

Authors' affiliations:

JITENDRA KUMAR PAL, Indian Institute of Vegetable Research, VARANASI (U.P.) INDIA

REFERENCES

Baulcombe D.C. (1996). Mechanisms of pathogen-derived resistance to viruses in transgenic plants. *Plant Cell,* **8** : 1833–1844.

Hofgen R. and Willmitzer, L. (1988). Storage for competent cells for *Agrobacterium* tumefaciens. *Nucl. Acids Res.,* **16**: 9877

Powell-Abel, P., Nelson, R.S., De B, Hoffman, N, Rogers, S.G., Fralley, R.T. and Beachy, R.N. (1986). Delay of disease development in transgenic plants that express the tobacco mosaic coat protein gene. *Sci.,* **232**: 138–143.

Sijen, T., Wellink, J., Hiriart, J.B. and Van Kammen, A. (1996). RNA mediated virus resistance: role of repeated transgenes and delineation of targeted regions. *Plant Cell,* **8**: 2277–2294.

Varma, A. and Malathi, V.G. (2003). Emerging geminiviruses problems: a serious threat to crop production. *Ann. Appl. Biol.,* **142**: 145–164

Yang, Y., Sherwood, T.A., Patte, C.P., Hiebert, E. and Polston, J.E. (2004). Use of tomato yellow leaf curl virus (TYLCV) gene sequence to engineer TYLCV resistance in tomato. *Phytopathol.*, **94** : 490–496

Zhang, C.X., Ho S.C., Chen Y.M., Fu J.H., Cheng S.Z. and Lin F.Y. (2009). Greater vegetable and fruit intake is associated with a lower risk of breast cancer among Chinese women. *Internat. J. Cancer,* **125** (1): 181–188.

****