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Original Article

Tomato and Tobacco Phytoene Desaturase Gene Silencing by Virus-Induced Gene Silencing (VIGS) Technique

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Abstract

Background and Aims: Virus-Induced Gene Silencing (VIGS) is a virus vector technology that exploits antiviral defense mechanism. By infecting plants with recombinant viruses containing host genes inserted in the viral genome, VIGS achieves the RNA silencing process. The purpose of this study was to investigate the possibility of tomato (*Lycopersicon esculentum* Mill.) and tobacco (*Nicotiana benthamiana)* phytoene desaturase (PDS) gene silencing, using VIGS technique by VIGS vector containing tomato PDS.

Methods: PDS gene encodes one of the important enzymes in the carotenoid biosynthesis pathway. In this method *Tobacco rattle tobravirus* (TRV) vectors including $pTRV_1$ ،$pTRV_2$ and $pTRV_2$PDS were used. The $pTRV_2$PDS vector carried inserts derived from tomato PDS gene. Vectors were transformed into *Escherichia coli* strain $DH_5\alpha$. *Agrobacterium tumefaciens* strain GV3101 cells were transformed by the vectors containing specific genes.

Results: Colony PCR with specific primers proved the presence of the PDS and RNA-dependent RNA polymerase genes in selected agrobacterium random colonies. Agrobactrium containing $pTRV_1$ ،$pTRV_2$ and $pTRV_2$PDS was inoculated into induction media culture. Finally, acetosyringone was added to $pTRV_1$ culture and then $pTRV_1$ and $pTRV_2$PDS cultures mixed in a 1:1 ratio. Mixed culture was infiltrated into the test plant seedlings using 1ml needless syringe.

Conclusion: Results showed the gene silencing in the form of photobleaching and mosaic occurred in tomato and tobacco plant leaves, respectively. However, plant infiltration with $pTRV_1$ and $pTRV_2$ (without PDS gene as control) mixed culture did not show any photobleaching.

Keywords: VIGS; *Tobacco rattle tobravirus* vectors; Gene silencing; Phytoene Desaturase Gene; Tomato

Introduction

Virus-induced gene silencing (VIGS) is a fast and efficient functional genomics tool (1, 2) and a method for rapid silencing of plant genes in order to discover their function (1, 3). VIGS is based on Post-Transcriptional Gen Silencing (PTGS) Plants exploit PTGS as their innate antiviral defense line to encounter viral multiplication, employing defense mechanism very similar to the pathways of RNA interference which refers to interference in gene expression, mediated by small RNA in a sequence specific manner (1, 4).

Vectors using VIGS were applied for *Agrobacterium tumefaciens* mediated plant transformation, contained host genes inserted

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in the viral infection clone. *Agrobacterium* based inoculation was used for excision of T-DNA containing the viral genome (1, 5) which was transcribed by the host's RNA polymerase. The RNA-dependent RNA polymerase generates dsRNA, using ssRNA viral transcripts (1). This dsRNA is recognized by Dicer-like proteins and cleaved into small interfering RNAs (siRNAs) which are approximately 21–24 nucleotides (nt) in length (1, 6, 7). The siRNAs are selected by RISC (RNA-induced Silencing Complex) and melted into ssRNAs which are used to target mRNAs and generate new siRNA (1, 8). In this case the VIGS viral RNAs and the mRNA of the target gene are cleaved. The single stranded siRNAs are generated (1, 9) and spread as silencing signals throughout the plant resulting in systemic gene silencing distant from the site of infection and lead to leaf photobleaching (1).

In this paper, we used *tobacco rattle virus* (TRV)-based VIGS vector [10] for efficient silencing of Phytoene desaturase (PDS) gene in tomato (*Lycopersicum esculentum* cv. Cal J). and tobacco(*Nicotiana benthamiana*). PDS encodes important enzymes in the carotenoid biosynthesis pathway (11) which reduce PDS gene expression and lead to markedly photobleaching phenotype (12).

Methods

Plant materials

Tomato (*L. esculentum* cv. Cal J) provided by Petoseed company were sterilized and used, for seedling germination in petri dishes under sterile conditions. They were transferred later into Murashige and Skoog (MS) media. After the cotyledons emerged, they were ready for infiltration. Similarly under sterilized conditions, Tobacco seeds were also used. Test plant seedlings used for silencing were at the stage of 2-4 true leaves.

Bacteria

Escherichia coli strain DH5α and *Agrobacterium tumefaciens* strain GV3101 were used in this study.

Transformation of *E. coli* using heat shock method

To propagate in *E. coli***,** three vectors including $pTRV_1$, $pTRV_2$ and $pTRV_2$tPDS provided by Dinesh-Kumar laboratory, were transformed. Five µl of each vector was mixed with competent cell of DH5α in a microcentrifuge tube and put on the ice for 30 min. Immediately vectors were submerged in to 42°C water for 90 sec and then were put on ice for 2 m. 800µl of LB (Luria-Bertani Brouth) were added to the microcentrifuge tube. The culture was grown in a shaking incubator for 1 hour at 37°C. Finally, 100µl of transformed cells were cultured on the LB plates containing 50µg/ml kanamycin. Plates were incubated for 16-18 hours at 37°C (13).

Transformation of *A. tumefaciens* using freeze thaw method

A. tumefaciens strain GV3101 was grown overnight at 28°C in presence of 50 µg/ml kanamycin, 5µg/ml tetracycline. Agrobacterium culture was spun down at 3500 rpm for 10 min and resuspended in the 100µl of 20mM $CaCl_2$. Five µl of vectors were added to the suspension and mixed culture were submerged in liquid nitrogen and then kept in for five min in a 37°C water bath. 1ml of LB was added the culture and incubated for 3 hours in a 28°C shaker. 100µl of transformed cells were cultured on the LB plates in presence of antibiotics. Plates were incubated for 2 days at 28°C (13).

Colony PCR

PDS and RNA-dependent RNA polymerase (RdRp) genes were PCR amplified using Taq DNA polymerase and specific primers. The specific primers were designed and synthesized from *L. esculentum* PDS gene (accession number X71023) and *Tobacco rattle tobravirus* RNA1 genes (accession number AF166084) sequence data at NCBI GeneBank. The nucleotide sequences of primers were as follow: Forward primer 5´-GAG AAA CAT GGT TCA AAA ATG G -3´ and reverse primer 5´-AAA CAC AAA AGC ATC TCC CTC -3´ for PDS gene and forward primer 5´-CTT GAA GAA GAA GAC TTT CGA AGT CTC-3´ and reverse primer 5´- GTA AAA

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TCA TTG ATA ACA ACA CAG AC AAA C -3´ for RdRp gene.

Agro-infiltration

Three methods were used in this study as described by Liu *et al*. 2003 [14], Lu *et al*. 2002 [12] and Velasquez *et al*. 2009 [15]. According to the described method by Velasquez *et al.*, for each experiment, *A. tumefaciens* harboring $pTRV_1$, $pTRV_2$ and $pTRV_2$tPDS were grown on LB plates supplemented with 50 µg/ml of kanamycin and 5µg/ml tetracycline. Three ml liquid culture of LB containing mentioned antibiotics was inoculated with each of the strains and incubated in a shaker at 30 °C for 18 hours. A 1: 25 dilution of the culture was inoculated into liquid induction media (IM) with kanamycin, tetracyclin and 200 µM acetosyringone. The cells were harvested by centrifugation for 10 minutes at 3000 rpm and were resuspend with 10 mM $MgCl_2$, 10 mM MES pH 5.5. Acetosyringone was added to a final concentration of 400 µM to $pTRV_1$ culture. Cultures containing the $pTRV_1$, $pTRV_2$, and $pTRV_2$tPDS were mixed in a 1:1 ratio. Using 1 ml syringe, mixed cultures were infiltrated into the tomato and tobacco seedlings.

Results

Presence of PDS and RdRp gene was proved by colony PCR technique (figures 1, 2).

Using method described by Liu *et al.*, VIGS symptoms appeared 20 days post-inoculation, but faded after 15 days. These symptoms were limited to white spots on the leaves. The second protocol which was explained by Lu *et al.* was applied and plants did not show any symptoms. When the third method of Velasquez *et al.* was used and VIGS knocked down the expression of PDS gene, consequently the infected plants showed photobleaching and symptoms. However, plant infiltration with $pTRV_1$ and $pTRV_2$ (without PDS gene as control) did not show any photobleaching and symptoms (fig. 3).

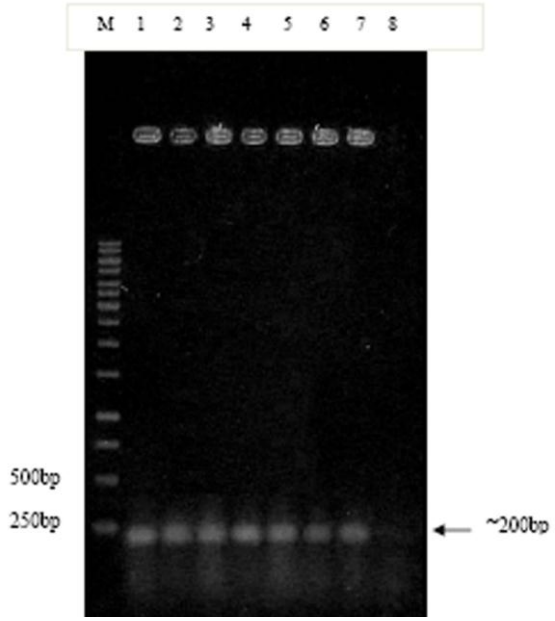

Fig. 1. Presence of PDS gene by colony PCR technique. M: 1kb marker. Lane 1-7: Random colonies selection. Lane 8: Negative control.

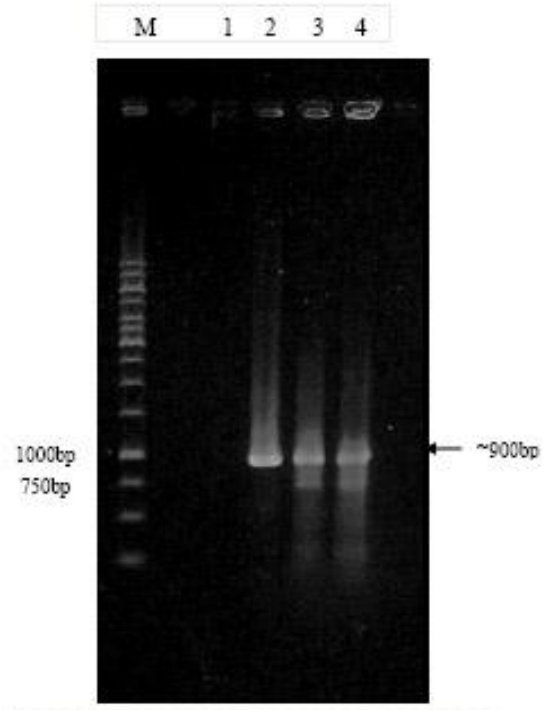

Fig. 2. Presence of RdRp gene using colony PCR technique. M: 1kb marker. Lane 1: Negative control. Lane 2: Positive control. Lane 3and 4: Selected random.

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Fig. 3. PDS gene silencing in Tomato. a: Control plant(infiltrated using $pTRV_1$ and $pTRV_2$ without PDS gene). b and c: PDS gene silencing in tomato showed photobleaching in leaves.

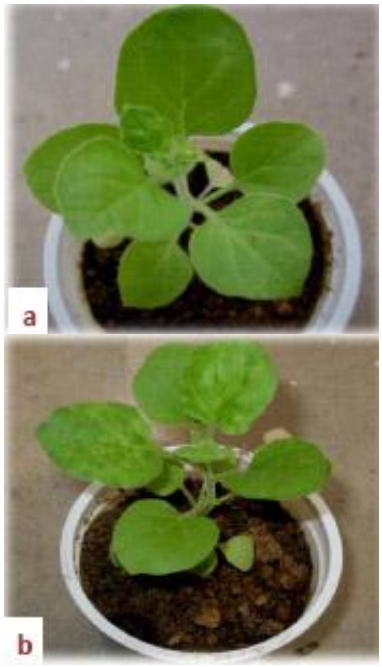

Fig. 4. PDS gene silencing in *N. benthamiana* by tomato PDS. a: Control plant(infiltrated using $pTRV_1$ and $pTRV_2$ without PDS gene). b: PDS gene silencing in tomato showed photobleaching in leaves.

Infiltration of tobacco plants using vectors carrying the tomato PDS gene, showed mosaic symptoms on leaves however no symptom was found on control plants (fig. 4).

Discussion

Virus-induced gene silencing is an excellent functional genomics tool for gene function analyses (14). In this report we showed that recombinant TRV vectors containing tomato PDS gene infect tomato and tobacco plants which can be used for gene silencing.

PDS gene which encodes the important enzymes in the carotenoid biosynthesis pathway was targeted. PDS silencing is an indicator for gene silencing in foliar tissue and shows a clear mutant phenotype (12).

Silencing of PDS expression in tomato plants was efficient close to about 4 weeks post-inoculation using Agro-infiltration method. Just one of the three employed methods was efficient for PDS gene silencing. The low strength answering of some Agro-infiltration methods may be due to presence of several viral resistance genes known in cultivated varieties of crops such as tomato (3).

We used recombinant TRV vectors containing tomato PDS gene for tobacco PDS gene silencing. There is 91% similarity between tomato and tobacco PDS gene. Tobacco plants infiltration was done, using TRV vectors that carry tomato PDS gene resulting in appearance of mosaic symptoms on the leaves. Such results have been obtained using the genes of different origins which used in gene silencing (16).

This method will be useful for functional analysis of many genes interest of tomato and will fulfill its promise of being a fast and efficient functional genomics tool.

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