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Tobacco Rattle Virus Vector-Based Virus Induced Gene Silencing - Optimization of A Functional Genomics Tool for *Hevea brasiliensis* using Reverse Genetics Approach

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Abstract

Hevea brasiliensis, an economically important industrial crop producing latex of commercial utility is widely cultivated in Southeast Asia. Various biotic and abiotic stresses affect plant growth and latex production, resulting in significant economic loss. A detailed knowledge of molecular defense signalling mechanism is essential to manage these stresses, which can be acquired through *in planta* studies. Establishment of an *in planta* functional genomics platform *via* stable plant transformation is difficult in non-model plants. In this study we optimized the technique of Virus Induced Gene Silencing (VIGS), an alternative approach to knockdown the gene function to study its functional role. Virus-induced gene silencing has been shown to be an effective tool for investigating gene functions in herbaceous plant species, but has rarely been applied in trees. The VIGS strategy relies on the recombinant virus vector carrying an inserted partial sequence of a target plant gene to initiate RNA-mediated Post-Transcriptional Gene Silencing (PTGS), leading to transcript suppression of corresponding homologous gene within the plant. In the present study, a Tobacco Rattle Virus (TRV) based VIGS system was optimized in *H. brasiliensis* for gene functional validation using Phytoene Desaturase (PDS) endogenous gene as a reporter. Various parameters such as age of the plant, concentration of inoculum, method of infiltration and time needed for effective silencing after treatment were optimized. To construct TRV-PDS vector, a 340 bp Hb:PDS gene was amplified from *H. brasiliensis* cDNA using primers designed from sequence of *H. brasiliensis* PDS gene already available and transferred to *Agrobacterium*. Out of the three agroinfiltration methods attempted, syringe infiltration method displayed significant down regulation of PDS gene expression. Young plants with 3 to 4 leaves infiltrated with an inoculum concentration, OD_{600} of 1.0 showed photo bleached phenotype with reduced expression of PDS gene, three to four weeks of post agroinfiltration. The optimized VIGS protocol can be used as

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a potential tool to validate the role of defense genes in *H. brasiliensis.* Further work is in progress towards construction of VIGS vectors of selected candidate defense genes, which has demonstrated differential gene expression in transcriptome profiling of resistant and susceptible clones of rubber tree. This will help us to understand defense mechanism of the plant which will facilitate to develop strategies for priming defense signalling during pathogen infection.

Introduction

Hevea brasiliensis, known as the Para rubber tree is an economically important industrial crop widely cultivated in Southeast Asia for the production of latex of commercial utility [1]. Various pathogens and pests cause threat to its growth and latex production, resulting in significant economic loss [2]. With the advancement in functional genomics, proteomics and host-pathogen interaction studies, molecular mechanisms of disease tolerance are being unravelled [3-5]. A considerable number of differentially expressed genes of *H. brasiliensis* involved in the post infection mechanism were identified using transcriptome sequencing [6]. Efficient disease control can be achieved by priming plant immunity towards the pathogen attack, which requires a detailed knowledge of the plant defense signalling programme obtained from *in-planta* functional genomic studies [7].

Functional genomic studies in model plants and several crop species have advanced using the available sequence information and various bioinformatics methods or tools such as next-generation sequencing, expressed sequence tags, microarrays, shotgun sequencing, RNA-sequencing, genome sequencing etc. has made major contributions in this area of research [8-10]. But, comprehensive understanding of the functional role of gene sequences cannot be revealed by all these analyses alone, except through stable up-regulation of the gene in mutant plants or down-regulation by knocking down the gene expression. However, such studies are time consuming, laborious and also restricted to plant species that are amenable to stable genetic transformation.

In planta gene functional studies through stable plant transformation is a labour-intensive, very difficult, less efficient and time-consuming procedure and sometimes, even not achievable in recalcitrant woody plants like *H. brasiliensis* [11-13]. Virus Induced Gene Silencing (VIGS) protocol serves as a powerful strategy in this regard to enable gene functional analysis for non-model plants bypassing the stable transformation process albeit in a transient way [14-16].

VIGS protocol relies on a plant's innate antiviral defense mechanism involved in the degradation of viral RNA by Post-Transcriptional Gene Silencing (PTGS) mechanism [16,17]. In this strategy, a recombinant viral vector carrying a small fragment of plant target gene is introduced into the plant which initiates PTGS leading to the suppression of corresponding homologous gene of the plant [15,16,18]. Inside the plant, viral replication leads to the formation of double stranded RNA which is recognized by DICER-like protein and gets cleaved into small interfering RNAs (siRNAs) of 21-23 nucleotides length. Later siRNAs get integrated with RNA Induced Silencing Complex (RISC) and target the complementary sequences for cleavage, resulting in the degradation of viral RNA and host's corresponding mRNA [17,19].

Many viral vectors have been reported for VIGS assay including Tobacco Mosaic Virus (TMV) [21], Potato Virus X (PVX) [22], Tobacco Rattle Virus (TRV) [17,22], Barley Stripe Mosaic Virus (BSMV) [24], Cucumber Mosaic Virus (CMV) [25] and Apple Latent Spherical Virus (ALSV) [26], among which the most commonly used VIGS vector is TRV based vector [17]. TRV has a broad host range, causes only mild symptoms and is capable of reaching the apical meristem within the plant. TRV based VIGS system has been reported in a wide range of plant species such as tomato [27], bell pepper (*Capsicum annum*) [28], *Arabidopsis* [29], maize (*Zea mays*) [30], barley (*Hordeum vulgare*) [31], wheat (*Triticum aestivum*) [30], strawberry [32], *Petunia* [33], *Gladiolus* [34] and tobacco [35]. However, successful VIGS strategy has been reported mostly in small herbaceous plants and only in a few woody plants.

Optimization of VIGS protocol specific for each plant species is necessary to achieve maximum silencing. Phytoene Desaturase (PDS), a key enzyme in the biosynthesis of protective carotene, is one of the commonly used reporter gene for optimizing VIGS method [36]. Silencing of PDS gene produces a characteristic photobleaching effect in the newly emerging leaves of infected plants following successful silencing [37].

In the present study, a TRV-based VIGS system for *Hevea brasiliensis* by down regulating Phytoene Desaturase (HbPDS) was optimized. We also demonstrated the importance of various factors such as age of plant, method of agro-inoculation and concentration of inoculum in determining the efficiency of gene silencing in rubber tree, *H. brasiliensis*.

Materials and methods

Plant material

Young *H. brasiliensis* plants were raised from seeds for VIGS studies. Mature fallen seeds were collected from rubber plantation fields of Rubber Research Institute of India, Kottayam, Kerala. The seeds were sowed into pots containing vermiculite mixture (peat moss: vermiculite: perlite, 1:1:1, v/v/v- Keltech Energies Ltd., Bangalore). The seedlings were grown in a greenhouse providing optimum growth conditions and young, healthy plants were selected for VIGS studies.

RNA isolation and cDNA synthesis

Leaves were collected from infiltrated plants and RNA was isolated from leaves following pine tree method [38]. Quality and quantity of the isolated RNA were assessed by Eppendorf UV-Visible Nano drop and integrity of isolated RNA was confirmed by checking on 2% agarose gel. Synthesis of first strand cDNA was performed using pure total RNA (1 µg), oligo d(T) primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA). Integrity of cDNA was checked using β-actin primers. List of all primers used in the study is given in Table 1.

Vector construction

HbPDS primers were designed using the sequence information from *H. brasiliensis* database (XM_021835824.1). HbPDS gene was amplified from the cDNA and cloned into pGEM-T easy vector (Promega, Madison, WI, USA) and transformed into $DH_5\alpha$ competent *E. coli* cells. Transformed colonies were screened by colony PCR using vector specific primers Tvect F and Tvect R. The parameters followed for PCR cycle were: initial denaturation at 95°C for 4 min., followed by 35 cycles of 95°C for 30 sec., 55°C for 30 sec. and 72°C for 1 min., and a final extension of 72°C for 5 min. Identity of the HbPDS sequence was

confirmed by sequencing.

Bipartite genome of TRV-VIGS vector, pBIN (TRV-RNA_1) and pTV00 (TRV-RNA_2) were obtained from Prof. David Baulcombe (Department of Plant Sciences, University of Cambridge). To construct TRV:HbPDS, pGEM-T vector carrying HbPDS and pTV00 (TRV-RNA_2) vector were digested using *HindIII* and *Spe*I (New England Biolabs) and ligated using T4 DNA ligase (Promega, Madison, WI, USA). The resulting construct TRV:HbPDS was transformed into $DH_5\alpha$ competent *E. coli* cells and plated on selective LB media containing 50 µg/ml Kanamycin. Colonies were PCR screened for the presence of modified constructs using the primers TRV F and TRV R which span the multiple cloning sites in pTRV-RNA_2. The parameters followed for PCR cycle were: initial denaturation at 95°C for 4 min., followed by 35 cycles of 95°C for 30 sec., 53°C for 30 sec. and 72°C for 1 min. and a final extension of 72°C for 5 min. Plasmids were isolated from selected positive colony and identity of the VIGS vector was verified by sequencing using TRV F and TRV R primers. TRV-RNA_1, TRV-RNA_2 and TRV:HbPDS plasmids were finally transferred into *Agrobacterium tumifaciens* GV3103 and positive colonies were identified by colony PCR.

Agroinfiltration

Agrobacterium tumifaciens GV3103 cultures carrying TRV-RNA_1, TRV-RNA_2, TRV:HbPDS plasmids, were grown separately for overnight in LB medium containing antibiotics (25 µg/ml Rifampicin and 50 µg/ml Kanamycin) at 28°C. Later, agrobacterial cells (OD_{600} of 1.5) were harvested and resuspended in infiltration media (10 mM $MgCl_2$, 10 mM 2-(N-morpholino) ethanesulfonic acid (MES; SIGMA), pH 5.7 and 200 µM acetosyringone (SIGMA), and incubated at room temperature for 3 hours without shaking. *Agrobacterium* cultures containing the *Agrobacterium* mixture of TRV-RNA_1 and TRV-RNA_2 or TRV:HbPDS (1:1 ratio) were infiltrated by agroinfiltration, vacuum infiltration and agrodrench methods.

Agroinfiltration was carried out using a needleless, 1 ml syringe on lower leaves of 4-5 leaf stage plants following the protocol of Liu *et al*. [32]. To ease infiltration, leaf surface was initially rubbed with carborundum and then *Agrobacterium* mix was slowly injected. For vacuum infiltration method, young plants were co-cultivated for 5 min. in *Agrobacterium* suspension taken in a 1 L side arm flask or vacuum flask (Schott Duran, Germany) connected to a vacuum pump. Mouth of the flask was closed with a rubber cork and vacuum was applied at desired level for 5 min. Vacuum was applied in a range from 100 to 600 mm Hg. The infiltrated plants were blotted dry on a filter paper and hardened.

For agrodrench method, a mixture of *Agrobacterium* strains containing TRV-RNA_1 and TRV-RNA_2 or TRV:HbPDS (1:1 ratio) were drenched into soil near the roots.

To confirm viral multiplication in infiltrated plants, fragments of RNA_1 and RNA_2 of TRV were amplified by plasmid specific primers (PBIN F and PBIN R; PTV F and PTV R). The parameters followed for the PCR cycle were: initial denaturation at 95°C for 4 min., followed by 35 cycles of 95°C for 30 sec., 50°C for 30 sec. and 72°C for 1 min. and a final extension of 72°C for 5 min.

Real time analysis

To determine the efficiency of silencing, new leaves were collected at different time intervals after infiltration from control and TRV:HbPDS infiltrated plants. First-strand of cDNA was synthesized as described earlier and all amplifications were performed on Light Cycler 480 II, Roche Real Time PCR System using RT PDS F and RT PDS R primers. Real Time PCR was performed in a 10 µl reaction mixture containing 0.5 µl template DNA from 1/10 dilution of first strand of cDNA reaction, 125 nM of each primer, and 10 µl of Light Cycler 480 SYBR Green I Master (Roche Diagnostics GmbH, Germany). The mixture was first incubated at 95°C for 7 min., followed by 40 cycles of 95°C for 20 sec. and 60°C for 30 sec. Subsequently, a melt curve analysis (95°C for 20 sec., 60°C for 1 min. and 95°C for about 5 min.) was conducted. Each experiment was repeated thrice and each PCR reaction was performed in triplicate with No Template Controls (NTC). β-actin gene was used as endogenous control. The Light Cycler 480 Software (release 1.5.0) was used for relative quantification analysis, and expression rate of each gene was represented as fold change. Statistical analysis was performed with single factor ANOVA using normalized expression data.

Table 1: Primers used in the study.

Primer Name	Sequence (5'– 3')
Hb PDS F	TGGCATGCAAAGTCTCTCTG
HbPDS R	TTAAATGGGATGTGGGCAAT
PBIN-F	GTTTGTGGACGGTAGGAG
PBIN-R	ATGAACCCAGGCGTATCT
PTV-F	CCTGCTGACTTGATGGAC
PTV-R	CAGTGTTCGCCTTGGTAG
Tvect F	GGGTTTTCCCAGTCACGACGT
Tvect R	CGCCAAGCTATTTAGGTGACAC
TRV F	GCTGCTAGTTCATCTGCAC
TRV R	GCACGGATCTACTTAAAGAAC
RT Act F	GATTCCGTTGCCCAGAAGTC
RT Act R	CACCACTCAGCACAATGTTACC
RT18S F	AAGCAAGCCTACGCTCTGG
RT18S R	GCTCCACCAACTAAGAACGG
RT PDS F	CAGGCAAAAGAAGCTTCAGG
Hb Actin F	CCAAGGCCAACAGAGAGAAG
Hb Actin R	ATCAGTGAGATCCCGACCAG

Results

Vector construction

Identity of TRV vector was initially confirmed by PCR amplification using TRV vector specific primers of TRV RNA_1 and TRV RNA_2. PCR products on agarose gel showed the expected amplicon size of 210 bp and 345 bp respectively for TRV RNA_1 and TRV RNA_2. A 340 bp phytoene desaturase gene was amplified from *H. brasiliensis* cDNA using HbPDS F and HbPDS R primers (Figure 1). HbPDS gene was initially cloned into pGEM-T easy vector and positive colonies were confirmed by colony PCR. Sequence analysis of pGEM-T HbPDS plasmid confirmed the insert as *H. brasiliensis* phytoene desaturase. Further, HbPDS cloned to TRV-RNA_2 and TRV-HbPDS plasmids were isolated from positive colonies and identified by colony PCR. TRV-HbPDS, TRV-RNA_2 and TRV RNA_1 plasmids were finally transferred to *Agrobacterium tumifaciens* GV3103 (Figure 1). Expected amplification of 340 bp confirmed the TRV-PDS positive colonies which were used for agroinfiltration studies.

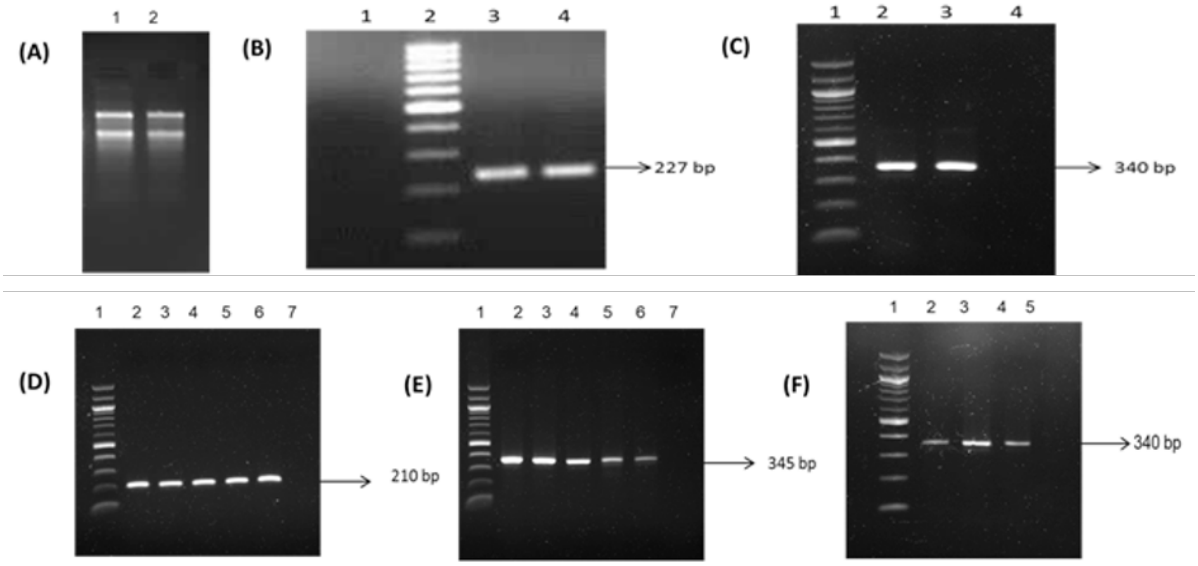

Figure 1: TRV: PDS vector construction -
(A) Agarose gel electrophoresis of RNA isolated from *H. brasiliensis* leaves;
(B) Amplification of β-actin from cDNA: 1- Negative control, 2- 100bp ladder, 3&4 - β actin amplified;
(C) Amplification of PDS gene: Lane 1-100 bp ladder, Lane 2&3 - PDS amplification, Lane 4 - Negative control;
(D) Colony PCR confirmation of TRV- RNA_1 transformation into *Agrobacterium:* Lane 1- 100 bp ladder, Lane 2-6-Positive colonies, Lane 7-Negative control;
(E) Colony PCR confirmation of TRV- RNA_2 transformation into *Agrobacterium:* Lane 1- 100 bp ladder, Lane 2-6- Positive colonies, Lane 7-Negative control;
(F) Colony PCR confirmation of TRV-PDS transformation into *Agrobacterium*: Lane 1- 100 bp ladder, Lane 2-4 - Positive colonies, Lane 5-Negative control.

Agroinfiltration

In order to confirm that TRV can propagate in *H. brasiliensis,* young plants were syringe infiltrated with *Agrobacterium* empty vector initially. After 21 days of infiltration, newly emerged leaves were checked for the presence of TRV particles by amplification of TRV RNA_1 and TRV RNA_2. Expected amplification of 210 bp for TRV RNA_1 and 345 bp for TRV RNA_2 confirmed the replication and movement of virus in *H. brasiliensis* (Figure 2).

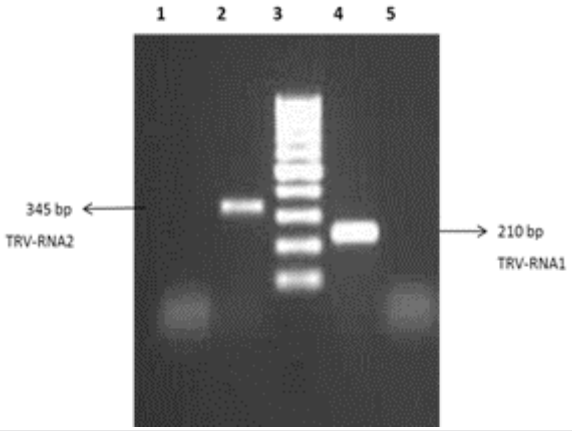

Figure 2: TRV movement in *H. brasiliensis*: Lane 1&5 - Negative control, Lane 3 - 100 bp ladder, Lane 2- TRV RNA_2, Lane 4- TRV RNA_1.

To optimize VIGS for *H. brasiliensis,* various parameters like method of agroinfiltration, age of plant and time needed for maximum downregulation were estimated. Of the three agroinfiltration methods attempted, both syringe and vacuum infiltration methods showed significant downregulation of PDS gene compared to agrodrench method (Figures 3&4). The newly emerged leaves showed photobleached phenotype 3 to 4 weeks of post agroinfiltration whereas there were no significant phenotypic changes for the plants infiltrated by agrodrench method (Figure 4).

One of the important factors affecting VIGS was found to be the growth stage of the plant at the time of agroinfiltration. To study the optimum age for infiltration, we tried to downregulate PDS expression at different growth stages of seedling. Young seedlings with 2 to 4 leaf stage showed maximum silencing. Also, vacuum infiltration was easy to implement when the seedlings were small in size. Agroinfiltrated plants were monitored for a period of 4 months following treatment. VIGS was found to initiate 3 to 4 weeks post agroinfiltration and persisted for up to 3 months. The effect was reduced after 3 months of infiltration.

It has been reported that the silencing efficiency is also affected by concentration of agroinoculum [28,29]. Accordingly, to optimize the best concentration for maximum silencing, we tried different concentrations of inoculum (OD_{600} in a range of 0.5 - 3). OD_{600} of 1.0 was found to be the best concentration of agroinoculum for gene silencing in *H. brasiliensis* seedlings.

Thus, it was confirmed that young plants with 2 to 4 leaves can be infiltrated with agroinoculum having OD_{600} of 1.0 to get efficient silencing, which gets noticeable as photobleaching on newly emerged leaves after 3 to 4 weeks of inoculation in *H. brasiliensis.*

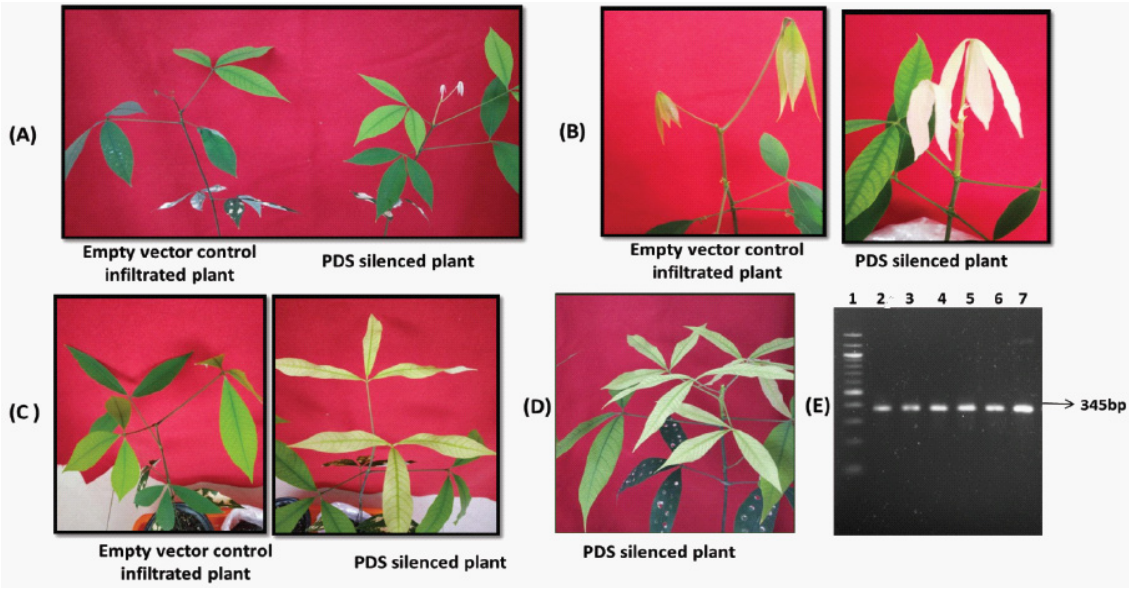

Figure 3: Phenotype of PDS silenced plants in *H. brasiliensis* by syringe infiltration method. Newly emerged leaves showed photobleached phenotype 3 to 4 weeks post agroinfiltration.
(A) PDS silenced plants 3 weeks after agroinfiltration;
(B) PDS silenced plants 6 weeks after agroinfiltration;
(C) PDS silenced plants 10 weeks after agroinfiltration;
(D) PDS silenced plants 3 months after agroinfiltration;
(E) RT-PCR confirmation of TRV replication: 1- 100 bp ladder, 2&3- empty vector control infiltrated plants, 4-6- TRV HbPDS agroinfiltrated leaves, 7-Positive control.

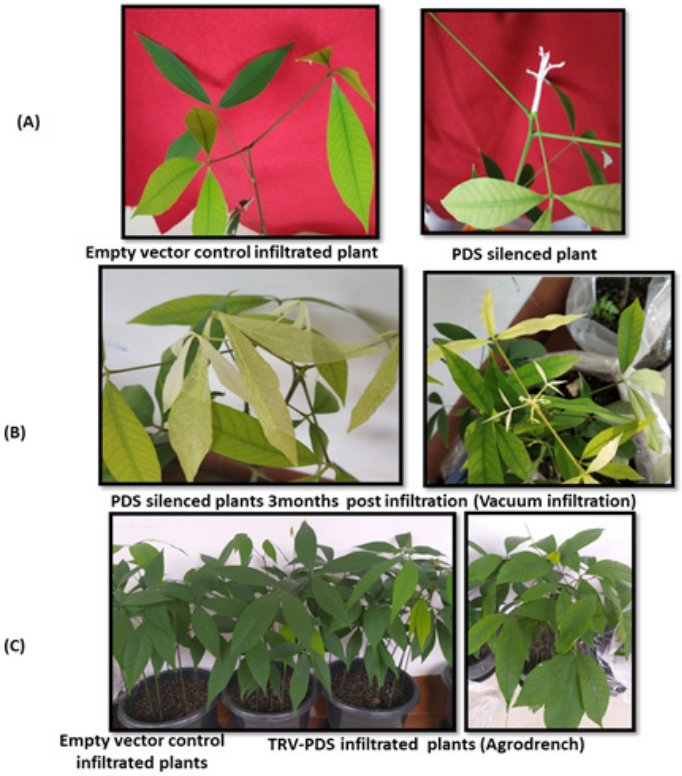

Figure 4: Phenotype of TRV- HbPDS agroinfilitrated plants by vacuum and agrodrench infiltration method
(A) Phenotype of PDS silenced plants 3 weeks after agroinfiltration by vacuum infiltration;
(B) Phenotype of PDS silenced plants 3 months after agroinfiltration by vacuum infiltration.
(C) Plants infiltrated by Agrodrench method.

Realtime PCR analysis

To confirm PDS downregulation at the molecular level, expression of PDS gene was evaluated by real time PCR using gene-specific primers. Transcript level of PDS gene was reduced by 85% by syringe infiltration and 74% by vacuum infiltration (Figure 5). There was no significant downregulation of PDS transcript by agrodrench method of infiltration. The expression of HbPDS gene was significantly reduced in silenced plants as compared to control plants which further confirmed that the optimized TRV-VIGS protocol can be used for functional gene validation in *H. brasiliensis*.

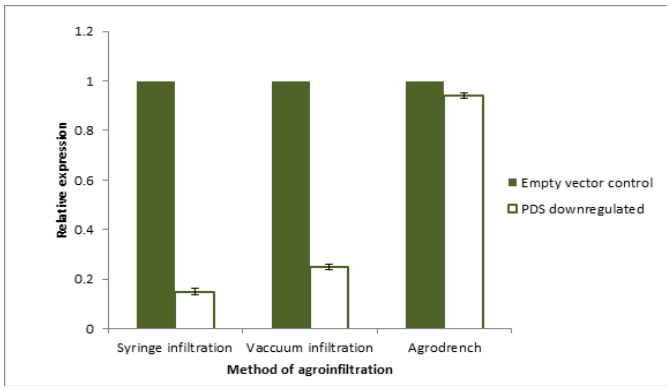

Figure 5: Validation of PDS transcripts downregulation by Real time PCR.

Discussion

Natural rubber obtained from *Hevea brasiliensis* is an indispensable commodity used worldwide for the production of a large number of products [39]. Rubber tree experiences various biotic and abiotic stresses leading to serious economic losses to rubber growers all over the world [40,41]. With the advancement in the field of "Omics", a large number of transcriptome and genome data has been generated by which the mechanism

of stress tolerance is being unravelled [6,42]. But, only a few genes have been tested for its functional role till date [43-47]. However, the molecular mechanism of rubber tree in response to these stresses remains unknown. Functional validation of genes is a difficult task in most perennial tree crops owing to lack of an efficient transformation protocol [48,49]. It has been reported that stable genetic transformation efficiency is very low in *H. brasiliensis* [50] and we have to adopt new techniques for genetic studies in this economically important tree crop.

VIGS, a transient transformation technique, has been proven to be an efficient reverse genetic tool in many plants which are recalcitrant to stable genetic transformation [20,51]. VIGS strategy does not require wide sequence information and it is a very simple technique compared to stable transformation. Success of VIGS depends upon the choice of the vector and optimization of the methodology [17]. In the present study, we have successfully optimized TRV-VIGS as a transient gene knockdown strategy and demonstrated that this method can be used as a tool for gene functional evaluation in *H. brasiliensis.*

Selection of vector is the key to get maximum silencing efficiency by VIGS method [17]. TRV is the most commonly used viral vector for VIGS because of its wide host range and uniformity in silencing without causing any visible viral disease symptoms [15]. Before constructing TRV-VIGS vector for gene functional studies in *Hevea*, we confirmed the movement of TRV within the plant. We could amplify viral particle from the newly emerged leaves as well as from those leaves without any visible symptoms of virus in *H. brasiliensis*. This is in accordance with the previous reports on TRV-VIGS in other plant species [30,52-56].

Use of an internal reference gene (marker gene) is essential to monitor the uniformity of silencing [17] and it allow easy detection of silenced and non-silenced tissues, thereby increasing the sensitivity of downstream analysis [57]. Commonly used VIGS markers are related to chlorophyll synthesis function since it allows evaluation of silencing efficiency by visible phenotypic effects. Phytoene Desaturase (PDS), Anthocyanin Synthase (ANS), Chalcone Synthase (CHS), Magnesium chelatase subunit I (Chl I) and Chloroplastos alterados 1 gene (CLA1) are some of the visible markers which helps to identify the location and extent of gene silencing through VIGS [21,58,59].

Phytoene Desaturase (PDS) gene is known to cause loss of chlorophyll and carotenoids when silenced [33,60-63]. PDS is a rate limiting enzyme in the carotenoid biosynthetic pathway [64] and downregulation of this gene leads to photobleached symptoms due to failure of chlorophyll synthesis [65]. Therefore, we used a TRV-HbPDS vector system to optimize VIGS in *H. brasiliensis*.

The efficiency of VIGS strategy varies with different plant species and even with different cultivars that necessitates the need to optimize the technique specifically for each plant [15,66,67]. Reports suggest that choice of vector, plant age and method of inoculation are the key parameters determining the efficiency of VIGS method [29,63,68,69]. Therefore, it was necessary to optimize these factors for VIGS studies in rubber tree [15]. There are various methods to introduce *A. tumefaciens* into plant tissues such as direct inoculation of bacterial colonies, needleless syringe infiltration, vacuum infiltration and agrodrench method [29,35,63,70,71]. In *H. brasiliensis*, syringe agroinfiltration was found to be more effective for VIGS compared to vacuum infiltration and agrodrench. Even though vacuum infiltration produced photobleached phenotype, transcript level downregulation was more with syringe infiltration. Syringe infiltration method is reported to be an effective method for agroinfiltration in many plant species and is relatively easy to carry out and requires only a small volume of culture for infiltration [63,72]. In *Nicotiana benthamiana,* agroinoculation of seedlings with a needleless syringe at four-leaf stage was found to be a more efficient method whereas in *S. lycopersicum* maximum silencing was achieved with vacuum infiltration [73] or by infiltration spray method [35].

In *H. brasiliensis*, downregulation of PDS gene was clearly visible phenotypically and genotypic transcript downregulation was also confirmed by real time PCR. The phenotypic changes were similar to reports in other plants like *N. benthamiana* [14], *Aquilegia vulgaris* [74], *Petunia* [33], tomato [35], *Arabidopsis thaliana* [29], *Thalictrum dioicum* [37], *Physalis floridana* [75] and *Gerbera hybrida* [61]. PDS can be chosen as an efficient reporter gene for VIGS as it does not produce any visible symptoms of viral multiplication and also does not cause any lethal effect on survival of the leaf/ plant in *H. brasiliensis.*

VIGS persist for about 3 months in *H. brasiliensis*, after which a slight reduction in efficiency was observed. Fu *et al*. [72] reported that various factors such as viral titre, age of the plant and environmental conditions that favour virus multiplication, influence the persistence of VIGS for long period. Studies are in progress to understand the exact duration of VIGS effect in *H. brasiliensis.*

Age of the plant is an important factor determining the success of VIGS technique. TRV is reported to infect and move effectively in younger tissues with meristematic cells [63]. Similar to other plant species, *H. brasiliensis* also showed effective silencing in young plants with 3 to 4 leaves. Our study optimized a rapid and efficient VIGS system in *H. brasiliensis* for functional genomics to be used for candidate defense gene validation.

Conclusion

TRV-VIGS serves as a rapid, easy and efficient gene validation tool for *H. brasiliensis*. The optimized strategy will contribute to functional genomics studies in *H. brasiliensis* by validating the role of different genes involved in various stress response mechanism which ultimately will help to develop strategies to control stress response by priming defense signalling.

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