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**Standardization of environmental conditions for induction and retention of post-transcriptional gene silencing using tobacco rattle virus vector**

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Post-transcriptional gene silencing technique (PTGS) using virus-induced gene silencing system (VIGS) is a highly useful gene knockout approach for functional analysis of endogenous genes in plants. Tobacco rattle virus based VIGS vector is a suitable vector for gene silencing in model plants like *Nicotiana benthamiana* and *Lycopersicum esculentum* (tomato). The effectiveness of Agroinfection and TRV–VIGS is influenced by environmental conditions. The vector having plant endogenous *Phytoene desaturase* (PDS) gene cloned either from *N. benthamiana* or tomato was used for targeted PTGS by Agro-infiltration approach. The extent of PDS RNA silencing was found to be highly temperature-sensitive and silencing was enhanced under low ambient temperature (less than 24°C), low light intensity (250 μmol m⁻² s⁻¹) and high humidity (85–90%). The efficiency of silencing was better in *N. benthamiana* than tomato. For examining the functional relevance of genes associated with specific physiological processes or abiotic stress, it is essential to retain the phenotype for longer period under greenhouse condition. *N. benthamiana* plants that were induced PTGS of PDS in growth room retained the phenotype for 30–35 days in the greenhouse maintained at high temperature (28–30°C) and light intensity (1000 μmol m⁻² s⁻¹). The silenced plants maintained low PSII quantum yield and did not show any photosynthesis.

**Keywords**: Agro-infiltration, post-transcriptional gene silencing, *Nicotiana benthamiana*, tobacco rattle virus, VIGS.

The genomes of the important crop plant *Oryza sativa*, and the model plant *Arabidopsis thaliana*, have been completed and the analysis predicts that there can be approximately 55,000 and 25,000 protein-coding sequences in the former and latter respectively1–3. The major focus is to evaluate the function of these genes, which can be further used for genetic manipulation of crops for better productivity. The most recent reverse-genetic approach of post-transcriptional gene silencing (PTGS) using Virus-Induced Gene Silencing (VIGS) system is considered as an attractive tool for gene function analysis4–6. VIGS can be effectively used to silence known gene sequences in

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any suitable virus host plant. Amongst many recombinant virus vectors used for VIGS assay, tobacco rattle virus (TRV)-based vectors are highly efficient in model plants like *Nicotiana benthamiana*. The virus will not produce any specific disease symptom, which might interfere with the phenotype of interest. The TRV-based silencing vector has been successfully used to examine the functional relevance of genes associated with biotic stress responses in *N. benthamiana*. However, there is no evidence of using this vector for studying the functions of genes related to abiotic stress (such as high temperature, light intensity, etc.) responses. Specific environmental conditions are required for studies related to abiotic stress responses and such conditions might affect TRV-infection. It is likely that the infection of TRV and activation of RNA silencing machinery is influenced by environmental conditions. From this context, we made an attempt to examine the conditions required for TRV infection and development of uniform silencing phenotype using *Phytoene dehydrogenase* (PDS) as reporter gene in model plants *N. benthamiana* and *Lycopersicum esculentum* (tomato). We report a method of developing and retaining silenced phenotype using TRV–VIGS, which would be useful for functional analysis of genes associated with abiotic stress responses in plants.

*N. benthamiana* and tomato seedlings were initially raised in a nursery and later transplanted to pots. Plants were irrigated twice a day to maintain 100% field capacity. For silencing experiments, the TRV-based VIGS vectors (pTRV1 and pTRV2) provided by S. P. Dinesh-Kumar, Yale University, USA was used. Recombinant VIGS vector carrying *PDS* gene fragment cloned either from *N. benthamiana* or tomato, was used for standardizing the conditions required for infection of *Agrobacterium* and activation of RNA silencing machinery. The vectors were mobilized into *Agrobacterium* strains GV3101 and GV2260 for inducing endogenous *PDS* gene silencing in tomato and *N. benthamiana* respectively. For agroinfiltration, two different *Agrobacterium* cultures carrying pTRV1 and pTRV2 vectors were grown overnight, suspended in infiltration medium (10 mM MES buffer, 200 μM acetosyringone, 10 mM MgCl₂, glucose 1%, sucrose 2%) and mixed in 1:1 ratio to an OD₆₀₀ = 0.8. The mixture was then incubated for 3 h and infiltrated onto the lower leaf of four-leaf stage *N. benthamiana* plants using 1 ml needleless syringe. For silencing experiments in tomato, cotyledonary leaves were infiltrated with the cultures. The infiltrated plants were maintained at different temperatures (20–22, 23–24 and 25–26°C for tomato and 20–24 and 25–26°C for *N. benthamiana*; ambient relative humidity of 85–90%; light intensity of 250 μmol m⁻² s⁻¹) and scored for the photo-bleached phenotype.

The extent of endogenous gene silencing was tested by quantifying the *PDS* gene transcripts using real-time reverse transcription polymerase chain reaction (RT–PCR; Opticon2, MJ Research, USA). About 10 μg of total RNA was reverse-transcribed using 200 U Moloney Murine Leukaemia Virus reverse transcriptase (MMLV–RT) at 42°C for 1 h. The reaction was primed with 2.5 μM digoT primers in the presence of a mix of 10 mM dNTPs. Real-time PCR was performed in the presence of SYBR-green fluorescence dye (DyNAmo SYBR-Green qPCR Kit FinNZYMES, Finland; www.finzzymes.fi) using equal amount of cDNA. The Ct value (the fractional cycle number at which fluorescence passes a fixed threshold) was used to compare target DNA in the sample. The silenced plants were examined for the variation in PSII quantum yield (ΦPSII) and photosynthesis using portable photosynthesis system (LiCOR, 6400, Nebraska, USA).

Success of post-transcriptional gene silencing with virus-based vectors by agroinfiltration depends upon the efficiency of *Agrobacterium* infection and spread of virus in candidate host plants. It is important to determine the optimum inoculum of *Agrobacterium* and plant growth conditions for successful gene silencing. If the conditions are not ideal, then there is a possibility of missing the expected phenotype and the infected plants may fail to induce uniform RNA silencing systemically. In addition, under such situations the degree of silencing can vary between the plants and experiments resulting in ambiguous conclusions. Developing uniform silencing phenotype is crucial for functional analysis of genes associated with specific physiological processes such as photosynthesis, a process related to abiotic stress response. Therefore, it is mandatory to standardize the conditions required for *Agrobacterium* infection and activation of RNA silencing system for developing uniform phenotype under a given greenhouse condition.

It has been reported in several PTGS studies that silencing of *PDS* involved in carotenoid biosynthesis results in photo-bleached phenotype and hence *PDS* is commonly used as a reporter gene in VIGS studies. The efficiency of the TRV–VIGS vectors in developing photo-bleached phenotype, used in this study, has been tested in earlier studies. Similar to the earlier studies, photo-bleached phenotype was used as an indicator of silencing while optimizing the environmental conditions for induction of PTGS.

Among the two model systems used, induction of phenotype in tomato was relatively difficult than in *N. benthamiana*, though both are host plants of TRV. Tomato plants could produce the expected phenotype at a frequency of 40–50% at low temperature (20–24°C), high humidity (85–90%) and low light intensity (250 μmol m⁻² s⁻¹) after 20–25 days post-infiltration (dpi; Figure 1c, d). The phenotype was uniform in young, developing leaves when the temperature was between 20 and 22°C (Figure 1d). However, as the temperature increased beyond 25°C, there were no clear photo-bleached symptoms, indicating the critical role of temperature in PTGS (Figure 1b). In case of *N. benthamiana*, systemic infection was seen under a relatively wide range of temperatures (20–24°C, Figure 1g) and the infection was more than 95%. At higher tempera-
ture between 25 and 26°C, poor infection was noticed with patchy phenotype (Figure 1f).

Plants grown in tropical climate are adapted to high temperature (beyond 28°C) and have high temperature optima for many of their physiological processes. Therefore, in most of the controlled experiments under greenhouse conditions, the temperature is set around 28–30°C. This study demonstrates that silencing is affected beyond 26°C and hence tropical greenhouse conditions are not ideal for induction of RNA silencing. In the case of tomato, uniform phenotype was seen only at a temperature between 20 and 22°C, whereas *N. benthamiana* exhibited uniform phenotype with high efficiency at a relatively wider range of temperature (20–24°C). Hence *N. benthamiana* is considered as a better model system than tomato for functional genomics with TRV–VIGS.

The function of most of the abiotic stress-related genes could not be studied under the specific environmental conditions required for the induction of RNA silencing using TRV–VIGS. It is necessary to examine the functional relevance of genes associated with specific abiotic stress responses under stressful environments. Hence, retention of the silenced phenotype for a specific period is essential and the phenotype should not revert back when a specific
Table 1. Determination of PDS mRNA levels by real-time polymerase chain reaction in mock-inoculated (control) and PDS gene-silenced N. benthamiana plants using DNA binding fluorescent dye, SYBR-Green.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Real-time Ct value</th>
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<tbody>
<tr>
<td></td>
<td>β-actin</td>
</tr>
<tr>
<td>Mock plants</td>
<td>15.12 ± 2.23</td>
</tr>
<tr>
<td>PDS-silenced plants</td>
<td>16.06 ± 1.19</td>
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Ct values (fractional cycle number at which fluorescence passes the fixed threshold) for PDS and β-actin (most abundant RNA, used as an internal control) gene products were determined simultaneously from the common cDNA pool.

n.d., Not detected; a single dissociation peak with a Tm within variation of 2°C of the expected temperature indicated amplification of the gene of interest. Each value represents the mean ± SD of three independent experiments.

Figure 3. Photosynthesis (µmol m⁻² s⁻¹; a) and quantum yield of PSII reaction centre (b), in mock-infected control and PDS gene-silenced plants of N. benthamiana. Silencing was induced under optimum temperature before transferring to greenhouse with temperature of 28–30°C. Measurements were made 35 dpi at light intensity of 1000 µmol m⁻² s⁻¹. Photosynthesis and chlorophyll a fluorescence were measured simultaneously. Vertical bars show SD.

Figure 4. Cartoon guide to functional analysis of genes associated with abiotic stress responses in N. benthamiana.

growth condition or stress is imposed. In this study we made an attempt to induce photo-bleached phenotype in growth room under optimum temperature (23–24°C) prior to transferring to greenhouse condition with temperature 28–30°C and light intensity 1000 µmol m⁻² s⁻¹. N. benthamiana plants that were induced PTGS of PDS gene in growth room retained the phenotype for 30–35 days in the greenhouse (Figure 2), whereas tomato plants failed to retain the phenotype. The extent of PDS mRNA silencing was assessed by quantitative real-time polymerase chain reaction using SYBR-Green DNA binding fluorescence dye. The real-time Ct values for PDS and β-actin gene products were compared between silenced and mock (vector control) inoculated plants. The most abundant RNA, β-actin was used as an internal control. There was no significant difference in Ct values between mock-inoculated and PDS-silenced samples. The PDS gene products were not detected in silenced plants as evidenced by the Ct values (Table 1). The process of photosynthesis was monitored in these plants 35 dpi to characterize the phenotype. As expected, there was no photosynthesis in the photo-bleached leaves and there was reduction in quantum yield of PSII reaction centre (Figure 3b). These findings suggest the usefulness of this approach (Figure 4) for functional analysis of genes associated with specific physiological process or abiotic stress response using TRV-VIGS in N. benthamiana.

Cation distribution in Cr-spinels from the Sittampundi layered complex and their intracrystalline thermodynamics

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High aluminium chromites occur as bands within anorthosite layered complex of Sittampundi (Tamil Nadu). Cation distribution of two chromite samples is determined by combined electron probe microanalysis and Mössbauer spectroscopy. In the deconvolution of the Mössbauer spectra, the suitability of the best model of spectral fitting has been established with the observation that both Fe2+ and Fe3+ ions occur at tetrahedral (A) and octahedral (B) sites and Fe3+/ΣFe found to be ranging between 0.45 and 0.48. Oxygen fugacity (fO2) has been determined to be about 10013. Thermodynamic parameters have been determined for the studied chromites using standard models.

Keywords: Cation distribution, chromite, Mössbauer spectroscopy, Sittampundi, thermodynamic parameter.

Spinels are used as a petrogenetic indicator because their chemical and structural variations are dependent on the paragenesis, pressure and temperature of crystallization. The widespread occurrence of spinels is in part a result of the large number of cations that the structure can accommodate. Many crystallographic1-4 and thermodynamic5-10 studies have been done on synthetic spinels because of their applications in materials science, metallurgy and earth sciences. However, studies on natural spinel are scarce due to difficulties in assigning major cations present in the tetrahedral (A) and octahedral (B) sites. Among the cations present in spinel, Fe commonly exists in multiple valence states and accurate knowledge of the $\text{Fe}^{3+}/\text{Fe}^{2+}$ ratio allows us to estimate the oxygen fugacity ($f_{\text{O}_2}$), which controls the magmatic crystallization path and composition of the resulting mineral phases. The aim of the present work is to determine the cation distribution of chromites (Cr-spinel) from the Sittampundi complex, Tamil Nadu, using electron probe microanalysis (EPMA) and room temperature $^{57}$Fe Mössbauer spectroscopy (MS) and the intracrystalline thermodynamic parameters of the natural samples.

Sittampundi layered anorthosite complex occurs as a layered igneous body11. The study area forms a part of the granulite terrain of South India. Major rock types are chromitite bearing meta-anorthosite, amphibolite, basic granulite, two pyroxene granulite, leptynite, biotite gneiss and pink granite. Chromitite occurs exclusively within the anorthosite as discontinuous bands/lenses. Samples used for crystallo-chemical investigation were culled from chromitite (chromite + rutile + calcic amphibole ± anorthophy- lite ± clinohlorite). Two samples, #30a and #56 were collected from conformable chromitite lenses on a foot track in the western part of Karungalpatti, Salem district, Tamil Nadu. These two samples are henceforth referred to as Ch1 and Ch2.

Chromite samples from the Sittampundi area were studied by the combined EPMA and MS. Samples were analysed by a JEOL-733 superprobe microanalyser with wavelength dispersive method at the Department of Geology, Yonsei University, Seoul. Room temperature (298 K) Mössbauer spectrum was recorded in a Wissel-make conventional constant acceleration spectrometer using a 10 mCi Co/Rh source. The spectrum was fitted to Lorentzian lines with a nonlinear least square fit programme. The velocity calibration was performed with respect to pure metallic iron (99.99%) standard. Detailed processing of the samples and the procedures adopted for data acquisition for the EPMA and MS studies are reported elsewhere12.

Natural chromite samples having complex compositions or crystallizing in an oxidizing environment, often exhibit disorder of $\text{Fe}^{2+}$ and $\text{Fe}^{3+}$ distribution between octahedral (B) and tetrahedral (A) sites. We have fitted the spectra considering both the normal and disordered distribution. The spectra fitted in the disordered distribution showed better acceptable $\chi^2$. For natural chromites best-fitting results were obtained using a three-doublet model by Wood and Virgo13 and a four-doublet model by Dyar et al.14. Fitting model with four doublets showed better results and

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