Assessing the tobacco-rattle-virus-based vectors system as an efficient gene silencing technique in Datura stramonium *(Solanaceae)*

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Abstract *Datura stramonium* is a well-known medicinal plant, which is important for its alkaloids. There are intrinsic limitations for the natural production of alkaloids in plants; metabolic engineering methods can be effectively used to conquer these limitations. In order for this the genes involved in corresponding pathways need to be studied. Virus-Induced Gene Silencing is known as a functional genomics technique to knock-down expression of endogenous genes. In this study, we silenced phytoene desaturase as a marker gene in *D. stramonium* in a heterologous and homologous manner by tobacco-rattle-virus-based VIGS vectors. Recombinant TRV vector containing *pds* gene from *D. stramonium* (pTRV2-*Dspds*) was constructed and injected into seedlings. The plants injected with pTRV2-*Dspds* showed photobleaching 2 weeks after infiltration. Spectrophotometric analysis demonstrated that the amount of chlorophylls and carotenoids in leaves of the bleached plants decreased considerably compared to that of the control plants. Semi-Quantitative RT-PCR results also confirmed that the expression of *pds* gene in the silenced plants was significantly reduced in comparison with the control plants. The results showed that the viral vector was able to influence the levels of total alkaloid content in *D. stramonium*. Our results illustrated that TRV-based VIGS vectors are able to induce effective and reliable functional gene silencing in *D. stramonium* as an alternative tool for studying the genes of interest in this plant, such as the targeted genes in tropane alkaloid biosynthetic pathway. The present work is the first report of establishing VIGS as an efficient method for transient silencing of any gene of interest in *D. stramonium.*

Keywords Alkaloids · *Datura stramonium* · TRV vectors · VIGS

Virus-induced gene silencing (VIGS) is known as a technique using recombinant viruses to knock-down expression of endogenous genes based on post-transcriptional gene silencing [1, 2]. In this technique, plants use a natural defense mechanism against viral attack and especially degrade RNAs derived from the viral genome [3]. VIGS is a very simple, fast, and strong method for functional genomics studies that do not involve stable transformation [4]. The VIGS technique is based on virus vectors which carry host-derived sequence homologous to endogenous genes to cause silencing of the corresponding genes in infected plants through a homology-dependent RNA degradation mechanism [5].

Datura stramonium, a plant from the *Solanaceae* family, is widely distributed in regions with different temperatures throughout the world [6, 7]. This plant has been known for its therapeutic and poisoning effects for many years. These effects are caused by tropane alkaloids of the plant [8, 9]. Major alkaloids of the plant are hyoscyamine and

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scopolamine [6, 7]. Alkaloid production has been proved to be hard due to restrictions such as the destruction of the plant habitats and geographical problems. Metabolic engineering is applicable in improving the alkaloid content or the supply of other sources of alkaloids. The isolation and characterization of the genes engaged in metabolic pathway is one of the primary steps in metabolic engineering [10].

In this study, we have employed tobacco-rattle-virus-based VIGS vectors (TRV) [11] for effective silencing of phytoene desaturase (*pds*) gene in *D. stramonium*. These have several advantages over other silencing vectors including a broad host range, uniform cell invasion, and relatively mild viral symptoms [12, 13].

D. stramonium seeds were collected from cultivated plants in the medicinal plants research station at Shahed University, Tehran, Iran, in September 2010. Total RNA was extracted from the leaves of *D. stramonium* seedlings. cDNA synthesis was performed as previously described by Vakili et al. [14]. A partial 344 bp fragment of *D. stramonium pds* (*Ds*pds) was obtained through RT-PCR using primers previously utilized by Enwu and Jonathan [15]. The primer sequences were (fw:5′TTATGAATTCATGCAGAACCTGTTTGG′3) and (rw:5′TTATGGATCCGTTAAGTGCCTTTGAC′3). The PCR product was cloned into pTRV2 vector (the TRV vectors purchased from Arabidopsis Biological Resource Center in Ohio, USA). The confirmed vectors were transformed into *Agrobacterium tumefaciens* strain GV3101 using electroporation [16]. The bacterial preparation and infiltration was performed according to the method previously described by Velasquez [17]. For leaf infiltration, IB (Infiltration Buffer)containing the bacteria with pTRV1 was mixed separately with IB containing the bacteria with pTRV2-*Dspds*, pTRV2-*Nbpds,* and empty-pTRV2 in a 1 to 1 ratio, respectively. Mock-treated plants were infiltrated with infiltration buffer without any bacteria. The mixtures were infiltrated into two leaves of 14-day-old *Datura* seedlings and 21-day-old *Nicotiana benthamiana* (as positive control) plants using a 1-ml needleless syringe. Infiltrated plants were maintained under 20 °C temperature overnight for plant recovery followed by maintaining under a temperature range of 23–25 °C for effective viral infection and spread.

The leaves and plants were photographed 4 weeks after infiltration. VIGS frequency and efficiency were estimated by the equation previously derived [4]. The pigments were extracted from the leaves using acetone 80 %. Absorbencies of the samples were read by UV spectrophotometer (Shimadzu UV visible-1601 PC) at 470, 645, and 663 nm, respectively. The chlorophyll and carotenoid contents were calculated by the following equations [18]:

$$\text{Chlorophyll a} = [12.7(D_{663}) - 2.6(D_{645})]$$

$$\text{Chlorophyll b} = [22.9(D_{645}) - 4.68(D_{663})]$$

Total RNA was extracted from the leaves 4 weeks after infiltration. The primers used for *pds* gene amplification were (FW:5′TTATGAATTCATGCAGAACCTGTTTGG′3) and (RW:5-TGGAGTGGCAAACACAAAAGCATCT-3) designed from external region of 344 bp cloned gene and the primers used for *actin* gene amplification (as a house-keeping gene) were (FW:5′-CCCACCACTGAGCACAATGTTCC-3′) and (RW:5′-GCAGGGATCCACGAGACCACC-3′). Total alkaloid was extracted using the method previously described [14].

The sequencing results of *D. stramonium pds* showed high nucleotide identity with *N. benthamiana pds* (92 %) and was submitted in gene bank (accession number: **JQ004869**). Initial signs of the silencing were observed in *D. stramonium* plants two weeks after agro-infiltration. Also the results suggest that Agrobacterium strains GV3101 can effectively deliver the vector to *D. Stramonium* cells.

As shown in Fig. 1a, b mock-treated plants did not show bleaching symptoms in any of the experiments while infection with empty-pTRV2 resulted in somewhat stunted plants and few crinkles in leaves. However, no signs of bleaching were observed in any of the plants.

The rate of silencing was measured by the analysis of the pigments contents. The chlorophylls a and b contents in plants infiltrated with pTRV2-*Dspds* were reduced significantly, which was more than that of plants injected with pTRV2-*Nbpds*. Similar results were also obtained in the case of carotenoid content. pTRV2-*Dspds*-treated plants showed a reduction of less than 76 % in total carotenoid compared to mock-treated plants (Fig. 1c–e).

The relative expression of *pds/actin* gene in silenced plants decreased significantly ($\sim$85 %) compared to mock plants. As shown in Fig. 1f, g, there were no significant differences between the expressions of *pds* in mock and empty-pTRV2-treated plants. Total alkaloid content in negative control plants infected just with empty vector increased 3.76 fold more than that of mock plants (Fig. 1h). The effectiveness and frequency of gene silencing in *D. stramonium* plants were 75 and 95 %, respectively.

In the past decade, there was remarkable interest to use medicinal plants which resulted in establishment of a relationship between studies in plant biochemistry/physiology and human health [19, 20]. VIGS is a powerful tool that has advantages such as simple methodology, rapid achievement of results, usefulness for analysis of lethal genes, needless of stable transformation, and the possibility of silencing multi-copied genes [1, 21]. The efficiency of TRV-based vectors was previously reported in various

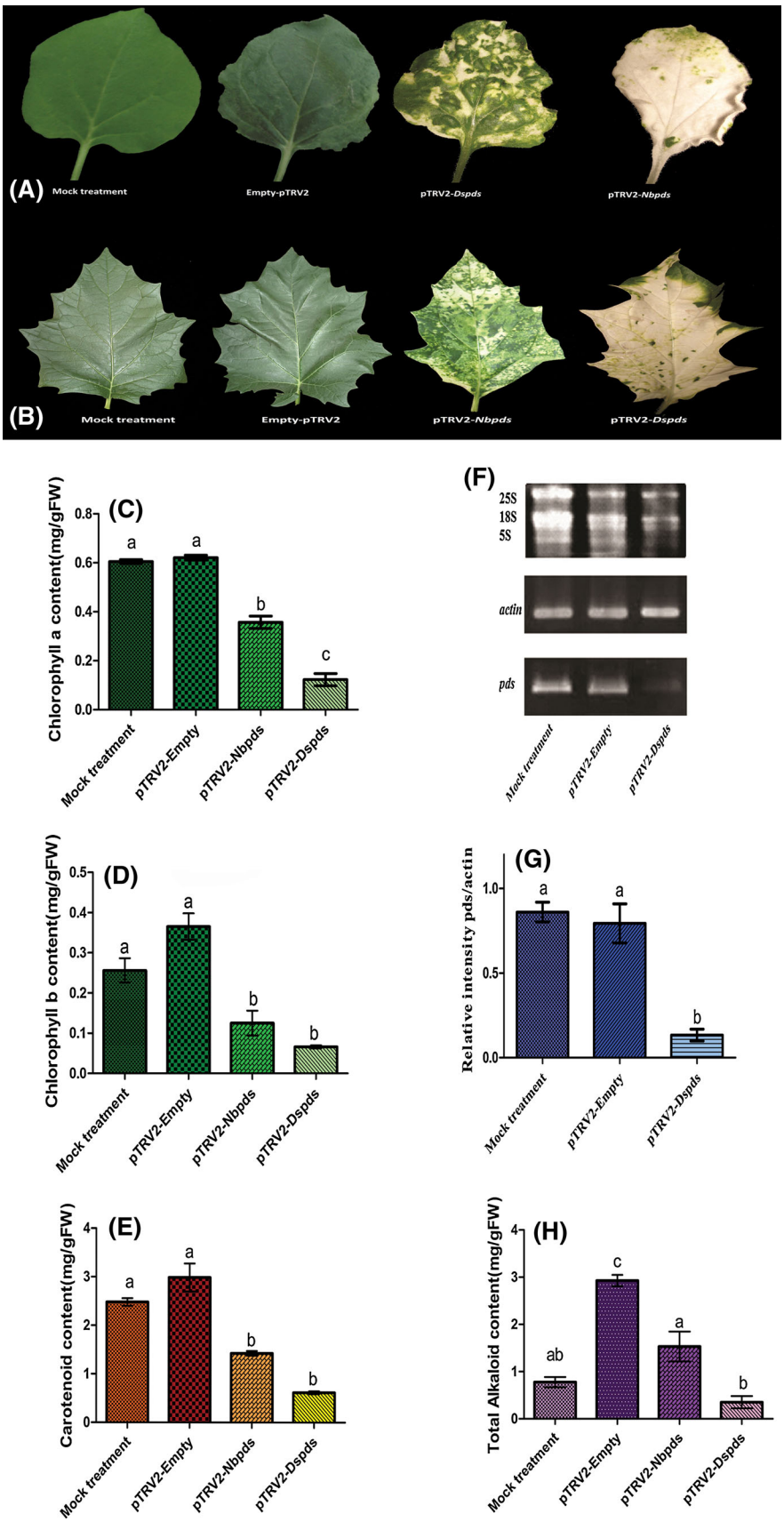

Fig. 1 Photobleached leaves of *N. benthamiana*
(**a**) Photobleached leaves of *D. stramonium,* (**b**) 4 week post-inoculation. Pigments contents in infected *D. stramonium* leaves: Chlorophyll a content (**c**) Chlorophyll b content (**d**) carotenoid content (**e**). Endogenous expression levels of *pds* gene: Semi-quantitative RT-PCR of *pds* expression: Relative expression intensity of endogenous *pds* transcripts in *D. stramonium* leaves (**f**, **g**). Total alkaloid contents in infected *D. stramonium* leaves (**h**). Mock treatment: inoculated with infiltration buffer, Empty-pTRV2: Negative control inoculated with *Agrobacterium* carrying empty-pTRV2 vector; pTRV2-*Nbpds*: Heterologous VIGS inoculation with *Agrobacterium* carrying pTRV2-*Nbpds*; pTRV2-*Dspds*: Homologous VIGS inoculated with *Agrobacterium* carrying pTRV2-*Dspds*; *Bars* represent mean ± SD (n = 3; $P < 0.05$)

solanaceous species. Some of these species are *Nicotiana benthamiana* [22, 23], *Hyoscyamus niger* [24], *Capsicum annuum* [25], *Solanum lycopersicum* [26], and many other plants from *Solanaceae* family [4]. According to our knowledge, there is no report about using VIGS for *D. stramonium* gene silencing. The present work is the first report of VIGS in this species, which can be used for characterization of interested genes in this plant along with the other species of the genus.

In our study, the level of mRNA reduced because when VIGS is applied, the target gene mRNA is widely degraded in plants [2]. Phytoene desaturase (*pds*) gene encodes an important rate-limiting enzyme in the carotenoid biosynthetic pathway. Therefore, the silencing of *pds* gene and reduction of its expression level can greatly reduce the carotenoid content of the plants [27, 28]. This can lead to obvious photobleaching phenotype because of the oxidation of chlorophylls [29]. Here, reduction in chlorophyll a and b contents was observed in plants silenced by TRV containing a partial fragment of *pds*. Higher degradation of chlorophylls shows increased silencing efficiency [4]. Reduction in pigment content that observed in homologous VIGS caused by pTRV2-*Dspds* was bigger than that of heterologous VIGS caused by pTRV2-*Ntpds*. The results were in accordance with other reports stating that VIGS efficiencies correlated with homology between the target gene and the inserted sequence as a trigger [4, 30, 31].

TRV vectors show mild symptoms compared to other viral silencing vectors, as shown in our study which mock-treated plants had slight signs of the viral disease. This is common in VIGS experiments. However, the symptoms did not complicate the results because the mild signs of viral attach are negligible against the severe symptoms of gene silencing. On the other hand, viral signs disappeared in a few weeks due to the plants' defensive activity [12, 21, 30, 32]. Nevertheless, it is also possible to reduce replication efficiency of the virus by insertion of a non-plant gene such as GUS or GFP in TRV2 which can reduce severity of the symptoms. The general negative controls often used for VIGS are empty TRV2 vector, infiltration buffer and TRV-GFP construct [15, 21, 33, 34].

The total alkaloid content was affected by the silencing of *pds* gene. It has been observed that alkaloids contents are increased in response to biotic and/or abiotic stresses [35]. The high level of alkaloid content in empty-pTRV2-treated plants might be a response to the *Agrobacterium* inoculation [10] or virus infection as biotic stresses. The reduced alkaloid content in highly photobleached leaves in pTRV2-*Ntpds* and pTRV2-*Dspds* treatments may be because of the reduction of photosynthesis and overall changes in the normal physiological status of the plants due to *pds* gene silencing [36]. This is followed by a lower carbohydrate content which results in other nutrients becoming different [29]. The alkaloid biosynthesis pathway was previously investigated by VIGS in some plants such as *Hyoscyamus niger* and *Chatarantus roseus* [10, 24].

Finally, this report showed that *D. stramonium* is compatible with VIGS by means of the pTRV2 vector system. We suggest that it can be used for *D. stramonium* as a primary step in metabolic engineering studies. The efficient and repeatable *pds* gene silencing proposes that there is an optimal condition for silencing other target genes [13].

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