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Evaluation of virus-induced gene silencing methods for forage legumes including alfalfa, sainfoin and fenugreek

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ABSTRACT

Virus-induced gene silencing (VIGS) is a rapid reverse genetics tool that has been developed in a wide variety of plant species for assessing gene function. However, while VIGS has been utilized successfully in the diploid model leguminous species, *Medicago truncatula* (barrel medic), such a platform has yet to be established in forage legume crop species. Therefore, we evaluated the effectiveness of this method in forage legumes using a previously developed PEBV (pea early browning virus) system whereby a fragment of the pea (*Pisum sativum* L.) PHYTOENE DESATURASE (*PDS*) gene was transferred into a range of alfalfa (*Medicago sativa* L.), sainfoin (*Onobrychis viciifolia* Scop.) and fenugreek (*Trigonella foenum-graecum* L.) cultivars using leaf infiltration and apical meristem injection. Barrel medic was used as a positive control. Gene silencing was observed after 10-15 days through the presence of a leaf bleaching phenotype, and was confirmed using quantitative real-time RT-PCR. Silencing of *PDS* was achieved in a selection of cultivars in all species assessed, with the highest silencing efficiency apparent in fenugreek. The introduction of a highly homologous gene fragment from a heterologous plant species to target endogenous genes for transient VIGS-based silencing in a range of species of interest represents a potentially useful strategy for the rapid functional characterization of candidate genes in forages.

Key words: barrel medic, alfalfa, sainfoin, fenugreek, VIGS, PHYTOENE DESATURASE

INTRODUCTION

Perennial forage legumes are widely used in the livestock industry as cut fodder or grazed pasture, in large part due to their high protein content and ability to fix nitrogen, which enriches
the soil and thus reduce the need for nitrogenous fertilizer. While alfalfa (*Medicago sativa* L.) is
the most extensively produced and well-studied of the perennial leguminous forage crops due to its high palatability and yield, sainfoin (*Onobrychis viciifolia* Scop.) is gaining popularity in
Western Canada because of the fact that it contains condensed tannins, which render it bloat-free
(Koivisto and Lane 2001; Acharya 2015). As is the case with sainfoin, fenugreek (*Trigonella
foenum-graecum* L.) is another bloat-free legume that can be used as a forage, although it is an
annual rather than a perennial crop (Acharya et al. 2008). Although genomic sequence
information is available for alfalfa at the diploid level (https://www.alfalfatoolbox.org/), sainfoin
and fenugreek genomes have not yet been sequenced, and genomics data in these forage crops
are limited to a small number of genetic markers and transcriptomics studies (Kempf et al., 2016;

Virus-induced gene silencing (VIGS) involves the induction of post-transcriptional gene
silencing (PTGS) in plants using a viral vector that generates double stranded RNA, leading to
degradation of the target mRNA (Baulcombe 1999). This method has been used effectively as a
relatively rapid transient reverse genetics tool to facilitate the functional analysis of genes
(Baulcombe 2004; Benedito et al. 2004) in many plant species, including legumes such as
soybean (*Glycine max*), pea (*Pisum sativum*) and cowpea (*Vigna unguiculata*) (Constantin et al.
2004; Igarashi et al. 2009; Yamagishi and Yoshikawa 2011). It is typically carried out through
the introduction of *Agrobacterium tumefaciens* carrying a viral vector (Liu et al. 2003). Various
methods have been employed for the inoculation of plants with *Agrobacterium* bearing a VIGS
vector in order to achieve optimum levels of silencing, including leaf infiltration, spraying,
vacuum infiltration, leaf/apical meristem injection, and root dipping (Liu et al. 2002; (Hileman et
In the majority of studies in which VIGS protocols have been developed for particular plant species, a fragment of the *PHYTOENE DESATURASE (PDS)* gene is included in the VIGS vector in order to target the corresponding homologous gene within the plant for silencing. This gene encodes an essential enzyme involved in carotenoid biosynthesis (Koschmieder et al. 2017) and thus elicits a bleached phenotype when down-regulated (Ruiz et al. 1998), allowing the simple visual identification of silencing. Intriguingly, it has been found previously that heterologous *PDS* sequences can be utilized to silence their corresponding ortholog in distantly related plant species, and that the extent of homology between the heterologous gene fragment and the endogenous target *PDS* gene does not necessarily correlate with VIGS silencing efficiency (Senthil-Kumar et al. 2007; Senthil Kumar and Mysore 2011). This ability to use heterologous sequences for targeting facilitates the use of VIGS technology in plant species for which genomic data is not readily available, making it an attractive option for functional genetic screens.

Although VIGS has become a powerful functional genomics tool used in many transient gene silencing studies, its application in leguminous forage crop species is lacking. This method, however, has been developed for the diploid model legume *Medicago truncatula* (barrel medic), which is a close relative of alfalfa, using VIGS vectors based on sunnhemp mosaic virus (SHMV; Várallyay et al. 2010) or pea early browning virus (PEBV; Constantin et al. 2004; Grønlund et al. 2008; Serwatowska et al. 2018). The infectivity of particular viral vectors has been found to be very genotype-specific, and in *M. truncatula* for instance, only 4 of 21 accessions tested using *Agrobacterium*-mediated leaf infiltration with the PEBV viral vector and a full-length β-glucuronidase (*GUS*) gene were infected systemically following infiltration (Grønlund et al. 2008). Similarly, the only *M. sativa* cultivar (Creno) assessed for PEBV
infectivity in this study did not display systemic infection using this viral vector (Grønlund et al. 2008), which indicates that a broader range of genotypes will need to be screened in order to develop a successful VIGS protocol for this species.

In the current study, a VIGS approach using a PEBV vector system developed previously (Constantin et al. 2004), which includes a fragment of the \textit{P. sativum PDS} gene, was applied to a selection of alfalfa, sainfoin and fenugreek genotypes, along with barrel medic as a control, using distinct \textit{Agrobacterium} infiltration/injection techniques, with the aim of developing a rapid transient reverse genetics tool to facilitate the functional analysis of genes in particular cultivars of a range of leguminous forage crop species. Silencing of endogenous \textit{PDS} genes, as evidenced by leaf bleaching and reductions in \textit{PDS} transcript levels, was successfully achieved in all four species, with the range of silencing efficiencies (the proportion of treated plants displaying a silenced phenotype) observed being species- and cultivar-specific. The results of this study provide a platform for the rapid functional characterization of candidate genes in three agronomically important leguminous forage crop species in the future.

**MATERIALS AND METHODS**

**Plant materials**

Seeds of different genotypes or cultivars of \textit{M. truncatula} were supplied by the South Australian Research and Development Institute (R108-1-C3 and Jemalong A17) and Plant Gene Resources Canada (PI307451, PI3077453 and PI307452). All cultivars of alfalfa (\textit{M. sativa}; AC Blue J, Grimm, Rambler, AC Meadowview, AC Bridgeview and Beaver), sainfoin (\textit{O. viciifolia}; AAC Mountainview, Melrose, AAC Glenview and Nova) and fenugreek (\textit{T. foenum-graecum}; Amber,
Tristar and L3312) were supplied by Agriculture and Agri-Food Canada’s Lethbridge Research and Development Centre.

Seeds were sown individually in foam trays until roots were visible and then the seedlings were transplanted to individual pots containing soil-free Cornell mix. Plants were grown in a greenhouse with 16/8 hr day/night photoperiod and a light intensity of approximately 150 µE/m²/s. Each experiment was repeated twice.

**Amplification of PDS coding regions**

Plant total RNA was extracted using the Sigma Spectrum Plant Total RNA kit (Sigma-Aldrich, Oakville) and reverse transcribed with the Superscript VILO cDNA synthesis kit (Thermo Fisher Scientific, Waltham USA). Partial-length (approximately 316 bp) coding regions of PDS homologs from alfalfa (Grimm), sainfoin (AAC Mountainview), fenugreek (Amber) and barrel medic (PI3077453) were amplified from 1 µl undiluted cDNA using Platinum SuperFi Green PCR Master Mix (Thermo Fisher Scientific) along with primers MTPDSf1 (5’- ATG GCT CTA TAT GGC TCT ATA TC – 3’) and MTPDSr1 (5’- GTT AAA CAG TAC TTG CTT GAG CTA – 3’) in a final volume of 50 µl. Since this region of the PDS coding sequence tends to be fairly well-conserved among leguminous species, these same primers were utilized for amplification from all four species in this study. Primers Tf1 (5’ – GCA ATG TTC CGT GGT AAG ATG – 3’) and Tr1 (5’ – GTA CCA ATG CAA GAA AGC TTG – 3’), which amplify a 252 bp region of the constitutively expressed tubulin gene, were utilized as a positive control in each case. Thermocycling parameters were as follows: Initial denaturation at 95°C for 2 minutes, 30 cycles of 95°C for 15s and 60°C for 60s and 5 minutes at 72°C extension. The resulting PDS-specific
RT-PCR products from each species were cloned into pGEM-T easy (Promega, Madison, USA) and sequenced.

**Bioinformatics analysis**

Nucleotide coding region and deduced amino acid sequences of the alfalfa, sainfoin, fenugreek and barrel medic *PDS* genes were aligned for homology determination using the Clustal Omega Multiple Alignment Tool (https://www.ebi.ac.uk/Tools/msa/clustalo/). For phylogenetic analysis, deduced amino acid sequences of PDS proteins from legumes representative of different subgroups within the family were selected using data available at GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and the Alfalfa Gene Index and Expression Atlas Database (https://plantgrn.noble.org/AGED/index.jsp). Partial carboxy terminal regions of the deduced amino acid sequences were aligned and a dendrogram was generated using the neighbor-joining algorithm with 1,000 bootstrap replications (http://evolution.gs.washington.edu/phylip.html).

**Virus-induced gene silencing**

The PEBV vector system described by Constantin et al. (2004) was used for the application of VIGS in various cultivars of barrel medic, alfalfa, sainfoin and fenugreek. In brief, PCAPE1 (a pCambia1300-derived plasmid containing the RNA1 expression cassette), PCAPE2 (a pCambia1300-derived plasmid containing the RNA2 expression cassette) and PCAPE-PDS (a pCambia1300-derived plasmid containing the RNA2-PDS expression cassette including the pea *PDS* fragment) vectors, respectively, were introduced into *A. tumefaciens* strain GV3101 via electroporation, and 30 mL overnight liquid cultures were used to inoculate 500 mL of Luria-
Bertani medium containing 10 mM 2-(N-morpholino) ethanesulfonic acid (MES), 20 μM acetosyringone, and 50 μg mL⁻¹ kanamycin. Cultures were grown overnight at 28°C with shaking at 200 rpm, and were then centrifuged at 3,000g for 15 min. The resulting pellets were re-suspended in infiltration buffer (10 mM MES, 200 μM acetosyringone and 10 mM MgCl₂) to an OD₆₀₀ of 2.5. Agrobacterium containing PCAPE1 was then mixed with that containing PCAPE2 (empty vector control) or PCAPE-PDS (containing a pea PDS fragment) in a 1:1 ratio (v/v) as described by Constantin et al. (2004).

Agrobacterium mixtures were then incubated at room temperature for 1 hr, after which time leaves from 2 to 3-week old alfalfa, sainfoin, fenugreek and barrel medic seedlings were infiltrated using a 1-cc needleless syringe. In every case, the abaxial surface of all true leaves on each plant was used for infiltration. Except in fenugreek, Agrobacterium mixtures were also injected into the apical meristem of each plant of barrel medic, alfalfa and sainfoin using a 1-cc syringe with needle. Ten to 20 plants of each cultivar were infiltrated/injected with buffer alone, empty vector and PDS vectors, respectively, and each experiment was replicated twice. The buffer and empty vector infiltrations were included as negative controls to ensure that phenotypic effects resulted from inclusion of the pea PDS fragment. Infiltrated/injected plants were grown in a greenhouse free of any other viral infection and were examined visually for bleaching phenotypes until they reached the flowering stage. Four to 5 weeks following infiltration/injection, plant tissues were harvested, immediately frozen in liquid nitrogen and stored at -80°C for subsequent RT-PCR and qRT-PCR analyses.

Plants infiltrated with empty vector and infected with PDS were subjected to PCR using Platinum SuperFi Green PCR Master Mix (Thermo Fisher Scientific) with USf (5’ – TGT ATT AAA GAC ATG GAG AGT GGA GTG – 3’) and USr (5’ – CTT AAA GAA CGA CCA CCA CAA
GTA CAG – 3’), which amplify 490 bp of the vector sequence upstream of the multiple cloning site. Conditions for thermocycling were initial denaturation at 95°C for 2 minutes, 30 cycles of 95°C for 15s and 60°C for 60s and the final extension at 72°C for 5 minutes. Following amplification, products were cloned into pGEM-T easy(Promega, Madison, USA) and sequenced to confirm their identity.

Quantitative real-time RT-PCR analysis of PDS gene expression

Quantitative real-time RT-PCR (qRT-PCR) was performed using a QuantStudio 6 Flex real-time PCR system (Thermo Fisher Scientific) to confirm that the bleached phenotype was due to silencing of the PDS gene in barrel medic (PI3077453), alfalfa (Grimm), sainfoin (AAC Mountainview) and fenugreek (Amber) infiltrated with A. tumefaciens harboring pCAPE1 and the pCAPE2-PDS vector construct. Plants infiltrated/injected with A. tumefaciens bearing pCAPE1 and empty pCAPE2 vector (EV), as well as infiltration buffer (MgCl₂) alone, were included as controls. Total RNA was extracted from leaves displaying a bleached phenotype from infiltrated plants, as well as unbleached leaves from controls. RNA extraction and first-strand cDNA synthesis were carried out as described in previous sections. Reactions were performed in 96-well plates in a final volume of 10 μl, which consisted of Power UP SYBR PCR mix (ThermoFisher Scientific), 0.5 μl of undiluted cDNA, and 5μM primers QPDSf1 (5’ – ACC TTT CGT GCT TCT CCT CGT CC – 3’) and QPDSr1 (5’ - CTT TCC ACC TAG AAC ATC TCT T – 3’). These primers were designed based on the legume PDS coding sequence alignment to amplify a 147 bp conserved region of alfalfa, sainfoin and fenugreek PDS cDNA. Primers MTEFf1 (5’ - CTG AAG TGA AGT CTG TGG AGA TGC A – 3’) and MTEFr1 (5’ - GTA AAT TCA GCA GCC TCC ATA GCT – 3’) were used to amplify 136 bp of the constitutively
expressed EF-1α coding sequence as an internal control. Thermal parameters were as follows: 2 min at 50°C and 2 min at 95°C, followed by 40 cycles of 95°C for 15s and 60°C for 60s. Three biological replicates and three technical replicates were used in each case, and the threshold value (Ct) for each gene was normalized against the Ct of the constitutive control. Mean relative gene transcript levels were quantified by comparisons between empty vector control plants and those in which PDS had been targeted for VIGS silencing using the 2-ΔΔct method (Livak and Schmittgen 2001).

Statistical analyses
Experimental data was analyzed using PROC GLM in SAS (SAS Institute, Cary, NC, USA). The least significant difference (P < 0.05) test was used to identify significant differences among treatment means.

RESULTS
Isolation of PHOTENOEN DESATURASE coding sequences from alfalfa, sainfoin, fenugreek and barrel medic
Partial-length PDS cDNA sequences for barrel medic, alfalfa, sainfoin and fenugreek were obtained by RT-PCR amplification (Supplementary Figure S1). Sequences from alfalfa, sainfoin and fenugreek have been deposited into GenBank (GenBank accession numbers MH078192, MH078190 and MH484048, respectively). Based on nucleotide alignments of the resulting sequences with the coding region of field pea PDS (AJ621573), 94%, 93%, 95% and 95% nucleotide identity was observed for alfalfa, sainfoin, fenugreek and barrel medic, respectively (Supplementary Figure S2). Similarly, alignment of deduced amino acid sequences of alfalfa,
sainfoin, fenugreek and barrel medic PDS with that of pea demonstrated 96%, 95%, 97% and 96% identity, respectively at the amino acid level.

Phylogenetic analysis of putative phytoene desaturase proteins from legumes

A dendrogram was constructed using deduced amino acid sequences of 15 putative PDS proteins from a selection of legume species, including alfalfa, sainfoin, fenugreek and barrel medic, as well as a PDS protein from *Vitis vinifera* as an outgroup. Two distinct clades were observed, with alfalfa and fenugreek PDS sequences falling into clade 1, along with those of *Vigna* spp., *Lupinus angustifolius* and *Glycine max*, while clade 2 comprised *P. sativum*, *Trifolium* spp., and *Cicer arietinum* PDS sequences. The PDS proteins of *Phaseolus vulgaris*, *Arachis* spp. and sainfoin did not fall into either of these clades, and appeared to be more diverged in their amino acid sequences (Figure 1).

Phenotypic evidence of VIGS in alfalfa, sainfoin, fenugreek and barrel medic

Leaf bleaching was observed in at least a proportion of cultivars from all legumes tested 15-20 days following infiltration with pCAPE1 in conjunction with pCAPE2 bearing a fragment of the pea *PDS* gene (Figure 2). The efficiency of VIGS was determined based on the percentage of treated plants displaying a bleached phenotype in each case. The highest VIGS efficiencies based on this phenotype (70-95% of treated plants) were seen in the three cultivars of fenugreek (Amber, Tristar and L3312), with Amber showing the maximum efficiency (90-95%). Alfalfa (Grimm) and sainfoin (AAC Mountainview and Nova) exhibited 15%-20% VIGS efficiencies, with AAC Mountainview displaying the highest efficiency in sainfoin. All other cultivars of alfalfa and sainfoin tested did not display any evidence of a leaf bleaching phenotype. Similarly,
while a VIGS efficiency of approximately 30%-40% was observed in barrel medic cultivars PI3077453 and PI307452, none of the other tested cultivars showed a leaf bleaching phenotype (Table 1). Alfalfa Grimm cultivar displayed this bleached phenotype for only 5-7 weeks following initial assessments, while the same phenotype was observed in sainfoin plants for 4-6 months following initial evaluation. The two annual species examined (barrel medic and fenugreek) exhibited the bleached phenotype for the remainder of their life span. No plants infiltrated/injected with pCAPE1 along with empty vector control pCAPE2, or buffer alone, displayed any bleaching of leaf tissues or other morphological alterations corroborated with previous evidence (Wijekoon and Facchini, 2012). Plants infiltrated with experimental VIGS vectors that exhibited a bleached phenotype also showed a slight reduction in growth compared to those infiltrated with buffer/empty vector (Figure 2F).

**Expression of PHYTOENE DESATURASE was suppressed in plants with a bleached phenotype**

In order to confirm that the bleached phenotype observed in plants infiltrated/injected with *Agrobacterium* containing pCAPE1 and pCAPE2-PDS (Constantin et al., 2004) was due to a reduction in endogenous *PDS* transcript, qRT-PCR was carried out using cDNA derived from total RNA isolated from barrel medic (PI3077453), alfalfa (Grimm), sainfoin (AAC Mountainview) and fenugreek (Amber). Bleached leaves from plants infiltrated/injected with *A. tumefaciens* harboring pCAPE1 and pCAPE2-PDS constructs were compared to unbleached leaves from plants infiltrated/injected with *A. tumefaciens* bearing pCAPE1 and empty pCAPE2, as well as infiltration buffer alone. The pCAPE-USER (viral vector) fragments were isolated from barrel medic (*M. truncatula*), alfalfa (*M. sativa*), sainfoin (*O. viciifolia*) and fenugreek (*T.*
foenum-graecum) and confirmed the presence of the vector sequence in empty vector controls and PDS-silenced plants (Figure 3A). PDS transcript levels were normalized against the amount of constitutively expressed EF1a transcript. Mean transcript levels of PDS were substantially reduced (between 10-fold and 20-fold) in bleached leaves from plants infiltrated with A. tumefaciens harboring the pCAPE2-PDS construct compared with unbleached leaves from plants infiltrated with either A. tumefaciens bearing the empty pCAPE2 vector or infiltration buffer alone (Figure 3B).

DISCUSSION

Virus-induced gene silencing has been used as a rapid, transient tool for gene functional characterization in many plant species to date (Robertson, 2004). Bean pod mottle virus-, white clover mosaic virus-, cucumber mosaic virus-, apple latent spherical virus-, SHMV- and PEBV-based vector systems have been used in the application of VIGS in a broad range of legumes such as soybean, common bean (P. vulgaris), cowpea (Vigna unguiculata), barrel medic and Lathyrus odorata (Grønlund et al. 2008; Igarashi et al. 2009; Várallyay et al. 2010; Zhang et al. 2010; Liu et al. 2010; Yamagishi and Yoshikawa 2011; Ido et al. 2012; Pflieger et al. 2013). The efficiency of silencing by VIGS, however, has not been previously evaluated in agronomically relevant forage legumes. Therefore, in the current study, we aimed to assess the effectiveness of VIGS in various cultivars of alfalfa, sainfoin and fenugreek through the suppression of PDS gene expression using a highly homologous field pea PDS gene fragment within a PEBV-based VIGS vector system developed previously for use in legumes (Figure 2). Barrel medic was used as positive control as PEBV-based VIGS has been developed previously in this species (Grønlund et al. 2008).
The main method of silencing genes via VIGS is through the introduction of Agrobacterium harboring the viral vector(s) of choice, and thus optimum introduction of bacterial cells is necessary for effective gene silencing. The degree of Agrobacterium infection in the leaf can be affected by leaf wetness, micro-morphology and surface chemistry (Kumar et al., 2004). Since the infiltration of fenugreek leaves occurred with ease due to their soft and thick nature, leaf infiltration of Agrobacterium cultures may have been effective for achieving VIGS-mediated silencing in this species. In contrast, leaf infiltration alone was not sufficient for the bacterial inoculation of alfalfa, sainfoin or barrel medic due to their leaf characteristics. Instead, the efficient delivery of the VIGS plasmid construct into these species therefore necessitated the use of a combination of leaf infiltration and apical meristem injection.

Endogenous plant genes are involved in viral resistance (Kang et al. 2005) and the viral vector used for VIGS should therefore be appropriate for the host to efficiently trigger the silencing of genes. In line with this, efficient VIGS-mediated gene silencing would not be achieved if a viral vector derived from a virus to which the plant was resistant were used. To the best of our knowledge, no previous research has been carried out with the aim of assessing VIGS efficiency in virus-resistant legumes. In the current study, none of the cultivars utilized are known to be resistant to any particular virus. However, although successful VIGS-mediated silencing was apparent in all three fenugreek cultivars tested, only a selection of the barrel medic, alfalfa and sainfoin cultivars assessed displayed silencing of the PDS gene Table 1; Figures 2 and 3). In addition, differences in VIGS efficiency (proportion of plants that displayed a bleached phenotype following infiltration/injection of Agrobacterium bearing the PDS-containing VIGS vector) were noted among the different species tested (15% for alfalfa, 20% for sainfoin, 70-95% for fenugreek and 30-40% for barrel medic). Similar differences were also
observed among cultivars, with the three fenugreek cultivars assessed exhibiting 70%, 80% and 95% efficiencies (L1312, Tristar and Amber, respectively). This suggests that species- and cultivar-specific effects play a role in silencing efficiencies and that the PEBV vector system used in this study may not be suitable for all genotypes. Previous studies with other plant species have yielded similar results (e.g., Senthil-Kumar and Mysoor 2011; Grønlund et al. 2008), although the particular differences between genotypes that are responsible for such specificity have yet to be elucidated.

The bleached phenotype observed in all species assessed in this study was associated with the presence of the pea PDS sequence within the VIGS vector used, since neither plants inoculated with buffer alone or empty VIGS vector exhibited such a phenotype in any instance. Furthermore, while the severity of visual phenotypes associated with VIGS-mediated silencing of PDS also varied among and within alfalfa, sainfoin, fenugreek and barrel medic cultivars (Figure 2), this was likely due to the fact that PDS transcripts were down-regulated to differing degrees, with the greatest reduction in transcript levels evidenced in fenugreek (Figure 3), which could have an effect on the phenotype.

Orthologs of PDS from distantly related plant species have previously been used for heterologous gene silencing in Nicotiana benthamiana (Senthil-Kumar et al. 2007; Senthil-Kumar and Mysore 2011). However, as of yet, there is no evidence of a correlation between gene silencing efficiency and percent homology of the heterologous and endogenous gene sequences (e.g., Senthil-Kumar and Mysore 2011). This also appeared to be the case in the present study given that although the PDS fragment from sainfoin appeared to be somewhat removed in a phylogenetic context from the rest (Figure 1), nucleotide identities between the PDS fragments from the four species assessed here and that from pea (included in the VIGS vector) were all
between 93%-95% (Supplementary Figure S2). Therefore, it appears that other species- and cultivar-specific factors that remain to be determined instead contribute to the differences in VIGS efficiency observed among alfalfa, sainfoin, fenugreek and barrel medic cultivars. However, the sequence analyses carried out in this study were based on partial PDS coding sequences, and therefore further clarification of homology will need to be accomplished using full length sequences.

In conclusion, a highly homologous PDS gene fragment from field pea was effectively used to silence PDS genes in two perennial forage legumes (alfalfa and sainfoin) and an annual forage legume (fenugreek). The highest efficiency of silencing was achieved in fenugreek, where plasmid delivery into the host plant was achieved with more ease than the other species assessed. The VIGS-mediated introduction of a highly homologous gene fragment of the endogenous target gene represents a useful loss-of-function approach for the future characterization of potential candidate genes in forage legumes, which could increase the pace of breeding and thus facilitate the improvement of various traits, including yield and nutritional attributes in the future.

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DATA ACCESS
The raw data of partial DNA and deduced amino acid sequences of PDS in *M. sativa* (MH078192), *O. viciifolia* (MH078190) and *T. foenum-graecum* (MH484048), and beta-tubulin in *M. sativa* (MH078191), *O. viciifolia* (MH078189) and *T. foenum-graecum* (MH484049) accessions have been deposited in GenBank.

REFERENCES


**Figure 1.** Phylogenetic analysis of predicted phytoene desaturase (PDS) amino acid sequences from legumes rooted with *Vitis vinifera* (XM010656055) PDS as an outgroup. The tree was constructed based on neighbor-joining calculations. The sequences were grouped into two major clades. Alfalfa (*M. sativa*; MH078192), sainfoin (*O. viciifolia*; MH078190) and fenugreek (*T. foenum-graecum*; MH484048) PDS sequences are circled in blue and were isolated in this study. *Phaseolus vulgaris*, *Arachis spp* and *O. viciifolia* Scop. do not belong to either of the clades.

**Figure 2.** Phenotypic effects of silencing *PHYTOENE DESATURASE (PDS)* genes in barrel medic (*Medicago truncatula*), alfalfa (*M. sativa*), sainfoin (*O. viciifolia*) and fenugreek (*T. foenum-graecum*). Plants were inoculated with *Agrobacterium* harboring PCAPE1 and PCAPE2-PDS vectors. After 10-15 days, barrel medic PI3077453 (A), alfalfa Grimm (B), sainfoin AAC Mountainview (C) and fenugreek Amber (D) infiltrated with PCAPE2-PDS showed a bleaching phenotype in the leaves. Different genotypes of fenugreek (Amber, Tristar and L3312) showing the bleached phenotype are shown (E). Comparisons of the phenotypes of fenugreek (F) infiltrated with buffer control (left), empty vector control (right) and the PDS construct (middle) are displayed.

**Figure 3.** Molecular assessment of plants displaying a bleached phenotype, as well as unbleached negative controls. A. Assessment of plant cDNAs for virus-induced gene silencing by agarose gel electrophoresis of pCAPE-USER amplicons in PDS-infected plants showing photo bleached phenotype. The pCAPE-USER (viral vector) fragment isolated from barrel medic (*M. truncatula*), alfalfa (*M. sativa*), sainfoin (*O. viciifolia*) and (fenugreek) *T. foenum-graecum* (lanes 2, 3, 4 and 5, respectively) is 490 bp. Lane 1 represents a 1kb DNA ladder (Thermo Scientific). B. Quantitative real-time RT-PCR using conserved *PDS* primers from *M. truncatula* and cDNA derived from total RNA isolated from bleached leaves of barrel medic (PI3077453), alfalfa (Grimm), sainfoin (AAC Mountainview) and fenugreek (Amber) infiltrated/injected with *A. tumefaciens* -harboring pCAPE1 and the pCAPE-PDS vector. Unbleached leaves from plants infiltrated/injected with *Agrobacterium* harboring empty pCAPE2 vector (EV) or infiltration buffer (MgCl2) alone were used as controls. Each bar represents the mean ± standard deviation of three technical replicates on each of three biological replicates from individual plants.
exhibiting the bleaching phenotype. Mean values of the empty vector in each is shown as 100%. Hatch marks at the y axis indicate 100% and 0%, respectively.
Table 1. Efficacy of PDS silencing in different forage legumes

<table>
<thead>
<tr>
<th>Forage legume</th>
<th>Population designations and cultivars</th>
<th>Number of plants inoculated</th>
<th>Number of plants showing bleaching phenotype</th>
<th>PDS silencing efficiency (%)</th>
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<tr>
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<td>5</td>
<td>30</td>
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<td>15</td>
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<td>L1312</td>
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<td>14</td>
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Data presented are means of two observations
Figures

Figure 1
Figure 3