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A Universal Primer for Isolation of Fragments of a Gene Encoding Phytoene Desaturase for Use in Virus-Induced Gene Silencing (VIGS) Studies

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Abstract

We have been using Virus-Induced Gene Silencing (VIGS) to test the function of genes that are candidates for involvement in floral senescence. Although VIGS is a powerful tool for assaying the effects of gene silencing in plants, relatively few taxa have been studied using this approach, and most that have are in the *Solanaceae*. We typically use silencing of phytoene desaturase (PDS) in preliminary tests of the feasibility of using VIGS. Silencing this gene, whose product is involved in carotene biosynthesis, results in a characteristic photobleaching phenotype in the leaves. We have found that efficient silencing requires the use of fragments that are more than 90% homologous to the target gene. To simplify testing the effectiveness of VIGS in a range of species, we designed a set of universal primers to a region of the PDS gene that is highly conserved among species, and that therefore allows an investigator to isolate a fragment of the homologous PDS gene from the species of interest. We report the sequences of these primers and the results of VIGS experiments in horticultural species from the *Asteraceae*, *Leguminosae*, *Balsaminaceae* and *Solanaceae*.

INTRODUCTION

Virus-induced gene silencing (VIGS) is a method of transiently interrupting gene function through RNA interference. The VIGS process is a natural plant defense mechanism and is initiated experimentally by *Agrobacterium*-mediated introduction of viral genomes containing a fragment of a targeted plant gene. When the virus replicates and spreads in the infected plants, the plant cells recognize the foreign double-stranded chimeric RNAs synthesized during viral replication and cut them into small oligonucleotides (siRNA). The siRNA molecules that serve as guides for an RNA-induced silencing complex (RISC) that recognizes homologous gene transcripts (Baulcombe, 1999; Chicas and Macino, 2001) targeting them for hydrolysis and thereby reduces expression of the target gene.

VIGS provides a rapid method for loss of gene function assay, avoiding the need for time-consuming transformation and regeneration processes. In addition, VIGS provides the opportunity to overcome functional redundancy by suppressing all or most members of a gene family (Burch-Smith et al., 2004). Vectors based on tobacco rattle virus (TRV) with a wide host range have been shown to function effectively in *Nicotiana benthamiana* (Liu et al., 2002a), tomato (Liu et al., 2002b), petunia (Chen et al., 2004), poppy (Wege et al., 2007) and *Aquilegia* (Gould and Kramer, 2007) for phytoene desaturase (*PDS*) silencing. Infected plants show characteristic photo-bleaching symptoms resulting from the decreased production of photoprotective carotenoid proteins and the subsequent breakdown of chlorophyll pigments. This phenotypic marker can be a potential reporter for examining the effect of silencing other target genes. In these previous studies, researchers reported that a highly homologous gene fragment was required to obtain high efficiency silencing. However, the sequence of PDS is not known for many species. In this paper, we describe a universal primer for isolation of *PDS* fragment from different plant species and report its successful use in a range of taxa.

MATERIALS AND METHODS

Plant Material

Seedlings of a range of species including petunia (*Petunia* × *hybrida*) 'Primetime Blue'; tomato (*Lycopersicon esculentum*) 'New Yorker'; Yesterday, today and tomorrow (*Brunfelsia glandiflora*); daisy 'Alaska'; gerbera (*Gerbera* × *hybrida*); chrysanthemum (*Dendranthema grandiflorum*) 'Veracruz'; lettuce (*Lactuca sativa*) 'Red Sails'; impatiens (*Impatiens wallerana*) 'Orange Bling'; Phalaenopsis orchid (*Phalaenopsis amabilis*); rose (*Rosa* × *hybrida*) 'Magnum'; four o'clock (*Mirabilis jalapa*); and bean (*Phaseolus vulgaris*) 'Kentucky Blue' and 'Golden Wax' were grown under 16h light/8h dark cycles at approximately 20-25°C.

Plasmid Construct

PDS constructs: A 520 bp fragment of the *PDS* gene was amplified from leaf cDNA of each species using primers 5'-GGTGGAAAG (g/a) T (a/g/t) GCTGC (a/t/c) TGG-3' and 5'-GCCAT (t/c) TT (g/a/t) GAACCATG (t/c) TT (t/c) TCCTG-3'. The resulting product was cloned into pGEM-T easy (Promega) for amplification, sequencing and cloning. The *PDS* cDNA fragment was excised using *Eco*RI digestion from the plasmid, then subcloned into pTRV2 to generate a pTRV2 *PDS* construct specific to each species.

Agrobacterium-Mediated Infection

The constructs, pTRV1 (TRV RNA1 construct), and pTRV2 (RNA2 (empty vector control) or RNA2+PDS fragment) constructs were transformed into *Agrobacterium* strain GV3101 by electroporation. The bacteria were cultured overnight at 28°C in LB medium with 40 mg/L Kanamycin and 20 mg/L Gentamycin. The *Agrobacterium* cells were then harvested and resuspended in inoculation buffer (10 mM $MgCl_2$, 10 mM MES, 200 µM acetosyringone) to an O.D. of 1~2 and left at room temperature for at least 4h. The bacteria containing pTRV1 and the bacteria containing pTRV2 or pTRV2 *PDS* were mixed together in a 1:1 ratio. The leaves of seedlings were inoculated with the mixed bacteria culture using a 1 ml disposable syringe without a needle (for daisy and tomato) or by vacuum infiltration (for lettuce and impatiens). Infected plants were incubated in growth chambers under 16h light/8h dark cycles at the temperature of 20-22ºC.

RT-PCR Analysis

Total RNA was extracted from lettuce leaves using TRIzol Reagent (Invitrogen) and treated with RNase-free DNase (Ambion) to remove any contaminating genomic DNA. The first strand cDNA was synthesized using 2 µg total RNA, oligo d(T) primer, random hexamer, and superscript reverse transcriptase (Invitrogen). This cDNA was normalized by real-time quantitative PCR using 18S rRNA primer (5'-CATGGCCGTTCTT AGTTGGTGGAG-3' and 5'-AAGAAGCTGGCCGCGAAGGGAT AC-3').

The primers for amplifying PDS transcripts by semi-quantitative PCR were designed outside the region targeted for gene silencing to avoid amplification from the pTRV *PDS* construct. These primers were 5'-CTTTGCAAGCCAATTGTTGA-3' and 5'-TTGTAGGGGTCGACATGGTT-3'.

RESULTS AND DISCUSSION

Design of Primers to Amplify *PDS* Gene Fragments from Different Plant Species

A BLAST search for PDS sequences from a wide range of taxa permitted us to design a set of degenerate primers that would amplify a PDS fragment from known species, and therefore, most probably from species where the gene's sequence is unknown. An approximately 520 bp fragment of the *PDS* gene was amplified by the degenerate primers from all tested species (Fig. 1). A comparison of the *PDS* fragment sequences from 9 different plant species reveals a similarity of higher than 80% but in most cases less than the 90% homology required for efficient VIGS (Fig. 2). It seems likely that most other species will include the highly conserved region of the *PDS* gene amplified by this pair of degenerate primers.

Host Range of TRV-Induced *PDS* Gene Silencing

Fragments isolated from the target species using our universal primers were very effective when used in VIGS silencing of PDS. Developing leaves of pTRV2-*PDS* infected plants showed the strong photobleaching phenotype that indicated movement of the virus and its effectiveness in silencing the native *PDS* gene in bean, impatiens, daisy, and tomato (Fig. 3). No photo-bleached phenotype was found in the H_2O or pTRV2 empty vector controls (data not shown).

PDS gene transcripts were clearly evident in RNA extracted from non-silenced leaves and vector control, but barely detected in that from photo-bleaching leaves of lettuce by RT-PCR (Fig. 4). The result suggested that the photo-bleaching phenotype was caused by the down-regulation of the *PDS* gene.

In previous VIGS studies of a wide range of different ornamentals (Chen et al., 2003), only *N. benthamiana* and *P. hybrida* (both from the *Solanaceae*) showed the *PDS* phenotype. We now suspect that this negative result reflected a lack of homology between the *PDS* of *N. benthamiana* (the source of the *PDS* fragment used in the study) and the *PDS* sequences in the other target species.

Efficiency of VIGS in Different Species

Although we were able to silence PDS in all of the species that we tested, efficiency (% plants showing phenotype), and extent of silencing varied among taxa. Increasing the concentration of the *Agrobacterium* inoculum (to an OD_{600} of 2.0) increased the frequency of silencing in impatiens plants (Fig. 5). In most tested species, however, the silencing symptom was local, confined to one or two leaves (Fig. 3). To obtain systemic symptoms in such species, it may be necessary to develop techniques to improve viral movement, and/or inhibit the plant's antiviral mechanism.

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Figures

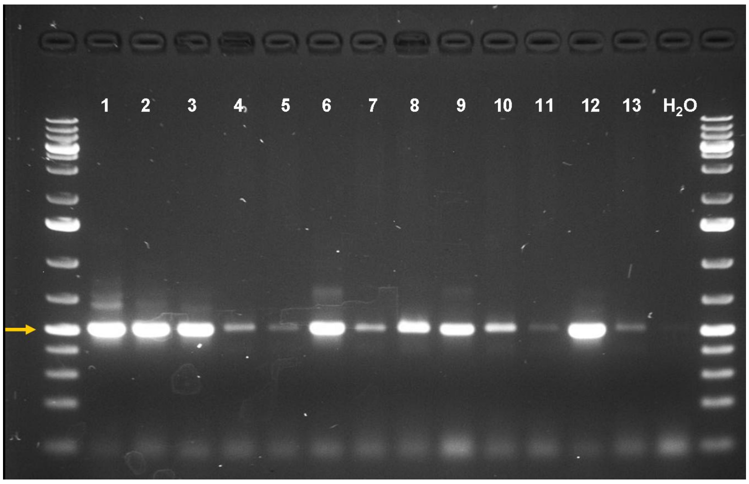

Fig. 1. *PDS* gene fragments were amplified by the universal primers from different species. 1: Petunia; 2: Tomato; 3: Brunfelsia; 4: Daisy; 5: Gerbera; 6: Chrysanthemum; 7: Lettuce; 8: Impatiens; 9: Orchid; 10: Rose; 11: Mirabilis; 12: Pole bean; 13: Bush bean. The size of the *PDS* fragment is approximately 520 bp (arrow).

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Rose          GGTGGAAAG-ATTGCTGCTTGGAAAGATAAAGATGGAGACTGGTATGAGACAGGCCTACA
Mirabilis     GGTGGAAAG-ATTGCTGCTTGGAAAGATGAAGATGGAGATTGGTATGAAACTGGACTACA
Impatiens     GGTGGAAAG-GTTGCTGCTTGGAAAGATGAGGATGGAGATTGGTATGAGACAGGACTACA
Bean          GGTGGAAAG-GTTGCTGCCTGGAAAGATGAGGATGGAGACTGGTATGAAACAGGTCTACA
Mum           GGTGGAAAGGATTGCTGCTTGGAAAGACGAGGATGGAGATTGGTACGAGACGGGTTTACA
Daisy         GGTGGAAAG-ATTGCTGCCTGGAAAGACAAGGATGGAGATTGGTACGAGACGGGTTTACA
Gerbera       GGTGGAAAG-ATTGCTGCTTGGAAAGATGATGATGGAGATTGGTACGAGACAGGTTTACA
Tomato        GGTGGAAAG-GTAGCTGCATGGAAAGATGATGATGGAGATTGGTACGAGACTGGTTTGCA
Brunfelsia    GGTGGAAAG-ATTGCTGCTTGGAAAGATGACGATGGAGATTGGTATGAGACTGGTTTGCA
              *********  * ***** ********  * ********* ***** ** ** **  * **

Rose          TATATTTTTTGGGGCTTATCCAAATATTCAGAACTTGTTTGGAGAGCTTGGTATCAATGA
Mirabilis     CATATTCTTCGGGGCTTACCCAAATATTCAGAATCTGTTTGGAGAGCTTGGTATTAATGA
Impatiens     CATATTCTTTGGGGCTTACCCGAATGTGCAGAACCTTTTTGGAGAACTTGGCATCAATGA
Bean          CATCTTCTTTGGGGCTTACCCTAATGTGCAGAACCTATTTGGAGAACTTGGCCTTAATGA
Mum           CATATTTTTTGGAGCTTACCCAAATGTACAGAACCTGTTTGGAGAGCTAGGCATTAACGA
Daisy         CATATTTTTTGGAGCTTATCCAAATGTACAGAACCTGTTTGGAGAGCTAGGCATTAACGA
Gerbera       CATATTCTTTGGGGCATACCCAAATATACAGAACCTCTTTGGAGAACTAGGCATTAATGA
Tomato        TATATTCTTTGGGGCTTACCCAAATATTCAGAACCTGTTTGGAGAATTAGGGATTAACGA
Brunfelsia    CATATTCTTTGGGGCTTACCCAAATATACAGAACCTGTTTGGAGAATTAGGGATTAATGA
               ** ** ** ** ** ** ** *** * *****  * ********  * **  * ** **

Rose          TCGATTGCAGTGGAAGGAACACTCTATGATATTTGCAATGCCAAACAAGCCAGGAGAGTT
Mirabilis     TCGGTTGCAATGGAAAGAGCACTCCATGATTTTCGCAATGCCAAGTAAGCCCGGTGAATT
Impatiens     CAGGCTCCAATGGAAGGAACATTCTATGATTTTTGCAATGCCAAATAAGCCTGGAGAATT
Bean          TCGGTTACAATGGAAGGAGCATTCCATGATTTTTGCTATGCCAAGTAAGCCCGGAGAGTT
Mum           TAGACTGCAGTGGAAGGAGCATTCTATGATATTTGCAATGCCAAACAAGCCTGGGGAATT
Daisy         TAGATTGCAGTGGAAGGAGCATTCTATGATATTTGCAATGCCAAACAAGCCTGGAGAATT
Gerbera       AAGATTGCAGTGGAAGGAGCATTCTATGATATTCGCAATGCCAAATAAGCCTGGAGAATT
Tomato        TCGATTGCAATGGAAGGAACATTCAATGATATTTGCAATGCCAAGCAAGCCAGGAGAATT
Brunfelsia    TCGATTGCAGTGGAAGGAGCATTCGATGATATTTGCAATGCCAAGCAAGCCAGGGGAATT
                *  * ** ***** ** ** ** ***** ** ** *******  ***** ** ** **

Rose          CAGCCGGTTTGATTTCCCTGAAGTTCTGCCAGCACCCTTAAATGGAATATGGGCCATATT
Mirabilis     CAGCCGCTTTGATTTTCCTGACGTCTTGCCTGCACCATTGAATGGCATTTGGGCTATCTT
Impatiens     CAGCCGATTTGACTTCCCCGATGTTTTGCCAGCACCATTAAATGGTATATGGGCCATCTT
Bean          TAGTCGATTTGATTTCCCCGAAATCCTTCCGTCCCCATTAAATGGAATCTGGGCCATATT
Mum           TAGTCGCTTTGACTTCCCAGATGTTCTGCCTGCACCATTGAATGGAATTTGGGCTATCTT
Daisy         TAGTCGCTTTGACTTCCCAGATGTTCTGCCTGCACCACTCAATGGAATATGGGCTATCTT
Gerbera       TAGTCGGTTTGACTTCCCAGACGTCCTGCCTGCACCATTAAATGGAATCTGGGCTATCTT
Tomato        CAGCCGCTTTGATTTCTCCGAAGCTTTACCCGCTCCTTTAAATGGAATTTTAGCCATCTT
Brunfelsia    CAGCCGCTTTGATTTTCCGGAAGCTCTTCCTGCTCCATTAAATGGAATTCTGGCGATCCT
               ** ** ***** **  * **     * **  * **  * ***** **    ** **  *

Rose          AAAGAACAATGAGATGCTGACATGGCCAGAAAAAGTGAAGTTTGCTATCGGACTTGTGCC
Mirabilis     AAGGAACAATGAAATGCTAACTTGGCCGGAGAAAGTGAGATTTGCTATAGGCCTCTTGCC
Impatiens     ACGGAATAATGAAATGCTTACATGGCCTGAAAAGGTCAAATTTGCAATTGGGCTGTTGCC
Bean          GAGGAACAATGAGATGCTGACATGGCCAGAGAAAATCAAATTTGCAATTGGGCTTCTGCC
Mum           GAGGAACAATGAAATGCTGACATGGCCAGAGAAAGTAAAATTTGCAATTGGGCTCTTGCC
Daisy         GAGGAACAATGAAATGCTGACATGGCCGGAGAAAGTAAAATTTGCAATTGGGCTCTTGCC
Gerbera       GAGAAACAATGAAATGCTGACGTGGCCTGAGAAAGTAAAGTTTGCGATTGGGCTCTTGCC
Tomato        AAAGAATAACGAAATGCTTACATGGCCAGAGAAAGTCAAATTTGCAATTGGACTCTTGCC
Brunfelsia    AAAGAACAATGAAATGCTTACATGGCCAGAGAAAGTCAAATTTGCAATTGGACTCTTGCC
                  ** ** ** ***** ** ***** ** **  * *  ***** ** ** **  ****

Rose          AGCAATTCTTGGTGGACAGGCTTATGTTGAAGCTCAGGATGGCTTGACTGTAAAGGAGTG
Mirabilis     TGCTATTGTCGGTGGACAAGATTATGTGGTGGCTCAAGATGGTTTGAGTGTCCAAGAGTG
Impatiens     AGCTATGATTGGTGGACAAGATTACGTGGAGGCTCAAGATGCTTTTAGTGTTAAAGACTG
Bean          AGCTATGCTTGGTGGACAGGCATATGTTGAGGCTCAAGATGGTCTTTCTGTTAAAGAATG
Mum           TGCAATGTTAGGTGGGCAGGCGTATGTTGAGGCTCAAGATGGGCTAAGTGTTCAAGACTG
Daisy         TGCAATGTTAGGTGGGCAGGCGTATGTTGAGGCTCAAGATGGCCTAAGTGTTCAAGACTG
Gerbera       AGCAATGTTAGGAGGACAGGCTTATGTTGAGGCTCAAGATGGTTTGAGTGTTCAAGACTG
Tomato        AGCAATGCTTGGAGGGCAATCTTATGTTGAAGCTCAAGATGGGATAAGTGTTAAGGACTG
Brunfelsia    TGCAATGCTTGGAGGGCAATCTTATGTTGAAGCTCAAGACGGGATAAGCGTTAAGGACTG
               ** **  * ** ** **    ** ** *  ***** ** *   *    **  * ** **

Rose          GATGACAAAACAGGGGATACCTGATCGAGTAACTACTGAGGTGTTTATTGCCATGTCAAA
Mirabilis     GATGAGGAAGCAAGGTGTACCTGATCGAGTGACTGATGAAGTATTTATTGCCATGTCAAA
Impatiens     GATGCAAAAACAAGGTATACCAGAACGTGTAACTACTGAAGTTTTTATTGCCATGTCAAA
Bean          GATGATAAAACAGGGTGTACCTGAACGAGTAACTGACGAGGTGTTCATAGCAATGTCGAA
Mum           GATGAGACAGCGAGGCATACCGGATCGAGTCACCACCGAGGTGTTTATTGCCATGTCAAA
Daisy         GATGAGACAGCGAGGAATACCGGATCGAGTTACCACCGAGGTGTTTATTGCCATGTCAAA
Gerbera       GATGAGAAAGCAAGGCATACCAGATCGGGTTACTACTGAGGTGTTTATTGCCATGTCAAA
Tomato        GATGAGAAAGCAAGGTGTGCCGGACAGGGTGACAGATGAGGTGTTCATTGCTATGTCAAA
Brunfelsia    GATGAGAAAGCAAGGTGTGCCAGATAGGGTGACAGATGAGGTGTTCATTGCCATGTCAAA
              ****    * *  **  * ** **  * ** **    ** ** ** ** ** ***** **

Rose          GGCCCTTAACTTTATTAATCCTGATGAGCTCTCAATGCAATGCATATTGATTGCTTTGAA
Mirabilis     GGCATTGAACTTTATAAATCCGGATGAACTCTCAATGCAGTGCATTTTGATTGCTTTGAA
Impatiens     GGCTTTAAACTTCATTAACCCTGACGAGCTTTCAATGCAGTGTATTTTGATTGCTCTGAA
Bean          GGCATTAAACTTCATCAATCCTGATGAACTTTCAATGCAATGTATATTGATTGCTTTAAA
Mum           GGCATTAAACTTCATAAATCCTGATGAACTGTCAATGCAATGTATTCTCATAGCTCTTAA
Daisy         GGCATTAAACTTCATAAATCCTGATGAACTGTCAATGCAATGTATTCTCATAGCTCTAAA
Gerbera       GGCGTTAAACTTCATAAATCCAGATGAACTTTCTATGCAATGTATTCTCATTGCTTTGAA
Tomato        GGCACTCAACTTTATAAACCCTGACGAACTTTCAATGCAGTGCATTTTGATCGCATTGAA
Brunfelsia    AGCACTTAACTTCATAAACCCTGACGAGCTTTCAATGCAGTGCATCTTGATTGCTTTGAA
               **  * ***** ** ** ** ** ** ** ** ***** ** **  * ** **  * **

Rose          TCGATTTCTTCAGGAAAAACATGGTTCAAAAATGGCA
Mirabilis     TCGATTTCTTCAGGAAAAGCATGGTTCAAAAATGAAT
Impatiens     TCGGTTTCTTCAGGAAAAACATGGTTCAAAGATGGCA
Bean          CCGATTCCTTCAGGAAAAACATGGTTCTAAAATGGCA
Mum           CCGGTTTCTTCAGGAAAAACATGGTTCAAAGATGGCA
Daisy         CCGGTTTCTCCAGGAAAAACATGGTTCTAAAATGGCA
Gerbera       CAGGTTTCTTCAGGAAAAACATGGTTCAAAAATGGCA
Tomato        CAGGTTTCTTCAGGAGAAACATGGTTCAAAAATGGCC
Brunfelsia    CAGATTTCTTCAGGAAAAACATGGTTCAAAAATGGCA
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Fig. 2. Multiple sequence alignment of the *PDS* fragments isolated from the 9 target plant species.

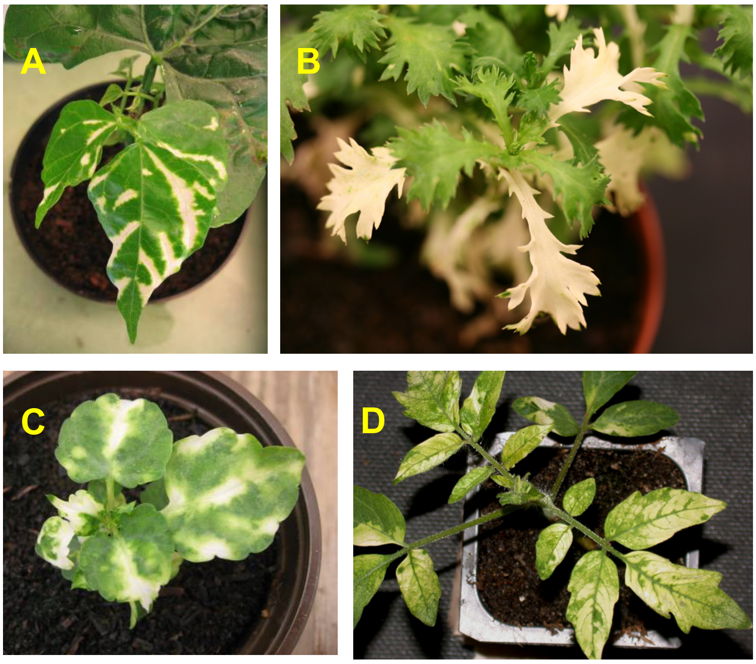

Fig. 3. Photobleaching phenotype resulting from VIGS silencing of *PDS* in different plant species using fragments isolated with the universal primers. A: Henderson's Baby Bush Lima Bean, B: Daisy 'Alaska', C: Impatiens 'Orange Bling', D: Tomato 'New Yorker'.

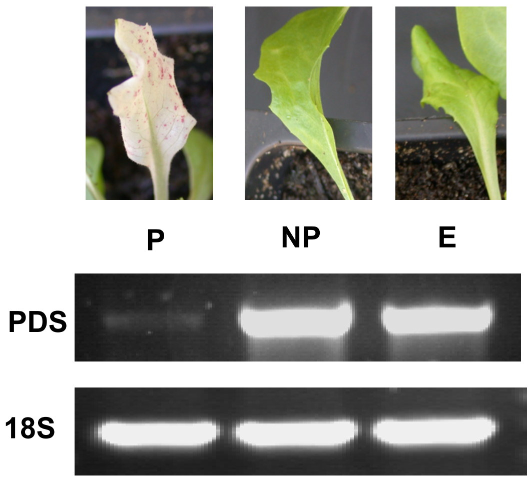

Fig. 4. Abundance of *PDS* transcripts in 'Red Sails' lettuce plants after VIGS silencing. P-silenced tissue, NP-non-silenced tissue, E-empty vector control.

Fig. 5. Effect of *Agrobacterium* concentration on silencing PDS in impatiens plants.