

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 *EcoRI* 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 MgCl₂, 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 20-22°C.

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 H₂O 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 OD₆₀₀ 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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Literature Cited

- Baulcombe, D.C. 1999. Fast forward genetics based on virus-induced gene silencing. *Current Opinion in Plant Biology* 2:109-123.
- Burch-Smith, T.M., Anderson, J.C., Martin, G.B. and Dinesh-Kumar, S.P. 2004. Applications and advantages of virus-induced gene silencing for gene function studies in plants. *Plant Journal* 39(5):734-746.
- Chen, J.C., Jiang, C.Z., Gookin, T.E., Hunter, D.A., Clark, D.G. and Reid, M.S. 2004. Chalcone synthase as a reporter in virus-induced gene silencing studies of flower senescence. *Plant Mol. Biol.* 55:521-530.
- Chen, J.C., Johnson, F., Clark, D.G., Gookin, T. and Reid, M.S. 2003. Potential

- application of virus-induced gene silencing (VIGS) in flower senescence studies. *Acta Hort.* 669:147-152.
- Chicas, A. and Macino, G. 2001. Characteristics of post-transcriptional gene silencing. *EMBO Reports* 2:992-996.
- Gould, B. and Kramer, E.M. 2007. Virus-induced gene silencing as a tool for functional analyses in the emerging model plant *Auilegia* (columbine, *Ranunculaceae*). *Plant Methods* 3:6.
- Liu, Y., Schiff, M., Marathe, R. and Dinesh-Kumar, S.P. 2002a. Tobacco Rar1, EDS1 and NPR1/NIM1 like genes are required for N-mediated resistance to tobacco mosaic virus. *Plant J.* 30:415-429.
- Liu, Y., Schiff, M. and Dinesh-Kumar, S.P. 2002b. Virus-induced gene silencing in tomato. *Plant J.* 31:777-786.
- Wege, S., Scholz, A., Gleissberg, S. and Becke, A. 2007. Highly efficient virus-induced gene silencing (VIGS) in California poppy (*Eschscholzia californica*): An evaluation of VIGS as a strategy to obtain functional data from non-model plants. *Annals of Botany* 100:641-649.

Figures

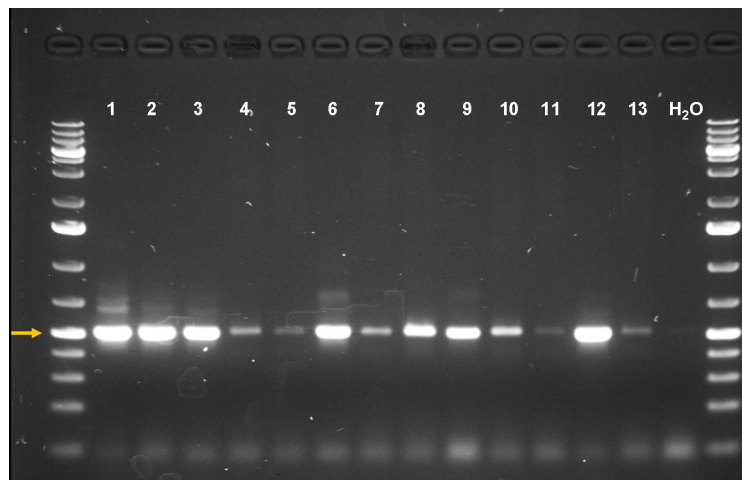


Fig. 1. *PDS* gene fragments were amplified by the universal primers from different species. 1: Petunia; 2: Tomato; 3: Brunfelsia; 4: Daisy; 5: Gerbera; 6: Chrysanthemum; 7: Lettuce; 8: Impatiens; 9: Orchid; 10: Rose; 11: Mirabilis; 12: Pole bean; 13: Bush bean. The size of the *PDS* fragment is approximately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

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Rose      GGTGGAAG-ATTGCTGCTTGGAAAGATAAAGATGGAGACTGGTATGAGACAGGCOCTACA
Mirabilis GGTGGAAG-ATTGCTGCTTGGAAAGATGAAGATGGAGATTGGTATGAACTGGACTACA
Impatiens GGTGGAAG-ATTGCTGCTTGGAAAGATGAGGATGGAGACTGGTATGAAACAGGCTTACA
Bean      GGTGGAAG-ATTGCTGCTTGGAAAGATGAGGATGGAGACTGGTATGAAACAGGCTTACA
Mum      GGTGGAAG-ATTGCTGCTTGGAAAGATGAGGATGGAGACTGGTATGAAACAGGCTTACA
Daisy    GGTGGAAG-ATTGCTGCTTGGAAAGATGAGGATGGAGACTGGTATGAAACAGGCTTACA
Gerbera  GGTGGAAG-ATTGCTGCTTGGAAAGATGAGGATGGAGACTGGTATGAAACAGGCTTACA
Tomato   GGTGGAAG-ATTGCTGCTTGGAAAGATGAGGATGGAGACTGGTATGAAACAGGCTTACA
Brunfelsia GGTGGAAG-ATTGCTGCTTGGAAAGATGAGGATGGAGACTGGTATGAAACAGGCTTACA
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Rose      TATATTTTTGGGGCTTATCCAAATATTCAGAACTGTTTTGGAGAGCTTGGTATCAATGA
Mirabilis CATATTTCTTGGGGCTTACCCAAATATTCAGAACTGTTTTGGAGAGCTTGGTATCAATGA
Impatiens CATATTTCTTGGGGCTTACCCAAATATTCAGAACTGTTTTGGAGAGCTTGGTATCAATGA
Bean      CATATTTCTTGGGGCTTACCCAAATATTCAGAACTGTTTTGGAGAGCTTGGTATCAATGA
Mum      CATATTTCTTGGGGCTTACCCAAATATTCAGAACTGTTTTGGAGAGCTTGGTATCAATGA
Daisy    CATATTTCTTGGGGCTTACCCAAATATTCAGAACTGTTTTGGAGAGCTTGGTATCAATGA
Gerbera  CATATTTCTTGGGGCTTACCCAAATATTCAGAACTGTTTTGGAGAGCTTGGTATCAATGA
Tomato   CATATTTCTTGGGGCTTACCCAAATATTCAGAACTGTTTTGGAGAGCTTGGTATCAATGA
Brunfelsia CATATTTCTTGGGGCTTACCCAAATATTCAGAACTGTTTTGGAGAGCTTGGTATCAATGA
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Rose      TCGATTGCAGTGGAAAGAACACTCTATGATATTTGCAATGCCAAACAGCCAGGAGATT
Mirabilis TCGATTGCAGTGGAAAGAACACTCTATGATATTTGCAATGCCAAACAGCCAGGAGATT
Impatiens TCGATTGCAGTGGAAAGAACACTCTATGATATTTGCAATGCCAAACAGCCAGGAGATT
Bean      TCGATTGCAGTGGAAAGAACACTCTATGATATTTGCAATGCCAAACAGCCAGGAGATT
Mum      TCGATTGCAGTGGAAAGAACACTCTATGATATTTGCAATGCCAAACAGCCAGGAGATT
Daisy    TCGATTGCAGTGGAAAGAACACTCTATGATATTTGCAATGCCAAACAGCCAGGAGATT
Gerbera  TCGATTGCAGTGGAAAGAACACTCTATGATATTTGCAATGCCAAACAGCCAGGAGATT
Tomato   TCGATTGCAGTGGAAAGAACACTCTATGATATTTGCAATGCCAAACAGCCAGGAGATT
Brunfelsia TCGATTGCAGTGGAAAGAACACTCTATGATATTTGCAATGCCAAACAGCCAGGAGATT
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Rose      CAGCCGTTTGATTTCCCTGAAGTCTCCGACACCCCTTAAATGGAATATGGCCATAT
Mirabilis CAGCCGTTTGATTTCCCTGAAGTCTCCGACACCCCTTAAATGGAATATGGCCATAT
Impatiens CAGCCGTTTGATTTCCCTGAAGTCTCCGACACCCCTTAAATGGAATATGGCCATAT
Bean      TAGTCGATTTGATTTCCCGAAATCCTTCCGTCACCAATTAATGGAATCTGGCCATCTT
Mum      TAGTCGATTTGATTTCCCGAAATCCTTCCGTCACCAATTAATGGAATCTGGCCATCTT
Daisy    TAGTCGATTTGATTTCCCGAAATCCTTCCGTCACCAATTAATGGAATCTGGCCATCTT
Gerbera  TAGTCGATTTGATTTCCCGAAATCCTTCCGTCACCAATTAATGGAATCTGGCCATCTT
Tomato   TAGTCGATTTGATTTCCCGAAATCCTTCCGTCACCAATTAATGGAATCTGGCCATCTT
Brunfelsia TAGTCGATTTGATTTCCCGAAATCCTTCCGTCACCAATTAATGGAATCTGGCCATCTT
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Rose      AAAGAACAATGAGATGCTGACATGGCCAGAAAAGTGAAGTTTGCATTCGGACTTGTGCC
Mirabilis AAAGAACAATGAGATGCTGACATGGCCAGAAAAGTGAAGTTTGCATTCGGACTTGTGCC
Impatiens AAAGAACAATGAGATGCTGACATGGCCAGAAAAGTGAAGTTTGCATTCGGACTTGTGCC
Bean      AAAGAACAATGAGATGCTGACATGGCCAGAAAAGTGAAGTTTGCATTCGGACTTGTGCC
Mum      AAAGAACAATGAGATGCTGACATGGCCAGAAAAGTGAAGTTTGCATTCGGACTTGTGCC
Daisy    AAAGAACAATGAGATGCTGACATGGCCAGAAAAGTGAAGTTTGCATTCGGACTTGTGCC
Gerbera  AAAGAACAATGAGATGCTGACATGGCCAGAAAAGTGAAGTTTGCATTCGGACTTGTGCC
Tomato   AAAGAACAATGAGATGCTGACATGGCCAGAAAAGTGAAGTTTGCATTCGGACTTGTGCC
Brunfelsia AAAGAACAATGAGATGCTGACATGGCCAGAAAAGTGAAGTTTGCATTCGGACTTGTGCC
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Rose      AGCAATTTCTTGGTGGACAGGCTTATGTTGAAGCTCAGGATGGCTTGAATGTAAGGAGTG
Mirabilis AGCAATTTCTTGGTGGACAGGCTTATGTTGAAGCTCAGGATGGCTTGAATGTAAGGAGTG
Impatiens AGCAATTTCTTGGTGGACAGGCTTATGTTGAAGCTCAGGATGGCTTGAATGTAAGGAGTG
Bean      AGCAATTTCTTGGTGGACAGGCTTATGTTGAAGCTCAGGATGGCTTGAATGTAAGGAGTG
Mum      AGCAATTTCTTGGTGGACAGGCTTATGTTGAAGCTCAGGATGGCTTGAATGTAAGGAGTG
Daisy    AGCAATTTCTTGGTGGACAGGCTTATGTTGAAGCTCAGGATGGCTTGAATGTAAGGAGTG
Gerbera  AGCAATTTCTTGGTGGACAGGCTTATGTTGAAGCTCAGGATGGCTTGAATGTAAGGAGTG
Tomato   AGCAATTTCTTGGTGGACAGGCTTATGTTGAAGCTCAGGATGGCTTGAATGTAAGGAGTG
Brunfelsia AGCAATTTCTTGGTGGACAGGCTTATGTTGAAGCTCAGGATGGCTTGAATGTAAGGAGTG
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Rose      GATGACAAAACAGGGGATACCTGATCGAGTAACTACTGAGGTGTTTATGCCATGTCAAA
Mirabilis GATGAGGAAGCAAGGTGTACCTGATCGAGTAACTACTGAGGTGTTTATGCCATGTCAAA
Impatiens GATGAGGAAGCAAGGTGTACCTGATCGAGTAACTACTGAGGTGTTTATGCCATGTCAAA
Bean      GATGAGGAAGCAAGGTGTACCTGATCGAGTAACTACTGAGGTGTTTATGCCATGTCAAA
Mum      GATGAGGAAGCAAGGTGTACCTGATCGAGTAACTACTGAGGTGTTTATGCCATGTCAAA
Daisy    GATGAGGAAGCAAGGTGTACCTGATCGAGTAACTACTGAGGTGTTTATGCCATGTCAAA
Gerbera  GATGAGGAAGCAAGGTGTACCTGATCGAGTAACTACTGAGGTGTTTATGCCATGTCAAA
Tomato   GATGAGGAAGCAAGGTGTACCTGATCGAGTAACTACTGAGGTGTTTATGCCATGTCAAA
Brunfelsia GATGAGGAAGCAAGGTGTACCTGATCGAGTAACTACTGAGGTGTTTATGCCATGTCAAA
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Rose      GGCCCTTAACTTTATAAATCCTGATGAGCTCTCAATGCAATGCATATTGATTGCTTTGAA
Mirabilis GGCCCTTAACTTTATAAATCCTGATGAGCTCTCAATGCAATGCATATTGATTGCTTTGAA
Impatiens GGCCCTTAACTTTATAAATCCTGATGAGCTCTCAATGCAATGCATATTGATTGCTTTGAA
Bean      GGCCCTTAACTTTATAAATCCTGATGAGCTCTCAATGCAATGCATATTGATTGCTTTGAA
Mum      GGCCCTTAACTTTATAAATCCTGATGAGCTCTCAATGCAATGCATATTGATTGCTTTGAA
Daisy    GGCCCTTAACTTTATAAATCCTGATGAGCTCTCAATGCAATGCATATTGATTGCTTTGAA
Gerbera  GGCCCTTAACTTTATAAATCCTGATGAGCTCTCAATGCAATGCATATTGATTGCTTTGAA
Tomato   GGCCCTTAACTTTATAAATCCTGATGAGCTCTCAATGCAATGCATATTGATTGCTTTGAA
Brunfelsia GGCCCTTAACTTTATAAATCCTGATGAGCTCTCAATGCAATGCATATTGATTGCTTTGAA
***** * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

Rose      TCGATTCTTCAGGAAAAACATGTTCAAAAATGGCA
Mirabilis TCGATTCTTCAGGAAAAACATGTTCAAAAATGGCA
Impatiens TCGATTCTTCAGGAAAAACATGTTCAAAAATGGCA
Bean      TCGATTCTTCAGGAAAAACATGTTCTAAAATGGCA
Mum      TCGATTCTTCAGGAAAAACATGTTCAAAAATGGCA
Daisy    TCGATTCTTCAGGAAAAACATGTTCTAAAATGGCA
Gerbera  TCGATTCTTCAGGAAAAACATGTTCAAAAATGGCA
Tomato   TCGATTCTTCAGGAAAAACATGTTCTAAAATGGCA
Brunfelsia TCGATTCTTCAGGAAAAACATGTTCAAAAATGGCA
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Fig. 2. Multiple sequence alignment of the *PDS* fragments isolated from the 9 target plant species.

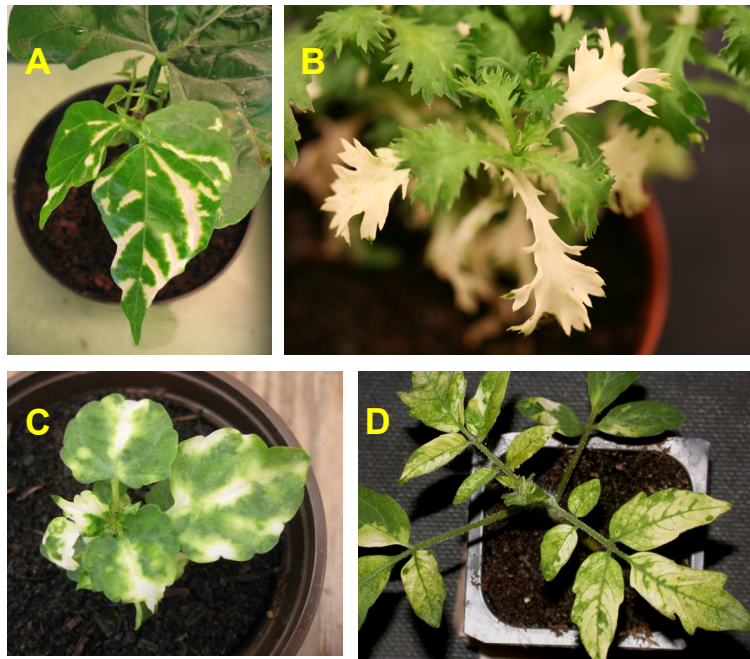


Fig. 3. Photobleaching phenotype resulting from VIGS silencing of *PDS* in different plant species using fragments isolated with the universal primers. A: Henderson's Baby Bush Lima Bean, B: Daisy 'Alaska', C: Impatiens 'Orange Bling', D: Tomato 'New Yorker'.

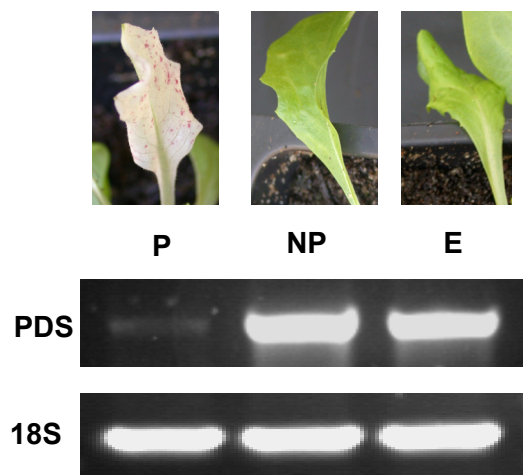


Fig. 4. Abundance of *PDS* transcripts in 'Red Sails' lettuce plants after VIGS silencing. P-silenced tissue, NP-non-silenced tissue, E-empty vector control.



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

