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Chalcone synthase as a reporter in virus-induced gene silencing studies of flower senescence

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Abstract

Agrobacterium-mediated infection of petunia (*Petunia hybrida*) plants with tobacco rattle virus (TRV) bearing fragments of *Petunia* genes resulted in systemic infection and virus-induced gene silencing (VIGS) of the homologous host genes. Infection with TRV containing a phytoene desaturase (*PDS*) fragment resulted in reduced abundance of *PDS* transcripts and typical photobleaching of photosynthetic tissues. Infection with TRV containing a chalcone synthase (*CHS*) fragment resulted in silencing of anthocyanin production in infected flowers. The silencing phenotype ranged from scattered white spots on the normal purple background to entirely white flowers. Symptoms in the V26 cultivar were a diffuse mosaic, but infection of some purple-flowered commercial cultivars resulted in large white sectors and even entirely white flowers. Abundance of *CHS* transcripts in the white flowers was less than 4% of that in purple flowers on the same plant. Infection with TRV containing a tandem construct of *PDS* and *CHS* resulted in leaf photobleaching and white patterns on the flowers. Transcripts of *CHS* and *PDS* were reduced both in leaves and in flowers confirming simultaneous silencing of both genes by the tandem construct. We tested the effects of infection with TRV containing *CHS* and a fragment of a petunia gene encoding for 1-aminocyclopropane-1-carboxylate oxidase (*ACO4*) Abundance of transcripts encoding *ACO4* and *ACO1* were reduced (by 5% and 20%, respectively) in infected flowers. Whether the flowers were treated with ACC or pollinated, the white (silenced) flowers or flower sectors produced less ethylene and senesced later than purple (non-silenced) tissues. These results indicate the value of VIGS with tandem constructs containing *CHS* as reporter and a target gene as a tool for examining the function of floral-associated genes.

Abbreviations: ACO, 1-aminocyclopropane-1-carboxylate oxidase; CHS, chalcone synthase; PDS, phytoene desaturase; PTGS, post-transcriptional gene silencing; PVX, potato virus X; TGMV, tomato golden mosaic virus; TMV, tobacco mosaic virus; TRV, tobacco rattle virus; VIGS, virus-induced gene silencing

Introduction

Senescence, the last stage of flower development, is a tightly regulated process that is initiated at a specific developmental phase or in response to environmental cues (Thomas *et al*., 2003). The elimination of the superfluous floral organs allows nutrient recycling to other developing portions of the plant (Rubinstein, 2000). Several plant hormones, particularly ethylene, have been shown to

be involved in flower senescence, and many flowers, including the commonly studied carnation, petunia, and morning glory, are classified as ethylene-sensitive (Woltering and Van Doorn, 1988). In these flowers, senescence is accompanied by a burst of ethylene production (Woodson *et al.*, 1992; Tang *et al.*, 1994). Exogenous ethylene treatment accelerates flower senescence in these flowers, and application of inhibitors of ethylene biosynthesis or action retards the process (Harkema, Dekker, and Essers, 1991; Reid and Wu, 1992; Serek, Sisler, and Reid, 1995). Abscisic acid (ABA) accelerates flower senescence of carnation and rose but this likely results from stimulated ethylene production (Ronen and Mayak, 1981; Muller *et al.*, 1999). In daylily, ABA has been suggested to be the primary hormonal regulator of flower senescence (Panavas *et al.*, 1998). In contrast, application of gibberellic acid (GA) or cytokinin delays senescence in a range of flowers (Van Staden *et al.*, 1990; Saks *et al.*, 1992; Lukaszewska *et al.*, 1994; Eisinger, 1977; Ferrante *et al.*, 2001), suggesting a complex regulation network controlling flower senescence.

A number of researchers have employed molecular tools in attempts to understand the regulation of flower senescence. cDNA libraries encoding genes whose transcript abundance changes during the onset of senescence have been reported from daylily (Valpuesta *et al.*, 1995), carnation (Lawton *et al.*, 1989), and daffodil (Hunter *et al.*, 2002). Recently, a major effort has identified more than 5,000 unique ESTs encoding genes associated with petal senescence in petunia (Clark, pers. comm.). Some of the putative proteins encoded by the genes identified in these studies are obvious candidates in the processes of petal senescence and resource remobilization. Cysteine proteases and other enzymes associated with protein turnover, nucleases, and cell-wall associated proteins commonly have been identified. However, many of the ESTs are homologous with 'putative proteins' identified in *Arabidopsis*, and some of them appear to encode DNA binding proteins that may be involved in senescence regulation.

In recent years, whole genome studies and gene sequencing from a wide variety of plant species under various environmental conditions and at different developmental stages have produced a massive amount of sequence information. A major issue for plant biologists is how to convert this sequence information into functional and biological understanding. In *Arabidopsis*, generation of T-DNA insertional mutants has proved useful for functional analysis of genes identified in the *Arabidopsis* genome project. This approach has some limitations such as requirements for stable transformation, the difficulty of producing knockouts of every gene in the genome, lack of visible phenotype where the T-DNA insertion is in one gene of a multi-gene family, and lethality of inserts in genes that are essential for survival. Alternative methods such as dsRNA-mediated suppression have been developed (Waterhouse *et al.*, 1998), but this PTGS approach requires generation of inverted-repeat constructs and stable transformation.

VIGS offers an attractively quick method for loss of function assay that may reveal the mature phenotype of embryo-lethal sequences and avoids the need for time-consuming (and sometimes problematic) processes of transformation and regeneration (Baulcombe, 1999; Ratcliff *et al.*, 2001). VIGS is a plant defense mechanism that limits the severity of virus infection (Baulcombe, 1999) by a process similar to PTGS (Chicas and Macino, 2001). During replication of the virus, double stranded chimeric intermediates are produced. The plant cell recognizes these intermediates as foreign, and cuts the double stranded RNA into small oligonucleotides (siRNA) that serve as guides in an RNA-induced silencing complex to degrade anything with identical homology. There are several advantages to VIGS over gene-silencing methods involving transgenic plants expressing inverted-repeat constructs. The constructs can be easily generated by directly cloning into the virus vector without involving any inverted repeats. The VIGS phenotype can be observed in a relatively short time after inoculation. More importantly, it can avoid the lethality due to its conditional nature.

Early VIGS vectors included tobacco mosaic virus (TMV) (Kumagai *et al.*, 1995), potato virus X (PVX) (Ruiz *et al.*, 1998) and TGMV (Kjemtrup *et al.*, 1998). All these viruses may cause strong symptoms of infection including chlorosis and leaf distortion. In addition, TMV and PVX are incapable of infecting the apical meristem and are therefore unlikely to provide information about genes involved in the identity and development of plant tissues and organs. Vectors based on TRV overcome many of the disadvantages of PVX, TMV and TGMV. For example, the TRV

vector induces very mild symptoms, infects large areas of adjacent cells and silences expression of genes in the meristem (Ratcliff *et al*., 2001). The system has been shown to function effectively in *Nicotiana benthamiana* and tomato (Ratcliff *et al*., 2001; Liu *et al*., 2002). These studies used *PDS* as a marker gene. Infected plants show characteristic photo-bleaching symptoms resulting from the inhibition of biosynthesis of protective carotene.

We sought to use the TRV-based VIGS system for studies in flower senescence. We were attracted by the patterns of silencing of the purple anthocyanin pigments in petunias by co-suppression achieved by stable transformation (Napoli *et al*., 1990) or with a geminivirus-based episomal vector (Atkinson *et al*., 1998). We hypothesized that *CHS* would be an ideal visible marker for gene silencing in floral tissues, and that tandem constructs of *CHS* and test sequences would enable us to quickly test the function of genes that have been isolated from senescing petals. In this paper, we describe the use of such tandem constructs in a tobacco rattle virus (TRV)-based vector for efficient silencing of target genes in petunia flowers.

Materials and methods

Plant material and growth condition

Petunia (*Petunia x hybrida*) seeds of cultivars Ultra Blue, Ultra Sky Blue, Storm Blue, Fantasy Blue, Primetime Blue, Cloud Blue, and Ramblin' Nu Blue were obtained from Goldsmith Seeds (Gilroy, CA). Plants were grown in growth chambers under 16 h light/8 h dark cycles with a day/night temperature regime of 25 °C/20 °C, respectively.

Plasmid construction

pTRV1 and pTRV2 VIGS vectors were kindly provided by Dinesh-Kumar, Yale University, and have been described in detail (Liu *et al*., 2002).

PDS constructs: A 138 bp fragment of the *PDS* gene was PCR-amplified from petunia cDNA sources using primers 5′-CAGTGCTTCTTGATC GCTTTGA-3′ and 5′-TCTGACTTGGCCACCT TTTGA-3′. The resulting product was cloned into pGEM-T easy (Promega) to form pGPhPDS for sequencing and then the *PDS* cDNA fragment was isolated from the pGPhPDS using *Eco*RI digestion and subcloned into pTRV2 to generate pTRV2 *PhPDS*.

CHS construct: A 194 bp fragment of the *CHS* gene corresponding to bases 654–847 of petunia *CHSJ* (genbank access number X14599) was PCR-amplified from petunia cDNA sources using primers 5′-gctctagaACCATTGGGCATTTCTG-3′ with an *XbaI* restriction site and 5′-cggaattcAGCCTTTCTCATTTCATCC-3′ with an *Eco*RI restriction site. The resulting product was cloned into pTRV2 to form pTRV2 *PhCHS*.

PDS/*CHS* construct: The 138 bp PDS cDNA fragment was isolated from the pGPhPDS using *Eco*RI digestion and cloned into pTRV2-*PhCHS* to generate a pTRV *PhPDS*/*CHS* construct.

ACC oxidase construct: A 447 bp 1-aminocyclopropane-1-carboxylate oxidase (*ACO4*) (genbank access number L21979) cDNA fragment corresponding to bases 695-1142 was isolated from a petunia EST collection and cloned into pTRV2-*PhCHS* to generate a pTRV2-*PhCHS*/*ACO* construct.

Agrobacterium-mediated infection

Virus infection was achieved by *Agrobacterium*-mediated infection of petunias. The constructs, pTRV1 (TRV RNA1 construct) and pTRV2 (TRV RNA2 construct) or its derivatives were transformed into *Agrobacterium* strain GV3101 by electroporation. Infection of plants with the transformed *Agrobacterium* was performed as described in Liu *et al*. (2002). The bacteria were cultured overnight at 28 °C in LB medium with appropriated antibiotics. 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 2.0 and left at room temperature for 3 h. The bacteria containing pTRV1 and the bacteria containing pTRV2 or its derivatives were then mixed together in a 1:1 ratio. The leaves of petunia plants were infiltrated with the mixed bacterial culture using a 1 ml disposable syringe without a needle.

RT-PCR analysis

Total RNA was extracted from petunia tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase (Promega, Madison, WI) to remove any contaminating genomic DNA. The first strand cDNA was synthesized

using 2 μg total RNA, oligo d(T) primer, random hexamer, reverse strand 18S rRNA primer (5′-AAGAAGCTGGCCGCGAAGGGATAC-3′), and superscript reverse transcriptase (Invitrogen). This cDNA was used as template for semi-quantitative and real-time quantitative PCR. The PCR primers for amplifying CHS transcripts were designed outside the region targeted for gene silencing to avoid amplification of virus RNA from the pTRV *PhCHS* construct. The primers were 5′-ATGGCTCCTTCTCTTGATG-3′ and 5′-AATCTTAGACTTGGGCTGGC-3′ for the CHS sequence. The primers were 5′ -CAGTGCTTCTT GATCGCTTTGA-3′ and 5′-TCTGACTTGGCC ACCTTTTGA-3′ for amplifying the *PDS* sequence. The amplification primers for ACO1 were 5′-TTTTACAATCCAGGAAGTGATGC-3′ and 5′-ATCTTGGCTCCTTAGCTTGAAAC-3′. The amplification primers for the ACO4 gene were 5′-TGCTGGGGGCATTAT ACTTCTCT-3′ and 5′-AATCACTGCATCACTT GCTGGAT-3′. The abundance of 18S rRNA was used as an internal control and the amplification primers were 5′-CATGGCCGTTCTTAGTTGGTGGAG-3′ and 5′-AAGAAGCTGGCCGCGAAGGGATAC-3′.

Flower longevity

For natural flower longevity studies, petunia flowers were emasculated prior to anther dehiscence and marked when flowers fully opened. For pollinated flower longevity studies, flowers were pollinated at the day of anthesis and marked at the same day. The flower longevity was determined as the time from anthesis until the corolla was completely wilted.

Ethylene measurements and ACC oxidase activity assay

Ethylene production by floral tissues and measurement of ACC oxidase activity were carried out as previously described (Pech *et al*., 1987).

Results

TRV VIGS silences endogenous genes in Petunia

Petunia seedlings were infiltrated with a mixture of *Agrobacterium* transformed with pTRV2 bearing a *PDS* cDNA fragment (TRV *PhPDS*). Fourteen days after infection, the photo-bleaching *PDS* phenotype was observed in the leaves of TRV infected plants (Figure 1) but not on control plants.

TRV VIGS silences floral genes in Petunia

Four–five weeks after petunia seedlings were infiltrated with a mixture of *Agrobacterium* transformed with the pTRV2-*PhCHS* vector (bearing a fragment of a petunia *CHS* cDNA) and *Agrobacterium* transformed with pTRV1, white sectors were observed on the normally purple corollas (Figure 2) indicating silencing of *CHS*. To test whether the flower phenotypes observed in the infected plants were associated with down-regulated *CHS* gene expression, semi-quantitative RT-PCR was used to compare the gene expression in purple and white flowers collected from the same plants. *CHS* gene transcripts were clearly evident in RNA extracted from purple flowers, but barely detected in that from white flowers (Figure 2). The RT-PCR observation was verified by quantitative real-time RT-PCR analysis, which showed that *CHS* transcripts were reduced (to about 4% of the control abundance) only in the white flower petals (Table 1A). Abundance of PDS transcripts was little affected in white tissues of the flowers from *TRV PhCHS*

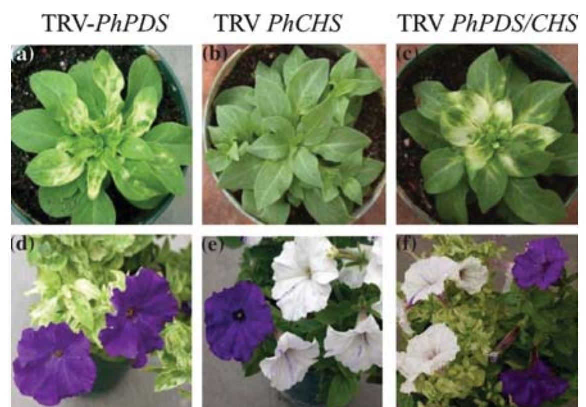

Figure 1. Virus-induced silencing of petunia genes using TRV vectors. Petunia plants were infected with *Agrobacterium* transformed with TRV carrying a fragment of petunia *PDS* (TRV *PhPDS*) a, d, with TRV carrying a fragment of petunia *CHS* (TRV *PhCHS*) b, e, or with TRV carrying both fragments in tandem (TRV *PhPDS/CHS*) (c, f). Photographs of the vegetative plants and of the flowers were taken 2 and 5 weeks after infection, respectively.

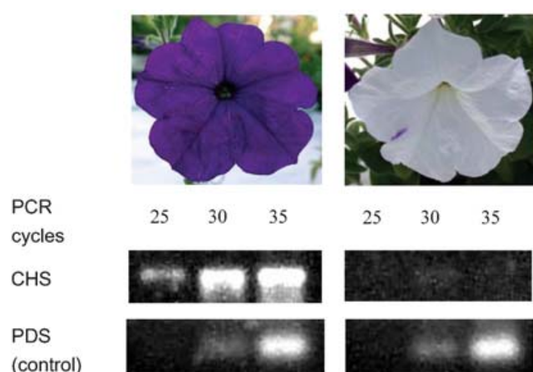

Figure 2. Effects of virus-induced silencing of *CHS* on *CHS* transcript abundance in petunia flowers. Petunia plants were inoculated with *Agrobacterium* transformed with TRV carrying a fragment of petunia *CHS* (TRV *PhCHS*). Total RNA was isolated from silenced (white) and non-silenced (purple) flowers. The abundance of *CHS* transcripts in the flowers was evaluated using 25, 30, and 35 cycles of RT-PCR with *CHS*-specific primers. *PDS*-specific primers were used in a control RT-PCR reaction. Non-RT controls gave no detectable signals (data not shown).

infected plants, but was markedly reduced (to 0.4% of the controls) in flowers from branches of *TRV PhPDS* infected plants showing the photo-bleaching phenotype (Table 1A).

TRV VIGS silences multiple genes in Petunia

To test whether the TRV VIGS system could silence multiple endogenous genes, petunia seedlings were infiltrated with a mixture of *Agrobacterium* transformed with the pTRV2-*PhPDS*/*CHS* vector (bearing both *PDS* and *CHS* cDNA fragments) and *Agrobacterium* transformed with pTRV1 and with appropriate controls. Fourteen days after infection, the photo-bleaching *PDS* phenotype was observed in the leaves of TRV *PhPDS* and TRV *PhPDS*/*CHS*-infected plants (Figure 1a, c) but not in TRV *PhCHS*-infected (Figure 1b) nor in un-inoculated control plants (not shown). Four weeks after infection, the flowers of TRV *PhPDS*/*CHS*-infected as well as TRV *PhCHS* infected

Table 1. Real-time PCR quantitation of the effects of VIGS silencing on transcript abundance in petunia plants. cDNA was prepared from RNA extracted from fresh corollas (or whole flowers for the ACO transcript analysis) from non-silenced and silenced flowers harvested from plants that had been infected with TRV bearing *CHS*, *CHS*/*PDS*, or *CHS*/*ACO*. Transcript abundance was determined using appropriate primers and SYBR green as reporter in an ABI7000 real-time PCR system (ABI, Foster City, CA) using the manufacturer's protocol. Data are reported as Δ threshold cycle (ΔCT), calculated with respect to the 18S transcript abundance in each sample. Analyses were conducted in triplicate, and means and standard deviations were calculated from determinations conducted on tissues from three replicate plants per construct.

Construct	Symptoms	Primer	ΔCT	S.D.	% of control
A.					
Vector control	Purple	CHS	8.15	0.89	100.0
CHS	Purple	CHS	7.98	0.87	112.5
CHS	White	CHS	12.98	2.00	3.5
Vector control	Purple	PDS	12.79	2.43	100
CHS	White	PDS	13.62	1.08	56.3
PDS	Purple*	PDS	20.76	0.23	0.4
B.					
Vector control	Purple	CHS	8.15	0.89	100.0
CHS/*PDS*	Purple	CHS	8.09	0.30	104.2
CHS/*PDS*	White	CHS	12.63	1.56	4.5
Vector control	Purple	PDS	12.79	2.43	100
CHS/*PDS*	Purple	PDS	12.22	0.09	148.5
CHS/*PDS*	White	PDS	20.42	1.35	0.5
C.					
Vector control	Purple	ACO1	6.38	0.64	100.0
CHS/*ACO4*	Purple	ACO1	6.46	0.25	94.6
CHS/*ACO4*	White	ACO1	8.51	0.75	22.8
Vector control	Purple	ACO4	9.38	0.65	100.0
CHS/*ACO4*	Purple	ACO4	9.41	0.04	97.9
CHS/*ACO4*	White	ACO4	13.77	0.40	4.8

*Purple flowers on a branch with photobleached leaves indicating silencing of PDS.

plants showed loss of anthocyanin pigmentation phenotype (Figure 1b, f) but the flowers of control plants and of TRV *PhPDS*-infected plants (Figure 1d) remained purple. Furthermore, white flowers of TRV *PhPDS/CHS*-infected plants were subtended by branches showing the photobleaching phenotype, while purple flowers in the same plants were subtended by normal green branches (Figure 1f).

Semi-quantitative RT-PCR was performed to compare the levels of gene expression for both *CHS* and *PDS* in leaves and corollas of infected plants, and real-time quantitative. RT-PCR was used to measure the observed changes in transcript abundance in corollas. *CHS* transcript levels were greatly reduced in leaves of plants infected with TRV *PhCHS* and with TRV *PhPDS/CHS* (Figure 3a), while *PDS* expression was greatly reduced in leaves of plants infected with TRV *PhPDS* and TRV *PhPDS/CHS*. *CHS* and *PDS* gene expression were both much reduced (to 4.5% and 0.5%, respectively) in white flowers from plants infected with TRV *PhPDS/CHS* (Figure 3b, Table 1B). Expression levels of both genes in the purple flowers of the infected plants were close to those in control flowers.

Silencing phenotype is cultivar dependent

We inoculated 4–6 leaf seedlings of V26 and commercial hybrid petunias with an *Agrobacterium* mixture containing pTRV1 and pTRV2 carrying the petunia *CHSJ* cDNA fragment (pTRV2-*PhCHS*). Silencing symptoms appeared four–five weeks after infection. Although many flowers on infected plants showed the gene-silencing phenotype, there were often some flowers that remained entirely purple (Figure 1). The silencing phenotype depended on cultivar (Figure 4). In 'V26', 'Ultra sky blue' and 'Storm blue', infection resulted in a mosaic-like phenotype of white spots and streaks on the purple corollas. In 'Fantasy blue', 'Ultra blue' and 'Ramblin Nu blue' silencing resulted in symmetrical or asymmetrical white sectors, and even entirely white corollas. The silencing phenotype in 'Cloud blue' was intermediate between these two types.

Silencing of ACC oxidase (ACO) delays senescence of pollinated petunia flowers

A 447 bp petunia ACC oxidase-4 fragment (*ACO*) was cloned from petunia cDNA into a

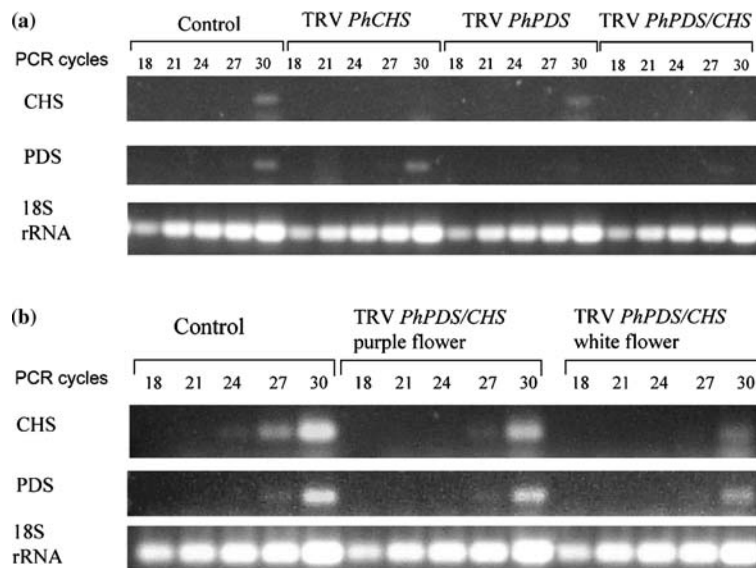

Figure 3. Abundance of *CHS* and *PDS* transcripts in petunia plants after VIGS silencing. Petunia plants were infected with *Agrobacterium* transformed with TRV carrying a fragment of petunia *CHS* (TRV *PhCHS*), with TRV carrying a fragment of petunia *PDS* (TRV *PhPDS*), or with TRV carrying both fragments in tandem (TRV *PhPDS/CHS*). Total RNA was isolated from young leaves harvested 2 weeks after infection (panel a) and from silenced (white) and non-silenced (purple) flowers, 5 weeks after infection (panel b). The abundance of *CHS* and *PDS* transcripts in the RNA samples was evaluated using RT-PCR with gene-specific primers. Controls included RNA extracted from leaves and flowers of uninfected plants. As a further control, we carried out RT-PCR on all samples with primers specific to 18S ribosomal RNA.

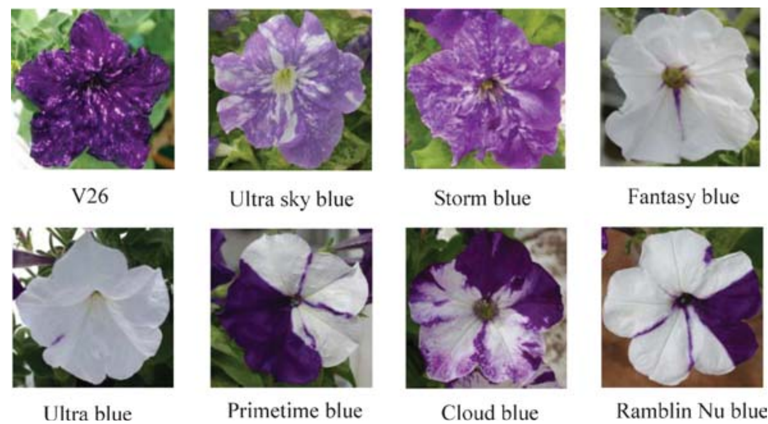

Figure 4. Phenotypes resulting from VIGS silencing of *CHS* in different petunia cultivars. Petunia plants were infected with *Agrobacterium* strains transformed with TRV carrying a fragment of petunia *CHS* (TRV *PhCHS*). Flowers showing typical phenotypes for each cultivar were photographed 5 weeks after infection.

pTRV2-*PhCHS* vector to test simultaneous silencing of *ACO* and *CHS*. The longevity of ACC-treated white flowers of TRV *PhCHS*/*ACO* was longer than that of purple flowers from the same plants, and than that of white flowers from TRV *PhCHS* plants as well as that of control flowers (Figure 5). A similar delay in flower senescence was seen in white flowers of TRV *PhCHS*/*ACO* infected plants 7 days after pollination (bottom panel, Figure 5). Flower longevity was not significantly altered in flowers of TRV-*PhCHS* infected plants (data not shown).

Figure 5. Effect of virus-induced silencing of *ACO* in petunia flowers on their responses to ACC and pollination. Petunia plants were infected with TRV *CHS* or TRV *PhCHS*/*ACO*. Flowers showing the white silencing phenotype and purple flowers from uninfected plants were excised and photographed immediately (a), then either placed in a solution of 2 mM ACC and photographed after 3 days (b) or pollinated, placed in water, and photographed after 7 days (c).

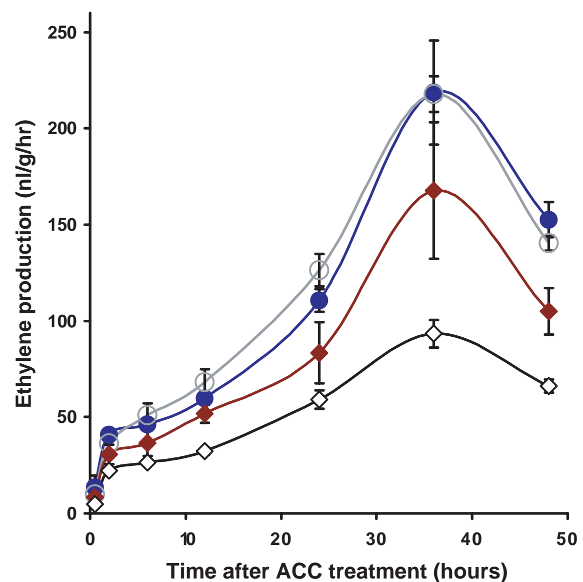

Figure 6. Effect of virus-induced silencing of *ACO* in petunia flowers on their ethylene production in response to ACC. Petunia plants were infected with TRV *CHS* (●, ○) or TRV *PhCHS*/*ACO* (◆, ◇). Flowers showing the white silencing phenotype (open symbols), and non-silenced purple flowers (solid symbols) were excised and placed with their pedicels dipping into a solution of 2 mM ACC. Ethylene production by the flowers was measured at intervals. Means +/− S.D. of four replicate flowers.

Ethylene production in response to applied ACC was reduced 2–3 fold in the white flowers of TRV *PhCHS*/*ACO* infected plants. ACC-dependent ethylene production by purple flowers of

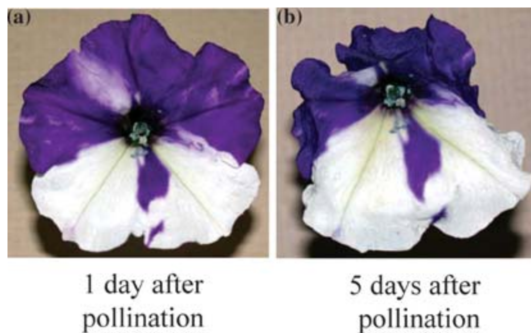

Figure 7. Effect of pollination on white and purple sectors of petunia flowers infected with TRV *PhCHS/ACO*. Petunia plants were infected with TRV *PhCHS/ACO*. Flowers showing purple and white sectors were excised, placed in DI water, and pollinated. Photographs were taken 1 day (a) and 5 days (b) after pollination.

TRV *PhCHS/ACO* infected plants was also lower than that of the controls but considerably higher than that of the white flowers (Figure 6). Similar differences in senescence response to pollination were observed in white and purple sectors of the same flower (Figure 7), and these differences were mirrored by differences in ACC oxidase activity in the different sectors (data not shown). Quantitative RT-PCR demonstrated that the abundance of transcripts encoding ACO1 and ACO4 was substantially reduced (to 23% and 5% of the controls, respectively) in the infected flowers.

Discussion

The efficacy of VIGS in other plant models has been demonstrated using silencing of phytoene desaturase (*PDS*). *PDS* catalyzes a key step in carotenoid biosynthesis, and loss of *PDS* function causes a characteristic photo-bleaching phenotype resulting from absence of protective carotenes (Kumagai *et al*., 1995; Ratcliff *et al*., 2001). Infection of petunia plants with TRV *PhPDS* resulted in leaf photobleaching similar to that demonstrated in other plants. Our data show the potential of VIGS as a tool for examining the function of genes associated with flower development and senescence. Observation of floral phenotypes resulting from loss of function of target genes using stable transformation and regeneration in petunia takes approximately 6 months per gene. In contrast, the VIGS system provides an opportunity for rapid throughput, since vegetative phenotypes may be observed within a week, and floral phenotypes within 3 weeks. Systemic viral movement typically results in a mosaic pattern of infection (Reid and Matthews, 1966). Knowing where VIGS-mediated gene silencing takes place is crucial for studying floral senescence since silencing of senescence-related genes may not result in an immediately visible phenotype.

Expression of *CHS* is required for production of the pigment in purple petunia flowers, and *CHS* has previously been used as a reporter gene in studies of co-suppression and PTGS in petunia (Atkinson *et al*., 1998; Napoli *et al*., 1990; Stam *et al*., 2000). We found *CHS* to be an ideal gene-silencing reporter for VIGS in floral tissues. Infection of petunias with TRV incorporating a fragment of *CHS* in the RNA2 resulted in striking patterns of highly visible white patches and sectors on purple corollas, and even entirely white flowers on purple-flowered cultivars. Napoli *et al*. (1990) used the V26 cultivar for their studies, but silencing of CHS with the VIGS systems in this cultivar resulted in a highly dissected mosaic pattern (Figure 4). In contrast, TRV *CHS* in other purple-flowered commercial hybrid cultivars generated large white sectors (Figure 4). Our results indicate that genetic background has a strong influence on the gene-silencing phenotype. Silencing of some flowers and not others on the same plant is consistent with non-uniform movement of RNA2. From the 8 cultivars that we tested, we chose ‘Fantasy Blue’ for later experiments because of speed of infection, because its naturally dwarf habit makes for easier handling of large numbers of plants, and because of its desirable silencing flower phenotype, displaying clean white sectors and entirely white flowers (Figure 4).

The key to our planned strategy for examining the role of unknown senescence-associated genes in flower senescence is the suggestion that silencing multiple genes within the same viral construct will allow silencing of a unknown gene to be linked to silencing of a gene with a visible phenotype (Turnage *et al*., 2002). To test this concept, we infected petunia plants with a TRV *PDS/CHS* construct. The results we obtained were as predicted by Turnage *et al*. (2002). RT-PCR analysis demonstrated that *CHS* was silenced in leaves

showing photo-bleaching (the phenotype for *PDS* loss of function), and that *PDS* was silenced in white petal sectors (the phenotype for *CHS* loss of function), clear proof that the TRV VIGS system results in silencing of multiple genes (Figure 1 and 3, Table 1B).

We finally examined a known senescence-associated gene in a tandem TRV construct by combining an *ACO* fragment and the *CHS* reporter. ACC oxidases catalyze the last step of ethylene production, converting ACC to ethylene (Kende, 1993). During flower senescence, increased ethylene production is associated with the increased ACC oxidase activity (Woodson *et al.*, 1992). In petunia, ACC oxidase is encoded by a small gene family consisting of 4 members with 80% similarity at the DNA sequence level (Tang *et al.*, 1993). One of the genes (*ACO*2) is a non-transcriptional pseudogene, and the other three are induced by ethylene (Tang *et al.*, 1993). Pollination of petunia flowers leads to a rapid increase in ACC oxidase activity followed by increased ethylene production by styles and subsequently induces increased ethylene production by the corolla, which accelerates flower senescence (Tang and Woodson, 1996). White sectors of flowers infected with the TRV *CHS*/*ACO* vector lasted longer, were less responsive to pollination, and showed reduced ethylene synthesis in response to applied ACC than purple sectors. We conclude that the senescence-associated ACC oxidase gene was silenced by the tandem construct. Quantitative RT-PCR analysis demonstrated a reduction in the abundance not only of the homologous sequence (ACO4), but also the highly similar ACO1 sequence. The reduced effect of stimulators of senescence (ACC and pollination) in the infected tissues presumably reflects a general down-regulation in the abundance of ACO.

Previous research has suggested that diffusion of ethylene through the intercellular space may be the means by which pollination of the stigma results in rapid corolla senescence (Woltering, 1996). Since we observed that contiguous control and silenced sectors in the *CHS*/*ACO* infected plants showed very different responses to pollination and to applied ACC (Figure 7), it appears that the response to these stimuli is a cell-specific phenomenon, probably not related to the diffusion of ethylene throughout the intercellular spaces of the flower.

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