

TECHNICAL ADVANCE

Virus-induced gene silencing is an effective tool for assaying gene function in the basal eudicot species *Papaver somniferum* (opium poppy)

Lena C. Hileman¹, Sinéad Drea¹, Gemma de Martino¹, Amy Litt^{1,†} and Vivian F. Irish^{1,2,*}

Departments of ¹Molecular, Cellular and Developmental Biology and

²Ecology and Evolutionary Biology, PO Box 208104, Yale University, New Haven, CT 06520-8104, USA

Received 31 May 2005; revised 5 July 2005; accepted 11 July 2005.

*For correspondence (fax 203 432 5711; e-mail vivian.irish@yale.edu).

†Present address: The New York Botanical Garden, 200th St. and Kazimiroff, Bronx, NY 10458-5126, USA.

Summary

Virus-induced gene silencing (VIGS) is an attractive method for assaying gene function in species that are resistant to conventional genetic approaches. However, VIGS has been shown to be effective in only a few, closely related plant species. Tobacco rattle virus (TRV), a bipartite RNA virus, has a wide host range and so in principle could serve as an efficient vector for VIGS in a diverse array of plant species. Here we show that a vector based on TRV sequences is effective at silencing the endogenous *phytoene desaturase* (*PapsPDS*) gene in *Papaver somniferum* (opium poppy). We show that this vector does not compromise the growth or reproduction of poppy and the plants did not display viral symptoms. The silencing of *PapsPDS* resulted in a significant reduction in *PapsPDS* mRNA and a concomitant photobleached phenotype. The ability to rapidly assay gene function in *P. somniferum* will be valuable in manipulation of the opiate pathway in this pharmaceutically important species. We suggest that our vacuum infiltration method used to deliver TRV-based vectors into poppy is a promising approach for expanding VIGS to diverse angiosperm species in which traditional delivery methods fail to induce VIGS. Furthermore, these studies demonstrate the utility of TRV-VIGS for probing gene function in a basal eudicot species that is phylogenetically distant from model plant species.

Keywords: TRV-VIGS, virus-induced gene silencing, *Papaver somniferum*, poppy, *phytoene desaturase*.

Introduction

Genetic transformation is a necessary prerequisite to determining the function of gene products in species where traditional forward genetic approaches are not possible. In plants, *Agrobacterium*-mediated transformation approaches are commonly used in a variety of model systems including *Arabidopsis* (Lindsey and Wei, 2000), rice (Hiei *et al.*, 1994) and tomato (Fillatti *et al.*, 1987). This strategy generally relies on transforming explanted plant tissue, and from these regenerating transgenic plants via tissue culture. For many species these approaches are limited due to difficulties in defining fast and effective means to regenerate large populations of transgenic plants.

The recent accumulation of plant genomic data, including expressed sequence tag (EST) library and genome sequencing projects (<http://www.ncbi.nlm.nih.gov/genomes/PLANTS/PlantList.html>), makes high-throughput functional studies in diverse angiosperm species an exciting prospect, but the difficulties faced in developing genetic transformation protocols for multiple plant species remain daunting. Virus-induced gene silencing (VIGS; Ruiz *et al.*, 1998) is an attractive alternative approach for determining the biological function of gene products in species that are not easily amenable to traditional *Agrobacterium*-mediated transformation protocols. Therefore, developing VIGS as a tool for

reverse genetics in diverse plant species will facilitate time-efficient large-scale functional studies.

VIGS takes advantage of the fact that plants induce homology-dependent defense mechanisms in response to attack by viruses. Therefore viruses can be engineered to target a host gene(s) of interest for silencing (Burch-Smith *et al.*, 2004; Robertson, 2004). VIGS probably acts through the same or similar mechanism as post-transcriptional gene silencing (PTGS; Robertson, 2004), also known as RNA interference or quelling in animal and fungal systems (Cogoni and Macino, 2000). VIGS or PTGS results in the degradation of endogenous RNA with extensive sequence complementarity to endogenous microRNA (miRNA), exogenous transgenes or viral RNA (Fagard and Vaucheret, 2000).

The trigger for PTGS is double-stranded RNA (ds RNA; Fire *et al.*, 1998). In VIGS, double-stranded viral RNA is generated by RNA-dependent RNA polymerase (RdRP) encoded in the viral genome. Double-stranded RNA is detected by the host plant genome as aberrant and is cleaved into small interfering RNA (siRNA) by the action of DICER (Hamilton and Baulcombe, 1999; Hammond *et al.*, 2000; Zamore *et al.*, 2000). Through sequence complementarity, siRNA in association with an RNA-induced silencing complex (RISC), directs the cleavage of endogenous RNA transcripts (Hammond *et al.*, 2000). Small interfering RNA is also responsible for amplifying the silencing signal by priming endogenous RNA which can be converted to dsRNA by the action of RdRP encoded in the plant genome (Makeyev and Bamford, 2002; Tang *et al.*, 2003).

Tobacco rattle virus (TRV) is one of a handful of plant viruses that have been modified to serve as a vector to facilitate the silencing of endogenous target genes in host plants (Liu *et al.*, 2002c; Ratcliff *et al.*, 2001). TRV is a bipartite single-stranded RNA (ssRNA) tobnavirus (MacFarlane 1999). To expedite cloning and transfer to plant hosts, cDNA versions of TRV sequences have been introduced into an *Agrobacterium* T-DNA binary vector. Multicloning sites (Liu *et al.*, 2002c) or recombination (Liu *et al.*, 2002b) are used to insert a fragment of an endogenous gene of interest, such that it is converted to dsRNA as part of viral replication. The presence of dsRNA complementary to endogenous transcripts results in the degradation of these dsRNAs along with the silencing of their endogenous mRNA counterparts. To date, TRV-based vectors have been used to silence endogenous genes in multiple Solanaceae genera including *Nicotiana* (Abbink *et al.*, 2002; Ahn *et al.*, 2004; Bouarab *et al.*, 2002; Jin *et al.*, 2002; Liu *et al.*, 2002a,b,c, 2004a,b; Peart *et al.*, 2002a,b; del Pozo *et al.*, 2004; Ratcliff *et al.*, 2001; Rivas *et al.*, 2004; Ryu *et al.*, 2004; Saedler and Baldwin, 2004; Valentine *et al.*, 2004), *Capsicum* (Chung *et al.*, 2004; Ryu *et al.*, 2004), *Solanum* (Brigneti *et al.*, 2004; Ryu *et al.*, 2004), *Lycopersicon* (Ekengren *et al.*, 2003; Liu *et al.*, 2002b; del Pozo *et al.*, 2004; Rivas *et al.*, 2004; Valentine *et al.*, 2004) and *Petunia* (Chen

et al., 2004; Ryu *et al.*, 2004), as well as endogenous genes involved in root development in *Arabidopsis* (Valentine *et al.*, 2004). Using VIGS in Solanaceae, a number of genes have been functionally characterized. The biological role of these genes ranges from pathogen defense (Bouarab *et al.*, 2002; Brigneti *et al.*, 2004; Ekengren *et al.*, 2003; Jin *et al.*, 2002; Liu *et al.*, 2002a,b,c, 2004b; Peart *et al.*, 2002a,b; del Pozo *et al.*, 2004; Rivas *et al.*, 2004; Valentine *et al.*, 2004) to metabolic processes (Abbink *et al.*, 2002; Ahn *et al.*, 2004; Chen *et al.*, 2004; Ryu *et al.*, 2004; Saedler and Baldwin, 2004) and developmental patterning (Liu *et al.*, 2004a; Ratcliff *et al.*, 2001; Valentine *et al.*, 2004), indicating that TRV-based VIGS can be used to target genes expressed at different stages, in different tissues and in different pathways.

Here we describe the novel use of TRV-mediated gene silencing in the basal eudicot species *Papaver somniferum* (opium poppy). We show a 3.2- to 7.5-fold reduction in endogenous transcript levels of the *P. somniferum phytoene desaturase* gene (*PapsPDS*; Demmig-Adams and Adams, 1992; Ruiz *et al.*, 1998), resulting in a photobleached phenotype, in VIGS-treated poppy plants. Poppy is of agronomic importance in that it is the primary source of opiate production (Allen *et al.*, 2004). Therefore, the ability to assess gene function in this species will have important ramifications, including the possibility of manipulation of the morphine biosynthetic pathway. In addition, the poppy family is a member of the basal eudicot clade which also contains buttercups, barberry and curare. While some members of this clade have significant pharmaceutical or ornamental value, there has been little progress in developing any of these species as a model system for functional genomics. Poppy diverged from Solanaceae and *Arabidopsis* at least 100 million years ago (Crepet *et al.*, 2004), and therefore represents a phylogenetically distant species from those that have previously been shown to be susceptible to TRV-mediated VIGS. This, along with the fact that TRV has a broad host range (<http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdb/index.htm>), spanning much of angiosperm diversity, and that TRV-mediated VIGS affects both vegetative and floral development in poppy, suggests that TRV-VIGS will be a powerful tool for investigating gene function in a wide range of species.

Results

TRV-based VIGS of *PapsPDS* in *P. somniferum*

To determine if endogenous gene silencing can be elicited by a TRV-based VIGS system in poppy we generated a TRV2 construct (Liu *et al.*, 2002c) that carries a 436 bp fragment of *PapsPDS* (Figure 1). The *PapsPDS* fragment was amplified from cDNA using primers corresponding to a conserved region present in *PDS* genes from diverse angiosperm species (see Experimental procedures). *PDS* is an ideal gene for

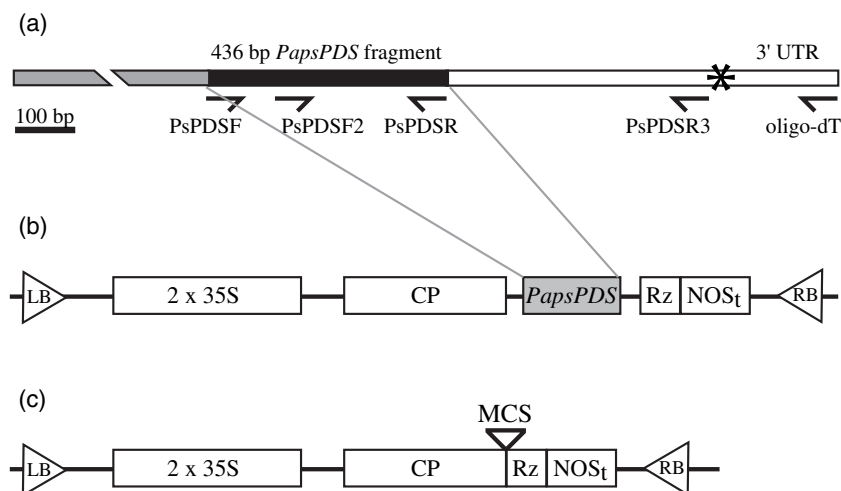


Figure 1. Constructs used in this study.

(a) Map of *PapsPDS*. The black area is the 436 bp fragment cloned into TRV2 and the gray area represents the 5' end. The primers used for amplification and sequencing are indicated below. The asterisk represents the stop codon.
 (b) Map of the TRV2-*PapsPDS* construct used for VIGS. The gray area is the 436 bp *PapsPDS* fragment.
 (c) Map of TRV2-E used for mock VIGS: LB, left border; RB, right border; CP, coat protein; Rz, self-cleaving ribozyme; NOS_t, NOS terminator.

assaying VIGS because it encodes an enzyme required for the biosynthesis of carotenoids, which in turn protect chlorophyll from photo-oxidation (Demmig-Adams and Adams, 1992). Silencing of *PDS* in several species has been shown to result in decreased carotene content and ultimately to the easily observed symptom of leaf photobleaching (Benedito *et al.*, 2004).

Endogenous gene silencing by TRV-based VIGS is facilitated by *Agrobacterium*-mediated delivery of TRV vectors into host plant cells (Lu *et al.*, 2003; Robertson, 2004). Once TRV1 and TRV2 have been introduced into plant cells, the viral machinery of TRV1 is responsible for the replication and movement of both TRV1 and TRV2 (Robertson, 2004). Standard methods for *Agrobacterium* inoculation of Solanaceae species include direct injection into leaves, misting (Dinesh-Kumar *et al.*, 2003) or agroinfiltration (exposing the crown of the plant to *Agrobacterium*; Ryu *et al.*, 2004). We assessed each of these methods for VIGS in poppy (data not shown) but found that only vacuum infiltration of *Agrobacterium*, transformed with TRV2-*PapsPDS*, into poppy plants successfully silenced endogenous *PapsPDS* expression, resulting in the expected photobleaching phenotype (Figure 2). Poppy plants at early (three to five leaves), mid (six to eight leaves) or late (eight to 11 leaves) stages of development were vacuum infiltrated with *Agrobacterium* (strain GV3101 or EHA105) carrying TRV1 and either TRV2-*PapsPDS* or a TRV2 empty vector (TRV2-E). Three independent trials for each condition were carried out. Fourteen to 17 days post-infiltration a photobleached phenotype was observed in the newly emerging leaves of 4.8 to 23.8% of TRV2-*PapsPDS*-treated plants (Table 1 and data not shown). The photobleached phenotype was easily detectable throughout subsequent stages of development (Figure 2). In mock-treated samples containing TRV1 and TRV2-E, plant growth and reproductive capabilities were unaffected by the presence of virus and no viral symptoms were noted (Figure 2, Table 1 and data not shown).

The photobleached phenotype is observed in P. somniferum flowers

Although many *PapsPDS* silenced plants showed a severe phenotype, with all newly emerging leaves being completely photobleached (e.g. Figure 2b), some *PapsPDS* silenced plants exhibited a less severe phenotype with a patchy pattern of photobleaching. Plants with severe photobleaching had high levels of early mortality, presumably due to extreme photo-oxidation. Many plants with less severe phenotypes survived to reproduction (about 20 weeks post-infiltration under growth chamber conditions). Strikingly, in these plants the photobleached phenotype persisted in flowers, specifically in the sepals (Figure 2e) and carpels (Figure 2c). This indicates that TRV-based VIGS is capable of eliciting a gene silencing phenotype in poppy for at least 20 weeks after inoculation. Petals in photobleached plants were indistinguishable in color from those of mock-treated or untreated poppy plants, since petal pigmentation is not dependent on carotenoids (Figure 2f,g).

Infiltration at earlier stages results in a higher percentage of photobleached plants

Poppy plants were infiltrated at early, mid and late stages of seedling development, and our results suggest that both TRV-VIGS-treated and mock-treated plants infiltrated at later stages were better able to survive the infiltration treatment (91.7–96.5% survival for later stage treatments compared with 58.5–85.6% survival for early and mid-stage treatments, Table 1). However, of the plants that survived, those infiltrated with *Agrobacterium* at earlier stages showed a higher percentage of the photobleached phenotype (15.2–23.8% for early and mid-stage treatments compared with 4.8–11.5% for late stage treatments; Table 1). Results from infiltration experiments using *Agrobacterium* strains GV3101 and EHA105 suggest that both strains were equally efficient at

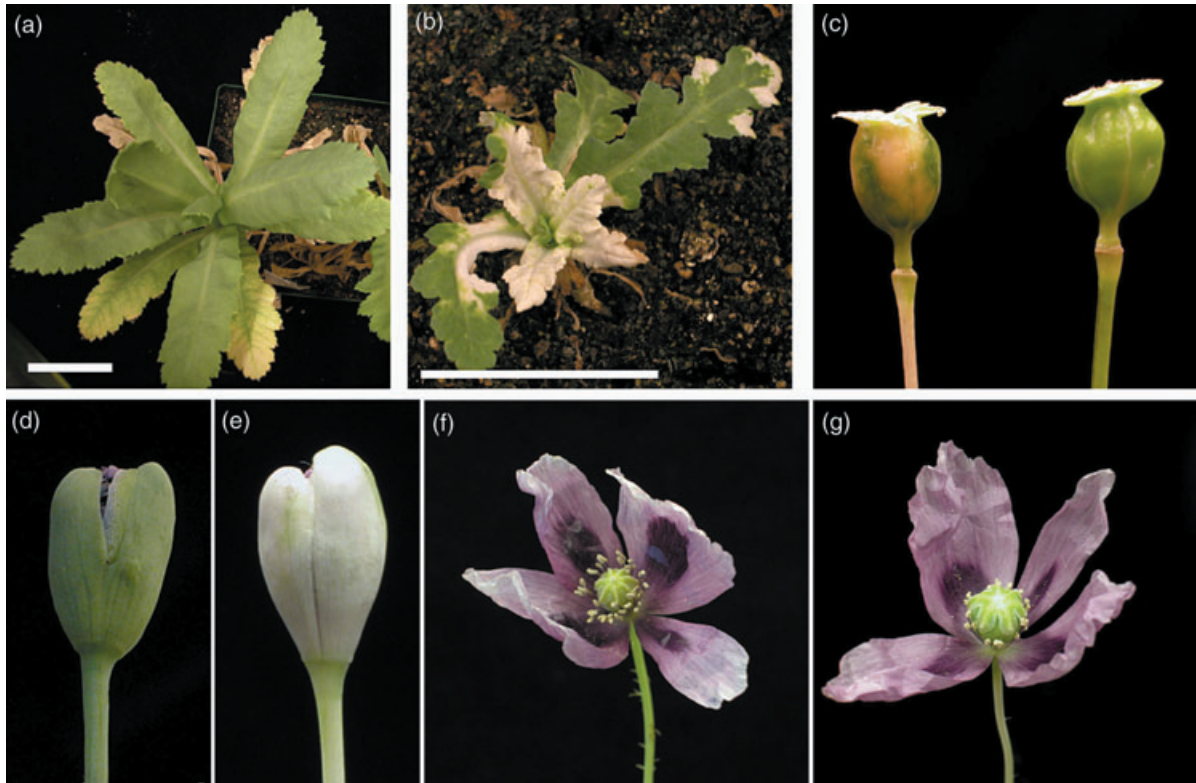


Figure 2. VIGS of *PapsPDS* results in photobleaching in *P. somniferum*.

(a) Mock-treated poppy plant 104 days post-TRV inoculation. Mock treatment does not result in a photobleached phenotype.

(b) VIGS-treated poppy plant 123 days post-TRV infiltration showing a photobleached phenotype. A photobleached phenotype is observed in the carpels (c, left) and sepals (e) of VIGS-treated poppy plants, but not in the carpels (c, right) or sepals (d) of mock-treated plants. Petals from VIGS-treated poppy plants (g) are indistinguishable from mock-treated plants (f) because carotenoid biosynthesis is not responsible for pigmentation in poppy petals. All plants were inoculated with *Agrobacterium* EHA105.

(c–g) Poppy plants 122–149 days post-inoculation. Scale bars = 3.5 cm.

Table 1 Rates of survival and silencing observed in TRV-VIGS-treated poppy plants

	No. of leaves at infiltration					
<i>Agrobacterium</i> strain	3–5		6–8		8–10	
GV3101						
Introduced construct	TRV2-E	TRV2-PapsPDS, <i>n</i> = 270	TRV2-E, <i>n</i> = 90	TRV2-PapsPDS, <i>n</i> = 270	TRV2-E, <i>n</i> = 86	TRV2-PapsPDS, <i>n</i> = 180
% survival	N/A	58.5	77.8	72.2	96.5	91.7
% phenotype ^a	N/A	17.7	0	15.2	0	4.8
	3–5		6–8		9–11	
EHA105						
Introduced construct	TRV2-E, <i>n</i> = 90	TRV2-PapsPDS, <i>n</i> = 117	TRV2-E, <i>n</i> = 234	TRV2-PapsPDS, <i>n</i> = 201	TRV2-E, <i>n</i> = 90	TRV2-PapsPDS, <i>n</i> = 81
% survival	85.6	76.9	82.9	62.7	93.3	96.2
% phenotype ^a	0	15.4	0	23.8	0	11.5

^aPer cent of surviving plants exhibiting phenotype.

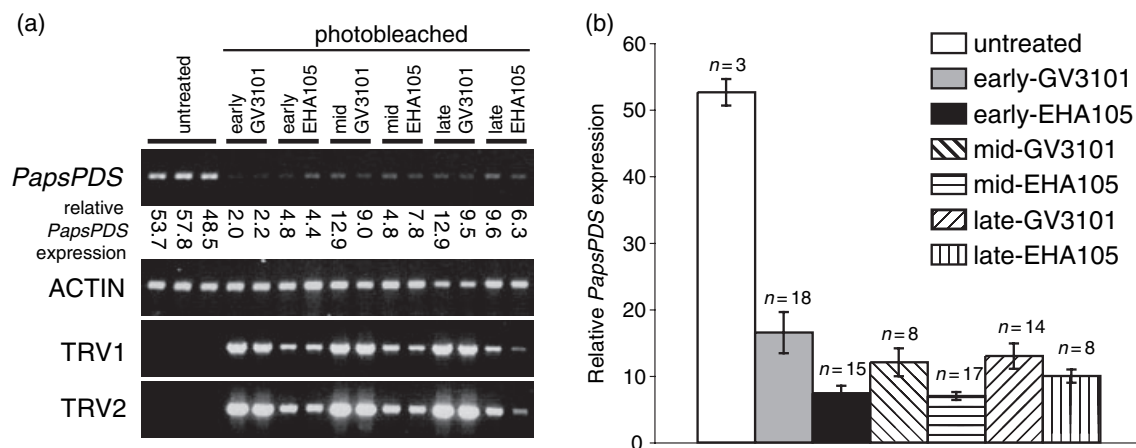


Figure 3. *PapsPDS* transcripts are reduced in VIGS poppy plants exhibiting a photobleached phenotype.

(a) Select semiquantitative RT-PCR results for *PapsPDS*. Relative *PapsPDS* levels were normalized against *ACTIN*. *TRV1* and *TRV2* RNA were detected in VIGS poppy plants exhibiting a photobleached phenotype, but not in untreated plants.

(b) Relative *PapsPDS* expression levels were determined by semiquantitative RT-PCR (as in A) for multiple VIGS poppy plants exhibiting a photobleached phenotype. *PapsPDS* expression levels are reduced by 3.2- to 7.5-fold compared with untreated plants. Standard errors are shown. Early, mid and late refer to the age of poppy plants at the time of infiltration. GV3101 and EHA105 are the strains of *Agrobacterium* used for infiltration.

delivering TRV vectors to poppy cells to elicit VIGS of *PapsPDS* (Table 1).

PapsPDS transcripts are reduced in photobleached *P. somniferum* plants

To confirm that the observed photobleached phenotype in TRV2-*PapsPDS*-treated poppy plants is due to silencing of *PapsPDS*, we assessed the relative abundance of *PapsPDS* mRNA using semi-quantitative RT-PCR in plants exhibiting a photobleached phenotype compared with untreated poppy plants. We found that photobleached plants exhibited a mean reduction of 3.2- to 7.5-fold in *PapsPDS* mRNA levels compared with untreated plants (Figure 3). In addition, the expression data suggest that when TRV vectors are introduced into poppy with EHA105, stronger *PapsPDS* suppression results (5.3- to 7.5-fold reduction on average for infiltration with EHA105 compared with 3.2- to 4.4-fold reduction on average for infiltration with GV3101).

To confirm that the observed photobleached phenotype is correlated with the presence of TRV vectors, we showed by RT-PCR that both TRV1 and TRV2-*PapsPDS* RNA was present in leaves from 100% of sampled plants exhibiting a photobleached phenotype (Table 2). On the other hand, TRV1 RNA was present in 3.1% of treated plants that do not show a photobleached phenotype, but both TRV1 and

TRV2-*PapsPDS* RNA were never detected in these plants (Table 2). The presence of both TRV1 and TRV2-E RNA was detected in 13.5% of sampled mock-treated poppy plants. None of these plants exhibited a photobleached phenotype, confirming that the TRV2-*PapsPDS* vector is responsible for *PapsPDS* silencing and the photobleached phenotype in VIGS-treated poppy plants.

Discussion

TRV-VIGS is effective in P. somniferum

We have shown that TRV-VIGS is a rapid and efficient method for assessing gene function in *P. somniferum*. We investigated several strategies for introducing TRV vectors via *Agrobacterium*-mediated transfer into host plant cells. Vacuum infiltration of *Agrobacterium* was far more effective than other strategies tested, with different *Agrobacterium* strains showing similar gene silencing results, although EHA105 may be slightly more effective in reducing endogenous mRNA levels (Figure 3). The levels of silencing that we observed are in line with those found in other studies, although it is clear that levels of silencing depend on a host of environmental variables, including temperature, growth conditions and stage of inoculation (Robertson, 2004). Infiltration of poppy at earlier stages gave higher percentages of

Table 2 Detection of viral RNAs by RT-PCR in treated poppy plants

	Infiltrated with TRV1 and TRV2- <i>PapsPDS</i> ; photobleached, <i>n</i> = 96	Infiltrated with TRV1 and TRV2- <i>PapsPDS</i> ; non-photobleached, <i>n</i> = 96	Infiltrated with TRV1 and TRV2-E; non-photobleached, <i>n</i> = 96
TRV1 and TRV2	100%	0	13.5%
TRV1 only	0	3.1%	5.2%

silencing (Table 1) and silencing could be observed throughout plant development, including in developing flowers and fruits (Figure 2). These observations indicate that gene products involved in a variety of developmental and biosynthetic processes in poppy should be amenable to functional dissection using TRV-VIGS. Furthermore, TRV-VIGS has been shown to be effective in silencing genes in meristematic regions (Liu *et al.*, 2004a; Ratcliff *et al.*, 2001), opening up the possibility of studying gene function at early stages of development.

Opium poppy is the main source of the analgesic alkaloids morphine and codeine (Kapoor, 1995). The biosynthetic pathways controlling the production of these alkaloids is largely understood (Allen *et al.*, 2004), but the chemical nature of these alkaloids makes it economically unfeasible to synthesize these compounds in the laboratory (Memelink, 2004). As such, purification from poppy latex remains the most efficient way to isolate these compounds. Modifications of the opiate biosynthetic pathways, and the properties of the resulting opiates, have thus relied on conventional plant breeding, forward mutagenesis procedures as well as stable *Agrobacterium*-mediated transformation technologies (Allen *et al.*, 2004; Kapoor, 1995; Millgate *et al.*, 2004). However, such approaches are in their infancy and are hampered by their relative inefficiency. Taking a TRV-VIGS approach to the study of opiate biosynthesis has the potential to facilitate pharmaceutical research without relying entirely on the laborious process of genetic transformation in poppy.

TRV-VIGS as a tool for functional analysis in a wide array of species

Most studies of TRV-VIGS have focused on the Solanaceae (reviewed in Burch-Smith *et al.*, 2004) and little work has been carried out to date to assess the potential of this method in diverse angiosperm species. EST databases and genomic sequencing efforts are currently ongoing for a variety of angiosperm species (see <http://www.ncbi.nlm.nih.gov/genomes/PLANTS/PlantList.html>), yet functional approaches to assess the endogenous roles of these newly identified genes lag far behind. By demonstrating the potential for functional dissection in *P. somniferum*, a species that has diverged from model systems such as *Arabidopsis* and tomato by at least 100 million years, we have provided a proof of concept that such strategies can be utilized in distantly related angiosperm species.

TRV-VIGS will most likely be effective in silencing endogenous genes in species that have previously been shown to act as host to TRV. Species that have been shown to be hosts include a variety of core eudicots and monocots (<http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/index.htm>; APG, 1998). In the core eudicots, host species are found in seven, eight and four families of the rosid, asterid and Caryophyllid

lineages, respectively. This accounts for the major diversity of core eudicot species. In the monocots, host species are found in five families representing much of monocot diversity. Host susceptibility has not been reported in other major angiosperm groups such as the basal eudicots (which include poppy), the paleoherbs or the basal angiosperms. The fact that we show TRV-VIGS is effective at silencing endogenous genes in poppy suggests that TRV-VIGS will be even more broadly applicable than expected from previously determined host susceptibility data.

We have also shown that vacuum infiltration of TRV vectors, a method rarely used in solanaceous species (Ekengren *et al.*, 2003), induced gene silencing in poppy where standard methods of delivery including injection, misting or agro-drench (Dinesh-Kumar *et al.*, 2003; Ryu *et al.*, 2004) failed. Many species may have various anatomies or physiologies that make them resistant to standard methods for TRV-VIGS, such as poppy with copious latex, plants with highly lignified leaves or plants in which silencing needs to be induced at later stages of development. Adding vacuum infiltration as a standard approach to applying TRV-VIGS to a new species expands the available tools for VIGS across angiosperms and increases the potential for high-throughput studies in non-model species.

Experimental procedures

Identification of PapsPDS from P. somniferum

RNA was isolated from *P. somniferum* var. *nigrum* (Sand Mountain Herbs, Fyffe, AL, USA) leaf tissue using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Complementary DNA was generated using Superscript III reverse transcriptase (Invitrogen) according to manufacturer's instructions. A 436 bp fragment of *PapsPDS*, corresponding to base pairs 778–1214 of the *Arabidopsis thaliana* *PDS* coding sequence, was amplified from cDNA using primers corresponding to conserved regions of PDS; PsPDSF (Figure 1; 5'-GAGGTGTTTCATCGCAATGTCAAAGGC-3') and PsPDSR (Figure 1; 5'-GTGTTGTTGAGCTTCGTCAAAC-CATATATG-3'). Conserved regions for primer design were determined using a nucleotide alignment that included PDS from *A. thaliana* (NM117498), *Lycopersicon esculentum* (M88683), *Cap-sicum annuum* (X68058), *Solanum tuberosum* (AY484445), *Pisum sativum* (AJ21573), *Glycine max* (M64704), *Gentiana lutea* (AB028665), *Citrus sinensis* (AJ319761) and *Zea mays* (U37285). The 436 bp *PapsPDS* PCR fragment was cloned into TOPO TA 2.1 (Invitrogen), and subcloned into TRV2 (Figure 1; Liu *et al.*, 2002c) using *EcoRI*. A sequence-confirmed TRV2-PapsPDS construct, with the 436 bp fragment in the sense orientation (Figure 1), was used for subsequent VIGS experiments.

The C-terminal end of the *PapsPDS* gene was amplified using PsPDSF2 (Figure 1; 5'-TGGTAATCCTCCCGAGAG-3') and an oligo-dT reverse primer. PCR products were cloned into the TOPO-TA 2.1 vector and sequenced. Based on the C-terminal sequence of *PapsPDS*, a reverse primer, PsPDSR3 (Figure 1; 5'-GCAGCAAGCATCTCACAATC-3') was designed for expression analysis. The poppy *PapsPDS* sequence has been deposited in GenBank (accession no. DQ116056).

Germination of *P. somniferum*

Poppy seeds were treated with 70% ethanol (EtOH) for 1 min, followed by sterilization in three 10 min changes of 50% bleach and rinsed three times in distilled water. Seeds were vernalized on vermiculite in the dark at 4°C for 10 days before being transferred to growth chambers for germination (21°C; 16 h light/8 h dark). The seeds germinated 3–4 days after transfer to growth chambers.

Preparation of *Agrobacterium*

Agrobacterium for VIGS was prepared according to a modified protocol (Dinesh-Kumar *et al.*, 2003). Two strains of *Agrobacterium tumefaciens* (GV3101 and EHA105) were used to introduce TRV-based VIGS vectors into poppy plants. Electrocompetent *Agrobacterium* was transformed with TRV1, TRV2-PapsPDS or an empty TRV2 vector (TRV2-E). Single colonies were used to inoculate 3 ml Luria Broth (LB) overnight cultures. These cultures were PCR screened for the presence of the appropriate construct using primers pYL156F (5'-GGTCAAGGTACGTAGTAGAG-3') and pYL156R (5'-CGAGAATGTCAATCTCGTAGG-3') for *Agrobacterium* transformed with TRV2, and primers OYL195 (5'-CTTGAAGAAGAA-GACTTTCGAAGTCTC-3') and OYL198 (5'-GTAAAATCATTGATAAC-AACACAGACAAAC-3') for *Agrobacterium* transformed with TRV1, and further used to inoculate 1 l LB (containing 10 mM MES (2-[N-morpholino]ethanesulfonic acid) and 20 µM acetosyringone). *Agrobacterium* from 1 l overnight cultures were pelleted at 3000 g and resuspended in inoculation solution (10 mM MES, 200 µM acetosyringone, 10 mM MgCl₂) to an absorbance at 260 nm (Abs₂₆₀) of 1.5. *Agrobacterium* resuspended in inoculation solution was incubated at room temperature overnight before being used in poppy vacuum infiltration.

Vacuum infiltration of *Agrobacterium* carrying TRV1 and TRV2 constructs into *P. somniferum*

Poppy plants were infiltrated at three different stages of seedling development: early stage, three to five leaves; mid stage, six to eight leaves; later stage, eight to 11 leaves. At each of these stages, seedlings were infiltrated with a 1:1 mixture of *Agrobacterium* carrying TRV1 and TRV2-PapsPDS. As a mock treatment, poppy plants at early, mid and late stages were infiltrated with a 1:1 mixture of *Agrobacterium* carrying TRV1 and TRV2-E.

Seedlings at early, mid and late stages were removed from the vermiculite, vermiculite was rinsed from the roots in distilled water, and rinsed seedlings were kept on wet paper towels before infiltration. Approximately 20 seedlings were infiltrated at a time. *Agrobacterium* (resuspended in infiltration solution) carrying TRV1 and *Agrobacterium* carrying TRV2-PapsPDS or TRV2-E, were mixed in a 1:1 ratio and Silwet was added to a final concentration of 0.05%. Seedlings were submerged into the 1:1 mixture of *Agrobacterium* + Silwet and vacuum infiltrated for 1 min. Following vacuum infiltration, seedlings were rinsed briefly in infiltration solution and moved to wet paper towels before replanting. Infiltrated seedlings were planted in a 1:1 mixture of vermiculite and soil (Super Fine Germinating Mix; Fafard Co., Agawam, MA, USA).

Analysis of PapsPDS, TRV1 and TRV2 expression

Two to 5 weeks following observed leaf photobleaching, early emerging leaves were collected from 96 poppy plants infiltrated with TRV1 and TRV2-PapsPDS exhibiting a photobleached phenotype, and from 96 plants infiltrated with TRV1 and TRV2-PapsPDS but not

exhibiting a photobleached phenotype. In additions, early emerging leaves were collected from 96 mock-treated poppy plants. RNA was extracted from these tissues using Trizol and cDNA was generated using Superscript III. The presence of TRV1 (primers OYL195, OYL198) and TRV2 (primers PYL156F, PYL156R) RNA in VIGS-treated and mock-treated plants was determined by RT-PCR. The relative mRNA levels of *PapsPDS* (primers PsPDSF2, PsPDSR3; Figure 1), and *ACTIN* (Prasad *et al.*, 2001) were determined in wild-type and VIGS-treated poppy plants using semiquantitative RT-PCR. Expression levels of *PapsPDS* relative to *ACTIN* were determined for eight to 18 VIGS-treated individuals showing a photobleached phenotype for each of the six experimental conditions (early, mid and older plants infiltrated with *Agrobacterium* GV3101 or EHA105). Relative *PapsPDS* expression in photobleached leaves was compared with relative *PapsPDS* expression in leaves from three untreated poppy plants. Semiquantitative RT-PCR was replicated 14 times for each of the three untreated leaf samples. The linear range of amplification for both *PapsPDS* and *ACTIN* in untreated poppy was determined to be between 24 and 34 cycles, and 26 cycles of PCR were used for all semiquantitative RT-PCR reactions. Semiquantitative RT-PCR gel images were scanned as jpeg documents and relative RT-PCR band intensities were analyzed using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>).

Acknowledgements

This work was supported by grant no. IBN-0110731 from the National Science Foundation to V.F.I. The authors thank Takudzwa Shumba and Lara Appleby for help with experiments, Drs S. P. Dinesh-Kumar and Yule Liu, Yale University, for providing TRV vectors, advice on applying standard VIGS protocols to poppy and comments on early versions of this manuscript, and Dr Zhanyuan Zhang, University of Missouri-Columbia, for providing the *Agrobacterium* EHA105 strain.

References

- Abbink, T.E.M., Peart, J.R., Mos, T.N.M., Baulcombe, D.C., Bol, J.F. and Linthorst, H.J.M. (2002) Silencing of a gene encoding a protein component of the oxygen-evolving complex of photosystem II enhances virus replication in plants. *Virology*, **295**, 307–319.
- Ahn, J.W., Kim, M., Lim, J.H., Kim, G.T. and Pai, H.S. (2004) Phyto-calpain controls the proliferation and differentiation fates of cells in plant organ development. *Plant J.* **38**, 969–981.
- Allen, R.S., Millgate, A.G., Chitty, J.A., Thisleton, J., Miller, J.A.C., Fist, A.J., Gerlach, W.L. and Larkin, P.J. (2004) RNAi-mediated replacement of morphine with the nonnarcotic alkaloid reticuline in opium poppy. *Nat. Biotechnol.* **22**, 1559–1566.
- APG (1998) An ordinal classification for the families of flowering plants. *Ann. MO Bot. Gard.* **85**, 531–553.
- Benedito, V.A., Visser, P.B., Angenent, G.C. and Krens, F.A. (2004) The potential of virus-induced gene silencing for speeding up functional characterization of plant genes. *Genet. Mol. Res.* **3**, 323–341.
- Bouarab, K., Melton, R., Peart, J., Baulcombe, D. and Osbourn, A. (2002) A saponin-detoxifying enzyme mediates suppression of plant defences. *Nature*, **418**, 889–892.
- Brigneti, G., Martin-Hernandez, A.M., Jin, H.L., Chen, J., Baulcombe, D.C., Baker, B. and Jones, J.D.G. (2004) Virus-induced gene silencing in *Solanum* species. *Plant J.* **39**, 264–272.
- 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 J.* **39**, 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.
- Chung, E., Seong, E., Kim, Y.C., Chung, E.J., Oh, S.K., Lee, S., Park, J.M., Joung, Y.H. and Choi, D. (2004) A method of high frequency virus-induced gene silencing in chili pepper (*Capsicum annuum* L. cv. Bukang). *Mol. Cell*, **17**, 377–380.
- Cogoni, C. and Macino, G. (2000) Post-transcriptional gene silencing across kingdoms. *Curr. Opin. Genet. Dev.* **10**, 638–643.
- Crepet, W.L., Nixon, K.C. and Gandolfo, M.A. (2004) Fossil evidence and phylogeny: the age of major angiosperm clades based on mesofossil and macrofossil evidence from cretaceous deposits. *Am. J. Bot.* **91**, 1666–1682.
- Demmig-Adams, B. and Adams, W.W. (1992) Photoprotection and other responses of plants to high light stress. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43**, 599–626.
- Dinesh-Kumar, S.P., Anandakshmi, R., Marathe, R., Schiff, M. and Liu, Y. (2003) Virus-induced gene silencing. *Methods Mol. Biol.* **236**, 287–294.
- Ekengren, S.K., Liu, Y.L., Schiff, M., Dinesh-Kumar, S.P. and Martin, G.B. (2003) Two MAPK cascades, NPR1, and TGA transcription factors play a role in Pto-mediated disease resistance in tomato. *Plant J.* **36**, 905–917.
- Fagard, M. and Vaucheret, H. (2000) Systemic silencing signal(s). *Plant Mol. Biol.* **43**, 285–293.
- Fillatti, J., Kiser, J., Rose, B. and Comai, L. (1987) Efficient transformation of tomato and the introduction and expression of a gene for herbicide tolerance. In *Tomato Biotechnology: Proceedings of a Symposium held at the University of California*, Davis, California, August 20–22, 1986 (Nevins, D. and Jones, R., eds). New York: A.R. Liss, pp. 199–210.
- Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. and Mello, C.C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, **391**, 806–811.
- Hamilton, A.J. and Baulcombe, D.C. (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science*, **286**, 950–952.
- Hammond, S.M., Bernstein, E., Beach, D. and Hannon, G.J. (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature*, **404**, 293–296.
- Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T. (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J.* **6**, 271–282.
- Jin, H.L., Axtell, M.J., Dahlbeck, D., Ekwenna, O., Zhang, S.Q., Staskawicz, B. and Baker, B. (2002) NPK1, an MEK1-like mitogen-activated protein kinase kinase kinase, regulates innate immunity and development in plants. *Dev. Cell*, **3**, 291–297.
- Kapoor, L.D. (1995) *Opium Poppy: Botany, Chemistry and Pharmacology*. New York: Haworth Press, Inc.
- Lindsey, K. and Wei, W. (2000) Tissue culture, transformation, and transient gene expression in *Arabidopsis*. In *Arabidopsis: A Practical Approach*. (Wilson, Z.A., ed.). New York: Oxford University Press, pp. 125–141.
- Liu, Y., Schiff, M., Serino, G., Deng, X.W. and Dinesh-Kumar, S.P. (2002a) Role of SCF ubiquitin-ligase and the COP9 signalosome in the N gene-mediated resistance response to Tobacco mosaic virus. *Plant Cell*, **14**, 1483–1496.
- Liu, Y.L., Schiff, M. and Dinesh-Kumar, S.P. (2002b) Virus-induced gene silencing in tomato. *Plant J.* **31**, 777–786.
- Liu, Y.L., Schiff, M., Marathe, R. and Dinesh-Kumar, S.P. (2002c) 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., Nakayama, N., Schiff, M., Litt, A., Irish, V.F. and Dinesh-Kumar, S.P. (2004a) Virus induced gene silencing of a DEFICIENS ortholog in *Nicotiana benthamiana*. *Plant Mol. Biol.* **54**, 701–711.
- Liu, Y.L., Schiff, M. and Dinesh-Kumar, S.P. (2004b) Involvement of MEK1 MAPKK, NTF6 MAPK, WRKY/MYB transcription factors, COI1 and CTR1 in N-mediated resistance to tobacco mosaic virus. *Plant J.* **38**, 800–809.
- Lu, R., Martin-Hernandez, A.M., Peart, J.R., Malcuit, I. and Baulcombe, D.C. (2003) Virus-induced gene silencing in plants. *Methods*, **30**, 296–303.
- MacFarlane, S.A. (1999) Molecular Biology of the Tobraviruses. *J. Gen. Virol.* **80**, 2799–2807.
- Makeyev, E.V. and Bamford, D.H. (2002) Cellular RNA-dependent RNA polymerase involved in posttranscriptional gene silencing has two distinct activity modes. *Mol. Cell*, **10**, 1417–1427.
- Memelink, J. (2004) Putting the opium in poppy to sleep. *Nat. Biotechnol.* **22**, 1526–1527.
- Millgate, A.G., Pogson, B.J., Wilson, I.W., Kutchan, T.M., Zenk, M.H., Gerlach, W.L. and Fist, A.J. (2004) Analgesia: morphine-pathway block in top1 poppies. *Nature*, **432**, 413–414.
- Peart, J.R., Cook, G., Feys, B.J., Parker, J.E. and Baulcombe, D.C. (2002a) An EDS1 orthologue is required for N-mediated resistance against tobacco mosaic virus. *Plant J.* **29**, 569–579.
- Peart, J.R., Lu, R., Sadanandom, A. et al. (2002b) Ubiquitin ligase-associated protein SGT1 is required for host and nonhost disease resistance in plants. *Proc. Natl Acad. Sci. USA*, **99**, 10865–10869.
- del Pozo, O., Pedley, K.F. and Martin, G.B. (2004) MAPKKK alpha is a positive regulator of cell death associated with both plant immunity and disease. *EMBO J.* **23**, 3072–3082.
- Prasad, K., Sriram, P., Kumar, C.S., Kushalappa, K. and Vijayraghavan, U. (2001) Ectopic expression of rice OsMADS1 reveals a role in specifying the lemma and palea, grass floral organs analogous to sepals. *Dev. Genes Evol.* **211**, 281–290.
- Ratcliff, F., Martin-Hernandez, A.M. and Baulcombe, D.C. (2001) Tobacco rattle virus as a vector for analysis of gene function by silencing. *Plant J.* **25**, 237–245.
- Rivas, S., Rougon-Cardoso, A., Smoker, M., Schauser, L., Yoshioka, H. and Jones, J.D.G. (2004) CITRX thioredoxin interacts with the tomato Cf-9 resistance protein and negatively regulates defence. *EMBO J.* **23**, 2156–2165.
- Robertson, D. (2004) VIGS vectors for gene silencing: many targets, many tools. *Annu. Rev. Plant Biol.* **55**, 495–519.
- Ruiz, M.T., Voinnet, O. and Baulcombe, D.C. (1998) Initiation and maintenance of virus-induced gene silencing. *Plant Cell*, **10**, 937–946.
- Ryu, C.M., Anand, A., Kang, L. and Mysore, K.S. (2004) Agrodrench: a novel and effective agroinoculation method for virus-induced gene silencing in roots and diverse Solanaceous species. *Plant J.* **40**, 322–331.
- Saedler, R. and Baldwin, I.T. (2004) Virus-induced gene silencing of jasmonate-induced direct defences, nicotine and trypsin proteinase-inhibitors in *Nicotiana attenuata*. *J. Exp. Bot.* **55**, 151–157.
- Tang, G., Reinhart, B.J., Bartel, D.P. and Zamore, P.D. (2003) A biochemical framework for RNA silencing in plants. *Genes Dev.* **17**, 49–63.
- Valentine, T., Shaw, J., Blok, V.C., Phillips, M.S., Oparka, K.J. and Lacomme, C. (2004) Efficient virus-induced gene silencing in roots using a modified tobacco rattle virus vector. *Plant Physiol.* **136**, 3999–4009.
- Zamore, P.D., Tuschl, T., Sharp, P.A. and Bartel, D.P. (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell*, **101**, 25–33.