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Virus-induced gene silencing (VIGS) in *Cysticapnos vesicaria*, a zygomorphic-flowered Papaveraceae (Ranunculales, basal eudicots)

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• *Background and Aims* Studies of evolutionary diversification in the basal eudicot family Papaveraceae, such as the transition from actinomorphy to zygomorphy, are hampered by the lack of comparative functional studies. So far, gene silencing methods are only available in the actinomorphic species *Eschscholzia californica* and *Papaver somniferum*. This study addresses the amenability of *Cysticapnos vesicaria*, a derived fumitory with zygomorphic flowers, to virus-induced gene silencing (VIGS), and describes vegetative and reproductive traits in this species.
• *Methods* VIGS-mediated downregulation of the *C. vesicaria PHYTOENE DESATURASE* gene (*CvPDS*) and of the *FLORICAULA* gene *CvFLO* was carried out using *Agrobacterium tumefaciens* transfer of *Tobacco rattle virus* (TRV)-based vectors. Wild-type and vector-treated plants were characterized using reverse transcription–PCR (RT–PCR), *in situ* hybridization, and macroscopic and scanning electron microscopic imaging.
• *Key Results Cysticapnos vesicaria* germinates rapidly, can be grown at high density, has a short life cycle and is self-compatible. Inoculation of *C. vesicaria* with a *CvPDS*-VIGS vector resulted in strong photobleaching of green parts and reduction of endogenous *CvPDS* transcript levels. Gene silencing persisted during inflorescence development until fruit set. Inoculation of plants with *CvFLO*-VIGS affected floral phyllotaxis, symmetry and floral organ identities.
• *Conclusions* The high penetrance, severity and stability of pTRV-mediated silencing, including the induction of meristem-related phenotypes, make *C. vesicaria* a very promising new focus species for evolutionary–developmental (evo–devo) studies in the Papaveraceae. This now enables comparative studies of flower symmetry, inflorescence determinacy and other traits that diversified in the Papaveraceae.

Key words: *Agrobacterium tumefaciens*, basal eudicots, *Cysticapnos vesicaria*, *FLORICAULA*, Papaveraceae, *PHYTOENE DESATURASE*, Ranunculales, *Tobacco rattle virus*, VIGS, zygomorphy.

INTRODUCTION

Investigations in the Ranunculales (the earliest-branched eudicot order; APG III, 2009) are important for understanding core eudicot floral diversification, as early diverging eudicots precede the extensive canalization of floral bauplans observed in core eudicots (Soltis *et al.*, 2002). The Ranunculales are also considered a 'playground for floral diversification', with, for example, more diverse perianth forms than in core eudicots (Litt and Kramer, 2010; Ronse de Craene, 2010). Within the order, Papaveraceae are especially notable for their range of flower and inflorescence morphologies (Hidalgo and Gleissberg, 2010, and references therein). The Papaveraceae (*sensu lato*) are a monophyletic lineage that consists of two subfamilies, Papaveroideae (the poppies) and Fumarioideae (the fumitories and bleeding hearts), together comprising about 770 species in 40 genera (Judd *et al.*, 2007; APG III, 2009). The phylogenetic position and taxonomic status of a putative third subfamily, the monotypic Pteridophylloideae, are currently questioned (Wang *et al.*, 2009). All poppies are united in having a dimerous perianth, which is actinomorphic (polysymmetric) in the subfamily Papaveroideae, and either disymmetric or zygomorphic (monosymmetric) in the subfamily Fumarioideae. Interestingly, the stepwise shifts in floral symmetry are paralleled by changes in inflorescence architecture. Actinomorphic or disymmetric flowers occur usually singly or clustered in determinate inflorescences, whereas zygomorphic flowers form in indeterminate inflorescences without a terminal flower. Further, unusual character combinations suggest incomplete coupling and/or reversals of the two traits (Hidalgo and Gleissberg, 2010). Other diversity patterns that qualify poppies for evolutionary–developmental (evo–devo) studies include a shift from oligandry to polyandry (Damerval and Nadot, 2007), diverse modes of leaf dissection (Gleissberg and Kadereit, 1999; Gleissberg, 2004) and alkaloid biosynthesis (Ziegler *et al.*, 2006).

The California poppy, *Eschscholzia californica*, is an emerging model plant for basal eudicots (Carlson *et al.*, 2006; Kramer, 2009; Zahn *et al.*, 2010). *Eschscholzia californica* and *Papaver somniferum* are amenable to virus-induced gene silencing (VIGS) (Hileman *et al.*, 2005; Wege *et al.*, 2007), permitting the investigation of gene function in these species (Drea *et al.*, 2007; Orashakova *et al.*, 2009; Yellina *et al.*, 2010; Bartholmes, 2011; Hands *et al.*, 2011). VIGS is now a widely used experimental procedure that allows the transient interruption of gene function through a process similar to RNA interference (for a recent review, see Becker and Lange, 2010). The technique takes advantage of an inherent

defence mechanism of plants against viruses (Baulcombe, 1999; Paroo *et al.*, 2007). Engineered viruses carrying one or more target genes are introduced into the plant, and double-stranded RNA produced during virus replication triggers the degradation of any RNA with sequence similarity, including the endogenous transcripts of the target gene(s). The *Tobacco rattle virus* (TRV)-based binary vector system (Liu *et al.*, 2002) is most commonly employed for VIGS in core eudicots, and it has also provided successful gene down-regulation in some basal eudicots and monocots (Becker and Lange, 2010). This system is also the one used thus far in VIGS studies applied to the early-branched eudicot genera *Aquilegia*, *Eschscholzia*, *Papaver* and *Thalictrum* (Drea *et al.*, 2007; Hileman *et al.*, 2005; Gould and Kramer, 2007; Wege *et al.*, 2007; Orashakova *et al.*, 2009; Di Stilio *et al.*, 2010; Yellina *et al.*, 2010).

VIGS-based evo–devo studies in Papaveraceae would greatly benefit from a zygomorphic-flowered model species, to contrast with the actinomorphic-flowered *E. californica* and *P. somniferum*. Gene expression data provided evidence that *CYCLOIDEA* homologues may be implicated in the establishment of flower symmetry in Papaveraceae (Kölsch and Gleissberg, 2006; Damerval *et al.*, 2007). However, these candidate genes still remain to be validated through functional studies. VIGS has been used to address the genetic control of floral zygomorphy in the core eudicot *Pisum sativum* (Wang *et al.*, 2008), and the technique is currently being developed in other zygomorphic-flowered species, e.g. in the monocot lineages Orchidaceae (Lu *et al.*, 2007) as well as in *Zingiber officinale* (Renner *et al.*, 2009), and in *Fedia cornucopiae*, a species in the core eudicot order Dipsacales (Boyden *et al.*, 2010). Here we report the application of VIGS in *Cysticapnos vesicaria*, a Papaveraceae with monosymmetric flowers. *Cysticapnos* belongs to a clade of derived fumarioid poppies that are characterized by monosymmetric flowers and open inflorescences (Hidalgo and Gleissberg, 2010, and references therein). Within this clade, molecular phylogenetic analysis placed *Cysticapnos* as sister to *Discocapnos* and *Trigonocarpos*, in the sub-tribe Discocapninae (Manning *et al.*, 2009). *Cysticapnos vesicaria* is part of a small genus of three species endemic to South Africa (revised by Manning *et al.*, 2009) of semi-succulent climbing plants with compound, apically tendrillate leaves. Stems end with terminal racemes of monosymmeric flowers presenting a dorsal nectary pouch. *Cysticapnos vesicaria* is polyploid, like most derived fumarioid poppies (Lidén, 1986); however, the species can be distinguished from its congeneric relatives by a chromosome number of $2n = 4x = 28$ ($2n = 4x = 32$ for *C. cracca* and *C. pruinosa*) based on $x = 7$, whereas almost all Fumarioideae have $x = 8$ (Lidén, 1986).

Cysticapnos vesicaria was selected because it is easy to grow in a laboratory setting from readily germinating seeds produced in abundance from self-fertile plants. Our assessment of this potential fumarioid poppy model species includes: (1) demonstrating the feasibility of cultivating *Cysticapnos* under laboratory conditions. Some aspects of vegetative and reproductive morphology are characterized that can be used as a reference for future evo–devo studies of the species. (2) We show the applicability of standard molecular techniques such as reverse transcription–PCR (RT–PCR) and *in situ* hybridization to *Cysticapnos*. (3) Finally, we evaluate the amenability of *Cysticapnos* to functional studies through TRV-based VIGS, using the marker gene *PHYTOENE DESATURASE* (*CvPDS*). Further, we use the *FLORICAULA/LEAFY* gene *CvFLO* to test whether silencing of a floral regulator that is known to be expressed in meristematic tissues results in morphological defects.

MATERIALS AND METHODS

Plant cultivation

Cysticapnos vesicaria seeds were provided by the Botanical Garden of Göttingen, Germany (index seminum 2007-981) and were grown for several generations to generate a seed pool. Seeds were sown in trays of 48 pots ($4 \times 6 \times 5{\cdot}5$ cm L × W × H) covered with a transparent lid and cold stratified at 4 °C. After 4 d, trays were transferred to a growth chamber at 22 °C in permanent light at 60–100 μmol of light m^2 s^{-1}. The first signs of germination were visible 6 d later. When the cotyledons were fully expanded or the first leaf had formed, seedlings were transplanted so as to have only one plant per pot, and the lid was removed. Two weeks after inoculating the plants with *Agrobacterium tumefaciens*, tap water was supplemented with 0·025 % liquid grow 7-9-5 (Dyna-Gro Co., San Pablo, CA, USA) as fertilizer.

Scanning electron microscopy

Flowers and inflorescences at different developmental stages were dissected under a stereomicroscope and fixed in 70 % ethanol. After dehydration through an ethanol series, samples were critical-point dried with CO_2 using a Balzers (CPD030), mounted on aluminium stubs on carbon conductive adhesive tabs and gold-coated with a Balzers sputter coater (SCD050). Micrographs were taken with a Cambridge Instruments Stereoscan 240 scanning electron microscope (SEM) equipped with digital image capture and the Orion32 6·53 software.

Expression pattern of a Histone H4 *gene by* in situ *hybridization*

A 215 bp fragment of a *Histone H4* gene, *CvH4*, was amplified with the primers H4F035 and H4R259 (Groot *et al.* 2005; GenBank accession no. JQ239046), and served as template to synthesize a DIG-11-UTP- (Roche, Indianapolis, IN, USA) labelled antisense RNA probe using T3 polymerase. *In situ* hybridization was carried out on seedling shoots, following the protocol described in Zachgo (2002). Development of signals with BCIP (5-bromo-4-chloro-3-indolyl phosphate) in 10 % polyvinyl alcohol was stopped after 2 d.

Isolation and sequencing of CvPDS *and* CvFLO

Total RNA from a tissue blend of *C. vesicaria* was isolated using the TRI Reagent (Sigma Aldrich, St Louis, MO, USA) according to the manufacturer's instructions. RNA was subsequently reverse transcribed to cDNA using the standard

oligo(dT) primer AB05-R (5'-GACTCGAGTCGACATC TG($T)_{18}$-3') and the enzyme M-MLV (Promega, Madison, WI, USA). *CvPDS* (GenBank accession no. JQ239047) was amplified by semi-nested PCR with the forward primers PDS-4F (5'-GATGGAGATTGGTATGAGACTGG-3') and PDS-5F (5'-CGAGTAACTGATGAGGTGTTTATTGC-3') and the reverse primer PDS-7R (5'-AAGAGGGGACTTC TGCTGA-3'). These primers were designed for use throughout Papaveraceae and possibly other Ranunculales. *CvFLO* (GenBank accession no. JQ239044) was isolated using the forward primers FLO-41F (5'-GCTGAGTTAGGGTTTA CTGTKAGCAC-3') and FLO-42F (5'-ACCCTATTGAC GCMCTCTCTC-3') and the reverse primers FLO-43R (5'-GCCCWACCAAGGTGACRAAYC-3') and FLO-44R (5'-C GCATTTTCGGCTTGTTTATGTAACTAGC-3'). Sequencing was done in the Ohio University Genomics Facility. The *CvFLO* sequence was aligned with other angiosperm *FLORICAULA/LEAFY* homologues, and a Neighbor–Joining tree was constructed using SplitsTree (Huson and Bryant, 2006) based on a 267 bp data set (Supplementary Data Fig. S1).

Vector construction, preparation of the inoculation medium and infection

A 485 bp fragment of *CvPDS* was amplified using PDS-8F (5'-AATCTAGACGAGTAACTGATGAGGTGTTTATTGC-3') and PDS-9R (5'-CCCGGGAAGAGGGGACTTCTG CTGA-3') and cloned into the *Xba*I and *Sma*I sites of pTRV2 (Liu *et al.*, 2002), resulting in the plasmid pTRV2-*CvPDS*. To test silencing of a meristematic gene, a 462 bp fragment of *CvFLO* was amplified with the primers FLO-42F and FLO-43R containing *Xba*I and *Xho*I restriction enzyme sites, respectively, and ligated at the corresponding sites of pTRV2, resulting in the plasmid pTRV2-*CvFLO*. The plasmids were sequenced to verify correct insertion of the fragment, and transformed into *A. tumefaciens* GV3101. *Agrobacterium tumefaciens* containing the appropriate plasmid (pTRV1, pTRV2-*CvPDS*, pTRV2-*CvFLO*, pTRV2-empty) were grown in standard LB medium for 24 h at 28 °C before harvesting them in a ratio of 1:1, pTRV1:pTRV2. Cells were resuspended in 1 mL of 5 % sucrose solution (i.e. half the volume of harvested cells), and the resulting inoculation media were left at room temperature for 30 min before infection.

Inoculation was carried out 25 d after sowing on seedlings at different developmental stages, with foliage leaf number between one and five. Leaves were considered when they reached an approximate petiole to blade length ratio of 1:1 and/or when the lamina was expanded flat. A 2 μL droplet of inoculation medium was applied onto needle scratches on the hypocotyl and leaf petiole base, allowing the *Agrobacterium* to enter the plant. Plants were left on the lab bench overnight and then returned to the growth chamber.

Expression analysis of CvPDS *by RT–PCR*

RNA of various tissues (vegetative shoot tips, whole inflorescences, floral buds and fruits) of *PDS*-VIGS and pTRV2-empty plants was extracted using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). cDNA synthesis was conducted as described above with 250 ng of total RNA. In the subsequent PCRs, 5 μL of the 1:50 diluted cDNA was used in a 25 μL reaction. *CvPDS* expression profiling was carried out with PDS4F and PDS7R primers, as they target a portion of the gene not included in the VIGS construct and permit avoidance of the amplification of virus-derived sequences. Furthermore, those primers were chosen because they span introns and could be used to discriminate endogenous *CvPDS* transcripts from any genomic DNA amplification. *CvPDS* was amplified for 40 cycles of 30 s at 94 °C, 30 s at 55 °C and 60 s at 72 °C. The housekeeping gene *GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE* (*GAPDH*) was used as reference control and for calculating relative expression intensities (Czechowski *et al.*, 2005). The amplification of a 377 bp region near the 3' end of *CvGAPDH* (GenBank accession no. JQ239045), carried out with the primers GAPDH-1F (5'-AAGGACTGGAGAGGTG G-3') and GAPDH-2R (5'-CCCCATTCGTTGTCGTACCA-3'), was done as described for *CvPDS*. Determination of presaturation cycles was assessed prior to quantification for the individual tissues and genes. A minimum of three technical replicates were run. ImageJ (Rasband 1997–2011) was used to convert gel images into relative intensities.

RESULTS

Cultivation of Cysticapnos vesicaria *plants*

Following a brief cold stratification, seeds germinated rather synchronously after 6 d, and could be readily transplanted to individual pots for further cultivation. Plants could be grown at high density in Conviron E8 growth chambers, and started to flower in permanent light conditions only a few weeks after germination. Plants started to die upon completion of fruit set around 3 months after germination. The dead capsules remain closed under our conditions, allowing easy and safe retrieval of seeds. A single plant produces several capsules, and each capsule contained 7–54 seeds (mean 24, $n = 28$). Seeds retained germination capability after storage at 4 °C for several months.

Morphological characterization of Cysticapnos vesicaria

The morphology of wild *C. vesicaria* (Harvey and Sonder, 1894; Manning *et al.*, 2009) is similar to that of plants cultivated in permanent light in growth chambers. *Cysticapnos vesicaria* is a small annual herb that uses tendriliform terminal leaflets, rachis and leaflet stalks for climbing (Fig. 1). Heteroblastic leaf series showed a characteristic pattern of increasing complexity also seen in other species (e.g. DeMason and Villani, 2001; Becker *et al.*, 2005). First-order leaflet pair numbers reached a stable maximum at node 7, while total segment number, including second-order leaflets, serrations and tendrils, peaked together with leaf length around node 11, and declined afterwards. Interestingly, the maximum leaf area was already reached within the six early-formed tendrilless leaves which had larger leaflet blades. Petiole length declines steadily along the shoot, and the leaves preceding

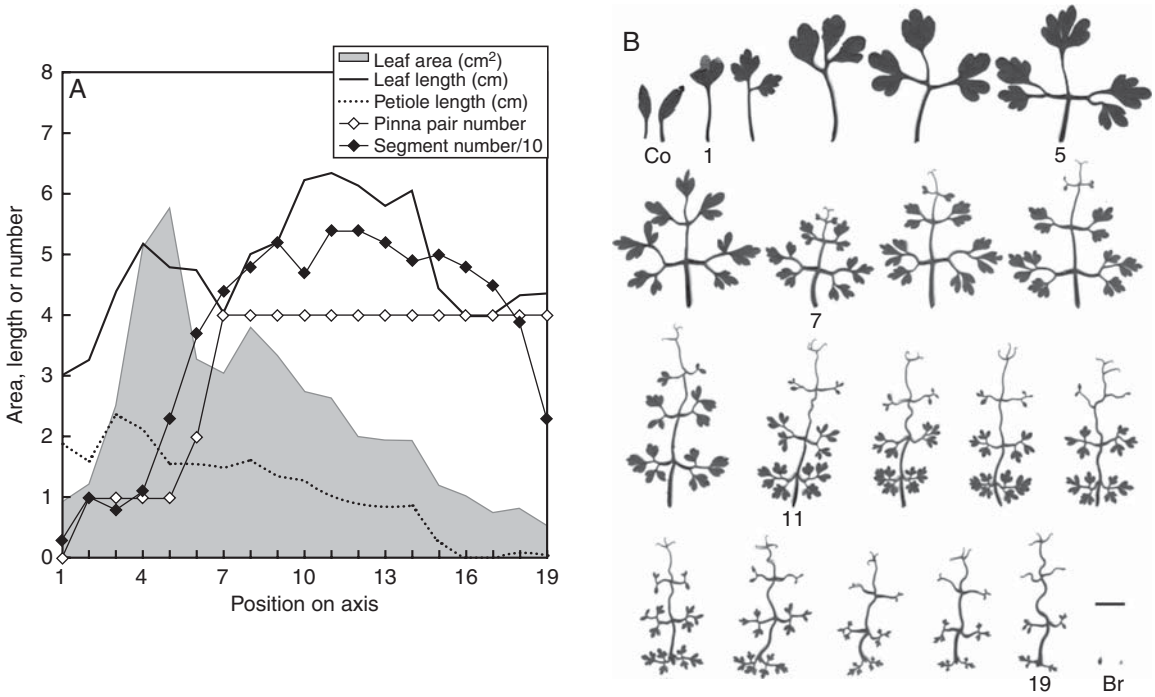

FIG. 1. Heteroblastic leaf series of wild-type *Cysticapnos vesicaria*. (A) Some characteristics of leaves along a primary axis, corresponding to the series of leaf silhouettes illustrated in (B). Units for lengths are in cm and for area in cm^2. (B) Some leaves are labelled: Co, cotyledons; 1, primary leaf; 5, leaf 5 with maximum area; 7, first leaf with tendrils; 11, leaf with maximum length and dissection; 19, leaf preceding terminal inflorescence; Br, inflorescence bracts subtending flowers. Scale bar = 1 cm.

the first inflorescence are sessile. A sharp transition divides the last foliage leaf and the minute inflorescence bracts.

After formation of the primary terminal inflorescence (Fig. 2A), sympodial branching from the axils of the preceding one or two foliage leaves gives rise to higher order inflorescences (Fig. 2A). The inflorescence is sessile, with a short internode below the first bract and flower (Fig. 2B,C). Inflorescence bracts are small, scarious and lanceolate. Flowers cluster in a raceme of 1–3 light-pink, zygomorphic flowers. The small number of flowers identifies our accession as *Cysticapnos vesicaria* subspecies *vesicaria* (Manning *et al.*, 2009). A pin-like structure forms the end of the inflorescence axis, and is sometimes preceded by an empty bract (Fig. 2D). Flowers initiate in a rapid sequence (Fig. 2G), and the sequence of maturation (Fig. 2E, F) and efflorescence (Fig. 2B) is acropetal. The calyx consists of two scale-like sepals that resemble bracts and are, like bracts, persistent (Fig. 3A). The corolla is composed of four petals in two whorls. Sepals are initiated in a medial position, in one plane with the subtending bract (Fig. 2E, F). Upon anthesis, pedicel torsion reorients the flower so that sepals assume a lateral position and the spurred outer petal is positioned upward (Figs 2B and 3A). Outer petals are identical at earlier developmental stages while in the lateral position (Fig. 2E), and epidermal cell shape remains similar even after zygomorphy establishment (data not shown). Inner petals are free at the base and partly connate at their tips, and have a translucent abaxial ridge (Fig. 3A). The androecium consists of six stamens in two bundles, and the bicarpellate gynoecium consists of a many-ovuled ovary, a linear style and a capitate stigma (Fig. 3A). Self-pollination occurs very effectively under our growth conditions, and plants produce many fruits and seeds. The fruit is an ovoid, membranous, vesicular and thin-walled capsule, that appears balloon-like inflated and grows up to about 30 mm in length. The numerous seeds are small lenticular, with a black shiny testa.

In situ hybridization of a Histone H4 *gene*

We tested whether *Cysticapnos* is amenable to gene expression characterization via *in situ* hybridization, an important component of evo–devo studies, including Ranunculales (e.g. Di Stilio *et al.*, 2005, 2009; Shan *et al.*, 2006; Liu *et al.*, 2010; Ballerini and Kramer, 2011) and Papaveraceae (e.g. Busch and Gleissberg, 2003; Groot *et al.*, 2005; Carlson *et al.*, 2006; Damerval *et al.*, 2007; Drea *et al.*, 2007; Orashakova *et al.*, 2009; Yellina *et al.*, 2010; Hands *et al.*, 2011). Vegetative shoot apices hybridized with an antisense *CvH4* RNA probe strongly labelled *CvH4* transcripts in individual cells in the shoot apical meristem and developing leaves (Supplementary Data Fig. S2), as expected (Groot *et al.*, 2005). Neighbouring cells remained unstained, demonstrating a good signal-to-background ratio.

Fast, strong and long-lasting CvPDS *silencing phenotype in infected* Cysticapnos vesicaria *plants*

PDS-VIGS-mediated photobleaching was detected in the young plants 9 d after infection in the second or third leaf formed after inoculation (87 % of 68 plants in three

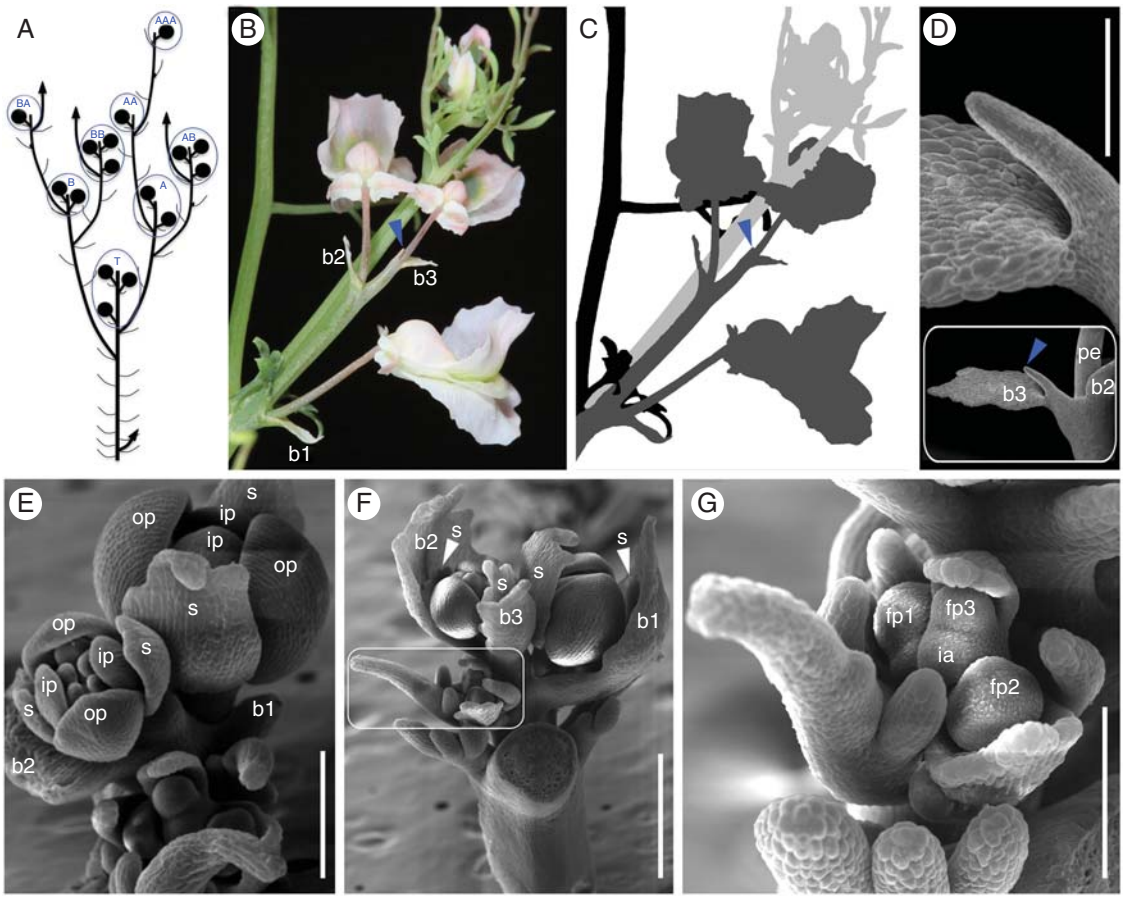

FIG. 2. Morphology and ontogeny of reproductive structures in *Cysticapnos vesicaria*. (A) Branching architecture and naming system for inflorescence position. Inflorescences are circled in blue. (B, C) Details of the inflorescence. (C) Black, last foliage leaf preceding the terminal inflorescence; dark grey, terminal inflorescence, corresponding to 'T' in (A). Three flowers are subtended by bracts b1–b3. The pin-like inflorescence apex is marked with a blue arrowhead; light grey, sympodial shoot and inflorescence arising from the foliage leaf axil, corresponding to 'A' in (A). (D–G) SEM micrographs of inflorescence ontogeny. (D) Pin-like ending of the mature inflorescence axis that may be preceded by an empty bract. (E) Two-flowered inflorescence; subtending bracts b1 and b2 removed. Perianth organs are labelled. (F) Terminal inflorescence with bracts b1 and b2 subtending flowers. Bract three is empty; inflorescence apex not visible. Sympodial inflorescence in the axil of the last foliage leaf is framed and shown enlarged in (G). (G) Early-stage inflorescence with three flower primordia. The inflorescence apex is still meristematic. Abbreviations: b1–b3, consecutive bracts; fp1–fp3, consecutive flower primordia; ia, inflorescence apex; ip, inner petal; op, outer petal; pe, pedicel; s, sepal. Scale bars: (D, F) = 500 μm, (E) = 250 μm, (G) = 200 μm.

experiments), more rarely in the first or fourth (Figs 4A and 5A). In one experiment in which detailed phenotypic scoring was undertaken, the proportion of individuals showing bleached parts was 100 % ($n = 38$; Fig. 5A), demonstrating a maximal penetrance capacity of the VIGS system in *C. vesicaria*. The transition from completely green to completely white leaves generally involved two intermediary leaves that were mostly green and mostly white, respectively (Fig. 5B). Not all individuals showed complete photobleaching, with some green leaflet and petiole parts occasionally formed. The *PDS*-VIGS phenotype remained remarkably strong and stable throughout further shoot development and included the stem, leaves and inflorescences (Figs 4B, C and 5A). Photobleached stem parts often appeared pinkish, probably due to exposed anthocyanin. Developing capsules were also photobleached, suggesting that silencing persisted throughout flower development (Fig. 4D). The photobleaching phenotype corresponded to effective downregulation of *CvPDS* transcripts, as shown by RT–PCR profiling (Fig. 4E). No difference between wild-type and negative control plants (pTRV2-empty) was noticeable for vegetative or reproductive parts, suggesting that TRV itself has minimal or no effects on development.

Phenotypic effects of CvFLO *silencing*

To test whether VIGS of a developmental gene results in morphological alterations, we conducted a separate experiment in which we inoculated seedlings with a pTRV2-*CvFLO* construct aimed to silence the *CvFLO* gene. Of the plants inoculated with pTRV2-*CvFLO* ($n = 48$) 12 abnormal flowers were analysed that suggested successful silencing during meristematic stages of flower development (Fig. 3). No abnormal flowers were found in control plants inoculated with pTRV2-E ($n = 40$) grown side by side with pTRV2-*CvFLO* plants. The morphological defects indicated that *CvFLO* function was affected during initiation and differentiation of all floral organs, consistent with the strong silencing effects seen in pTRV2-*CvPDS* inflorescences (Fig. 3B). pTRV2-*CvFLO* flowers exhibited perturbed floral phyllotaxis

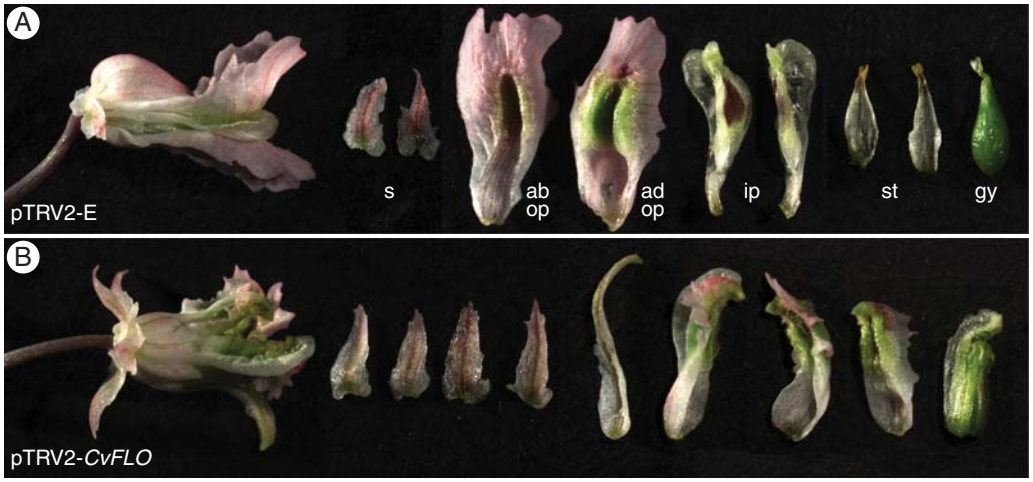

FIG. 3. *CvFLO*-VIGS phenotypes (A) Control plants inoculated with pTRV2-E showed, from left to right, wild-type flowers that were zygomorphic and had two sepals, an abaxial and a spurred adaxial outer petal, two inner petals, two stamen bundles (adaxial faces shown) and a gynoecium. (B) A pTRV2-*CvFLO* flower with altered floral phyllotaxis and symmetry. Dissection of this flower revealed four sepals, one sepaloid organ, three organs with mixed outer and inner petal identities and a composite organ with carpel, stamen and inner petal characteristics. Abbreviations: ad op, adaxial outer petal; ab op, abaxial outer petal; gy, gynoecium; ip, inner petals; s, sepals; st, stamen bundles.

and symmetry, altered organ numbers and mosaic organ identities, reflecting partial defects in flower meristem identity also known from *FLORICAULA/LEAFY* mutants in rosid core eudicots. Mutants in *Arabidopsis* and *Lotus* develop mosaic flower/shoots with sepals and carpels (Schultz and Haughn, 1991; Huala and Sussex, 1992; Dong *et al.*, 2005), while equivalent mutations in the asterid core eudicots *Antirrhinum* and *Solanum* show a full conversions of flowers into shoots (Coen *et al.*, 1990; Allen and Sussex, 1996). A more detailed characterization of the *CvFLO*-VIGS phenotype is currently underway that will allow a better understanding of the role of this developmental regulator in this zygomorphic-flowered basal eudicot.

DISCUSSION

Research in basal eudicots is crucial to help decipher major evolutionary transitions and associated histories of developmental genes between distant angiosperm lineages, such as basal angiosperms, monocots and core eudicots (Kramer, 2009; Zahn *et al.*, 2010). In this sense the Papaveraceae are of special interest as this family occupies a basal position within Ranunculales (the sister clade to all other eudicots) together with the earliest-diverging monogeneric Eupteleaceae (Worberg, 2007; Wang *et al.*, 2009). The Ranunculaceae, on the other hand, are considered a derived core Ranunculales family (Wang *et al.*, 2009). Furthermore, Papaveraceae flowers exhibit a separate morphological canalization within Ranunculales, with whorled flowers, a clear differentiation of calyx and corolla, and well-defined expression domains of floral organ identity genes, potentially providing a model for core eudicot flower evolution (Chanderbali *et al.*, 2009; Voelckel *et al.*, 2010; Zahn *et al.*, 2010). Finally, unique diversification patterns regarding floral symmetry and inflorescence (see Introduction) well qualify Papaveraceae for the study of correlated traits in a defined phylogenetic framework.

We have identified *C. vesicaria* as a new model system in the zygomorphic-flowered subfamily Fumarioideae that will enable comparative evolutionary developmental studies of reproductive traits in the poppy family to be conducted. The combination of relative phylogenetic proximity and contrasting morphology makes *C. vesicaria* and *E. californica* a very promising pair of species for comparative evo–devo studies. Recent VIGS-based studies in papaveroid poppies (Orashakova *et al.*, 2009; Yellina *et al.*, 2010; Bartholmes, 2011; Hands *et al.*, 2011; S. Wreath *et al.*, Ohio University, unpubl. res.) provide interesting starting points for comparative studies using *C. vesicaria*.

Extending developmental genetic studies from isolated model systems to morphologically divergent relatives minimizes phylogenetic noise in comparative studies (Baum *et al.*, 2002; Kramer, 2009). For example, the *Arabidopsis thaliana* and *Cardamine hirsuta* models have been useful in the study of leaf dissection (Hay and Tsiantis, 2006; Canales *et al.*, 2010). Within Ranunculales, VIGS technology has been recently developed for related species of the genera *Aquilegia* (Gould and Kramer, 2007) and *Thalictum* (Di Stilio, 2010). Our study provides proof of the value of a VIGS-based approach to comparative functional studies in basal eudicots.

The application of VIGS in *Cysticapnos* requires no vacuum infiltration, and results in maximal post-treatment survival and maximal phenotypic penetrance. In these respects, VIGS in *Cysticapnos* is more effective than in other Ranunculales systems such as *P. somniferum* (Hileman *et al.*, 2005) or *Aquilegia vulgaris* (Gould and Kramer, 2007). The ability of TRV to trigger silencing in whole shoots and inflorescences, and the persistence of silencing until the fruit set will allow the study of a broad range of reproductive and vegetative developmental traits, with the only exclusion being pre-treatment stages of germination and early seedling establishment. In addition, the apparent absence of seed dormancy, easy cultivation under laboratory

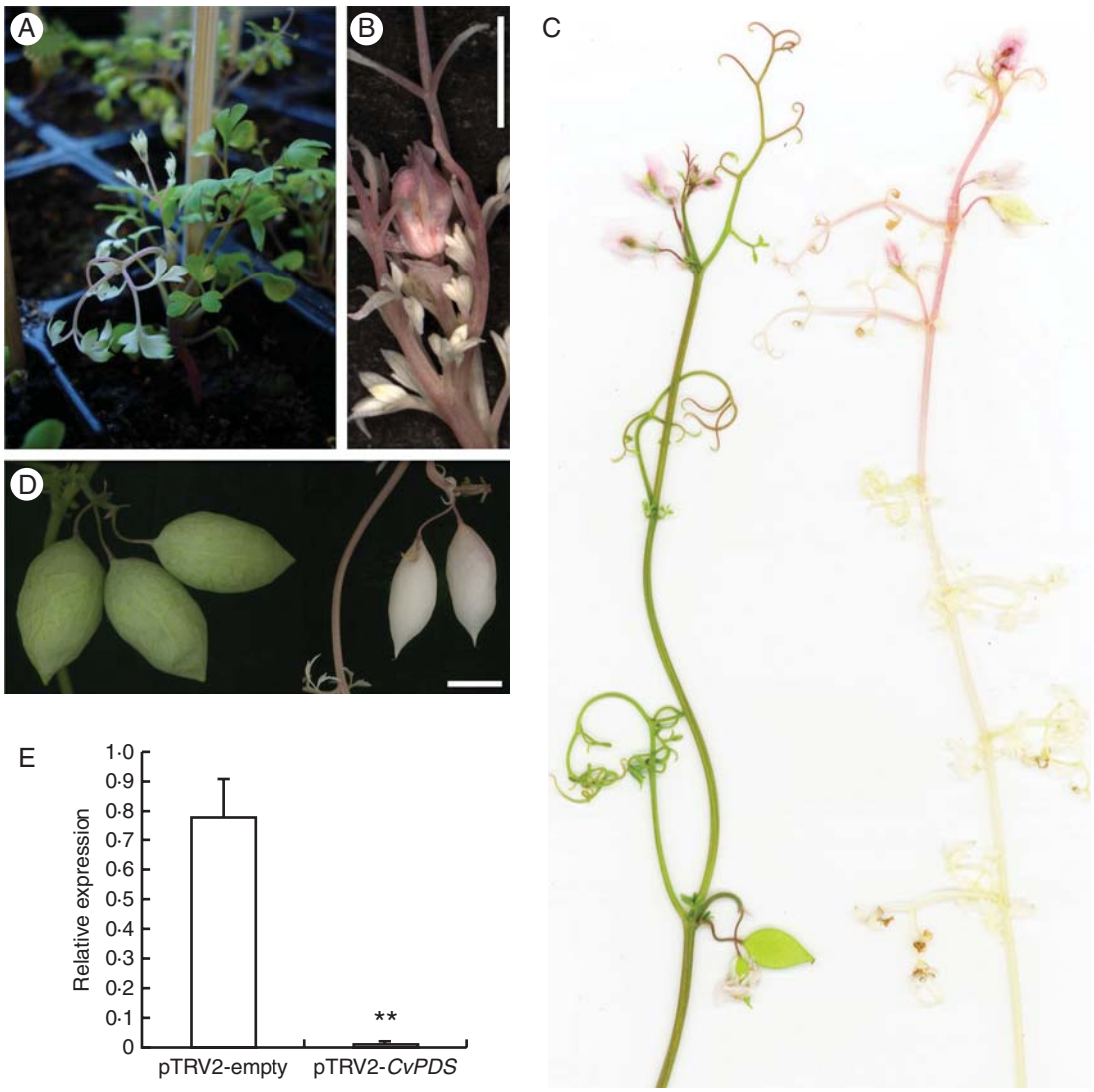

FIG. 4. *CvPDS* silencing symptoms in vegetative and reproductive parts of *Cysticapnos vesicaria*. (A) Young plant showing beginning of photobleaching. (B) Young inflorescence of a *CvPDS*-VIGS plant with complete photobleaching. (C) Mature reproductive shoots of a control (pTRV2-empty; left) and a *CvPDS*-VIGS plant (right). (D) Capsules of a control (left) and a *CvPDS*-VIGS plant (right). (E) RT–PCR showing the strong reduction of *CvPDS* transcripts in *C. vesicaria* plants infected with pTRV2-*CvPDS* ($n = 6$), compared with a pTRV2-empty control ($n = 2$). Expression levels are shown relative to *GAPDH*. Asterisks indicate a significant difference compared with the control group at 99 % confidence intervals after ANOVA. Scale bars = 1 cm.

conditions at high density, a short life cycle and self-fertilization facilitate experimentation with *Cysticapnos*. The fact that *Cysticapnos* is tetraploid could potentially make knock-down studies more difficult in the presence of paralogues. In this study, we have identified only one copy of *PDS* and *FLO*, and silencing constructs based on these showed strong phenotypic consequences. This could be due to the absence of duplicated gene copies, or to co-silencing of paralogues. VIGS, in comparison with mutant-based studies, can be particularly suitable for studying polyploids (Scofield and Nelson, 2009) as it permits the simultaneous silencing of a set of paralogues, either directly when they share high sequence similarity, or by using a specific construct design that incorporates multiple genes. The copy number of target genes can be better assessed when genomic information become available for this and other fumarioid poppies.

Conclusions

We have demonstrated that *Cysticapnos* is amenable to laboratory culture and poses no challenge for gene isolation and expression studies. The emerging availability of transcriptomics data for *Eschscholzia* as well as other Papaveraceae and Ranunculales species (Chanderbali *et al.*, 2009; Voelckel *et al.*, 2010; Zahn *et al.*, 2010) will greatly facilitate the identification of genes and their orthology in *Cysticapnos*. More importantly, *Cysticapnos* is the first species with zygomorphic flower symmetry in the Papaveraceae and in basal eudicots for which gene function studies are now available. We developed this system primarily to enable comparative functional studies of inflorescence determinacy and flower symmetry in basal eudicots. However, *Cysticapnos* is also a promising system with which to investigate other traits, including its climbing habit and compound, tendril-bearing leaves. Tendrilled

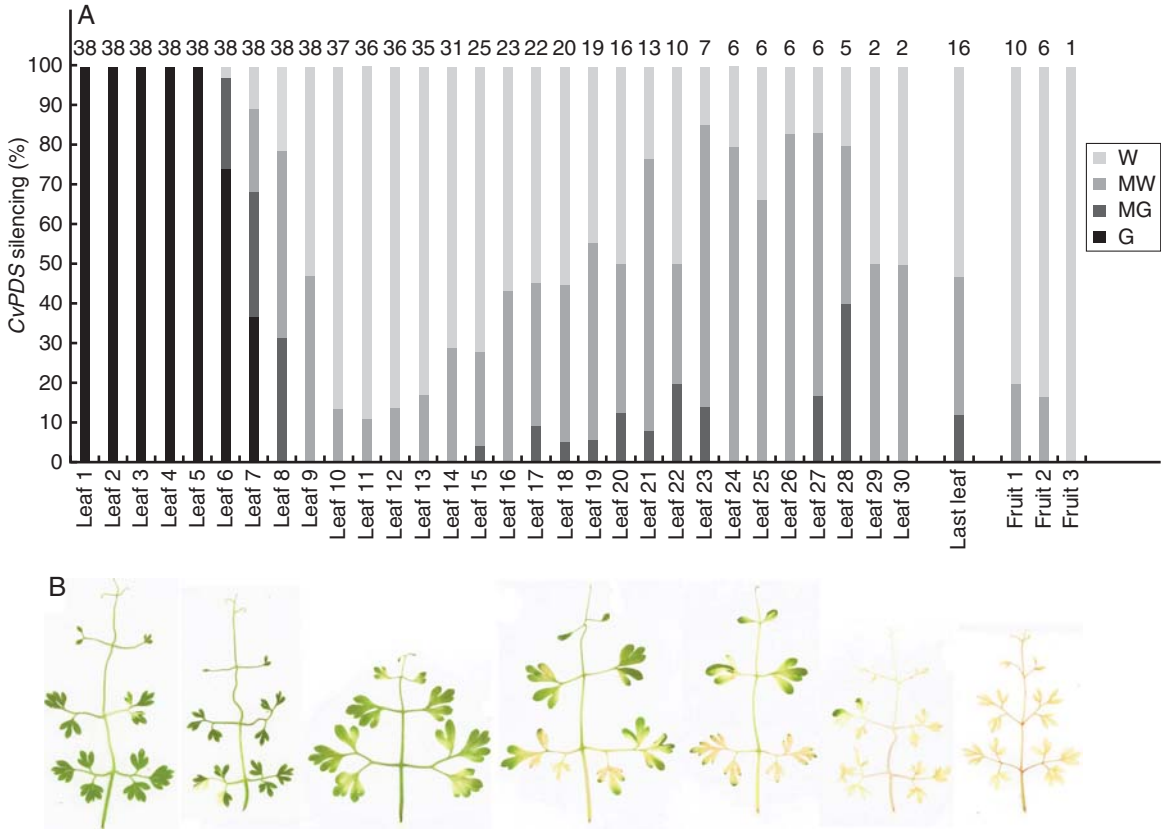

FIG. 5. Degree of *CvPDS* silencing. (A) Intensity of leaf and fruit bleaching symptoms in the primary axis. 'Last leaf' = last leaf before inflorescence. Abbreviations: W, white; MW, mostly white; MG, mostly green; G, green. Sample sizes are indicated above each column. Note that *CvPDS* silencing has a negative effect on plant robustness and survival, leading to an over-representation of partially silenced plants. (B) Different intensities of *CvPDS* silencing are reflected in a gradient from almost completely green (left) to completely white (right).

compound leaves have thus far only been studied in *P. sativum* (garden pea) and to some extent in *Lathyrus odoratus* (sweet pea) (Gould *et al.*, 1994; Hofer *et al.*, 2009), and it is unknown whether any underlying genetic networks are shared between these rosid core eudicots and *Cysticapnos*. The addition of a first fumarioid poppy to the VIGS toolbox in poppies makes it likely that other Fumarioideae are amenable to this TRV-based silencing method as well. As comparative insights into the role of genetic networks in morphological evolution of poppies become available, future studies may extend to more basal fumitory species with disymmetric flowers.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: Neighbor–Joining tree of *FLORICAULA/LEAFY*-like genes including *CvFLO*. Figure S2: *in situ* hybridization of the *Histone H4* gene.

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