**PREMISE:** Cleomaceae is emerging as a promising family to investigate a wide range of phenomena, such as C₄ photosynthesis and floral diversity. However, functional techniques are lacking for elucidating this diversity. Herein, we establish virus-induced gene silencing (VIGS) as a method of generating functional data for *Cleome violacea*, bolstering Cleomaceae as a model system.

**METHODS:** We leveraged the sister relationship of Cleomaceae and Brassicaceae by using constructs readily available for *Arabidopsis thaliana* to provide initial information about the feasibility of VIGS in *C. violacea*. We then developed endogenous constructs to optimize VIGS efficiency and viability for fruit development.

**RESULTS:** PHYTOENE DESATURASE was successfully downregulated in *C. violacea* using both heterologous and endogenous constructs. The endogenous construct had the highest degree of downregulation, with many plants displaying strong photobleaching. FRUITFULL-treated plants were also successfully downregulated, with a high rate of survival but less effective silencing; only a small percentage of survivors showed a strong phenotype.

**DISCUSSION:** Our optimized VIGS protocol in *C. violacea* enables functional gene analyses at different developmental stages. Additionally, *C. violacea* is amenable to heterologous knockdown, which suggests that a first pass using non-endogenous constructs is a possible route to test additional species of Cleomaceae.

**KEY WORDS** Cleomaceae; *Cleome violacea*; emerging model; FRUITFULL; PHYTOENE DESATURASE; virus-induced gene silencing.
only one study has used stable transformation in Cleomaceae to empirically examine mechanisms regulating C₄ photosynthesis in G. gynandra (Williams et al., 2015). An effective alternative to stable transformation is virus-induced gene silencing (VIGS), which is a well-established post-transcriptional gene silencing (PTGS) tool used in many model and non-model species (Burch-Smith et al., 2004, 2006; Gould and Kramer, 2007; Becker and Lange, 2010; Di Stilio et al., 2010; Becker, 2013). VIGS uses RNA interference to exploit plant defenses, which greatly reduces endogenous mRNA transcription without the need for stable transformation over multiple generations (Ruiz et al., 1998; Baulcombe, 1999; Burch-Smith et al., 2004). Thus, VIGS provides the opportunity to quickly downregulate individual or multiple genes and infer function via the observation of modified phenotype (Becker and Lange, 2010; Becker, 2013). Notwithstanding the potential of VIGS for examining gene function in a range of species, protocols must be tailored for each new species (Senthil-Kumar et al., 2007; Becker, 2013). Tailoring of protocols can be challenging because of factors like developmental timing, susceptibility to Agrobacterium tumefaciens infection, and strength of PTGS response. Furthermore, not all species are amenable to reverse genetic approaches like VIGS.

Cleome violacea is a species of note for investigations within Cleomaceae (Mabry et al., 2020) and an ideal candidate for VIGS. It has a published genome and exhibits morphological traits that are important for pollinator interactions, e.g., floral monosymmetry and prominent nectaries (Fig. 1). Furthermore, C. violacea displays features that facilitate its study, including small stature, high fecundity, self-fertilization, and short generation time. Here, we report on testing and optimizing the VIGS methodology in C. violacea. Specifically, the goals of this study are threefold: (1) establish that constructs with heterologous sequences are an informative first screen to determine efficacy of VIGS in C. violacea, (2) optimize the VIGS protocol for C. violacea, and (3) demonstrate that this protocol is effective for multiple developmental stages. We selected two genes that would maximize comparison of C. violacea to other VIGS models and that would adequately test different developmental processes. First, PHYTOENE DESATURASE (PDS) is commonly used to determine the efficacy of VIGS in many systems due to its easy-to-score bleaching phenotype when downregulated (Gould and Kramer, 2007; Senthil-Kumar et al., 2007; Wege et al., 2007; Velasquez et al., 2009; Fujita et al., 2019). Second, the MADS-box transcription factor FRUITFULL (FUL) has an established role in fruit development in A. thaliana (Gu et al., 1998; Ferrández et al., 2000a, 2000b; Ferrández and Fourquin, 2014; Eldridge et al., 2016), a species with similar fruit morphology to C. violacea (Fig. 2B). Furthermore, fruit development is the last stage in reproduction and, as such, successful downregulation of FUL would demonstrate the efficacy of VIGS across multiple developmental stages in C. violacea.

METHODS

Plant growth conditions

Inbred lines of C. violacea were grown from lab seed stock, and a voucher was deposited in the Vascular Plant Herbarium (ALTA) at the University of Alberta (Hall & Bolton s.n., 20 Feb 2008; #813 from Hortus Botanicus, Amsterdam). Seeds were sown in groups of 10 using 10-cm-wide square pots, and individual plants were transferred after treatments to 7.5-cm-diameter round pots. Growth substrate was a 2 : 1 mixture of sterilized (liquid cycle, 121.1°C, 20 min) Sun Gro Sunshine Mix (Sun Gro Horticulture, Agawam, Massachusetts, USA) and perlite. All plants were grown in a growth chamber at the Department of Biological Sciences at the University of Alberta, with 16 h of light and 8 h of darkness at 24°C. This temperature was chosen for C. violacea because it falls within the optimal range for VIGS silencing reported in Arabidopsis thaliana (22–24°C) (Wang et al., 2006) while maintaining fast and efficient plant growth. However, it is important to note that C. violacea grows well under a range of growth conditions (e.g., small to large pots, temperatures between 20–26°C, and growth medium without perlite).

Cloning and viral vector construct design

Viral vector construct design and inoculation were carried out in accordance with previous VIGS protocols (Gould and Kramer, 2007; Kramer et al., 2007). Tobacco rattle virus vectors pTRV1 (donor stock no. YL192), pTRV2 (donor stock no. YL156), and pTRV2-APDS (donor stock no. YL154) were obtained from The Arabidopsis Information

FIGURE 1. Cleome violacea whole plant (A), ventral view of flower (B), and lateral view of flower (C).
Resource (TAIR; https://www.arabidopsis.org) using their stock center (Arabidopsis Biological Resource Center [ABRC], Ohio State University, Columbus, Ohio, USA; https://abrc.osu.edu). The pTRV1 vector is required for viral movement (Ziegler-Graff et al., 1991), and the pTRV2 vector contains the gene of interest inserted into the multiple cloning site (MCS) (Ratcliff et al., 2001). Three endogenous constructs were also generated using *C. violacea* mRNA: pTRV2-*CvPDS*, pTRV2-*CvFUL*, and a pTRV2-*CvPDS-CvFUL* hybrid (Appendix S1).

RNA was extracted from *C. violacea* leaves, and separately from whole flowers, using a Concert Plant RNA Reagent Kit (Invitrogen, Carlsbad, California, USA), and was then treated with DNase I (Fermentas, Hanover, Maryland, USA) and enriched using an RNeasy Mini Kit (QIAGEN, Germantown, Maryland, USA). The mRNA was isolated using a Dynabeads mRNA Purification Kit (Invitrogen). The cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen), poly(T) primers, and random hexamer primers, following manufacturer instructions. *Arabidopsis thaliana* PDS3 degenerate primers (Appendix S1) were used to amplify a 953-bp fragment from leaf cDNA. This fragment was then cloned into a TOPO-TA plasmid vector (Invitrogen) following the manufacturer instructions, and sequenced using BigDye Terminator sequencing (Applied Biosystems, Foster City, California, USA) with vector-specific primers M13F and M13R (Applied Biosystems). From that sequence, new primers were designed to amplify a 441-bp fragment with XbaI and BamHI restriction sites on the 5′ and 3′ fragment ends, respectively (Appendix S1). Alternatively, *CvFUL* primers with 5′ XbaI and 3′ BamHI restriction enzymes were designed using an unpublished transcriptome (Appendix S1); the primers amplify from the fifth exon to the eighth exon of the coding sequence, which avoids silencing other MADS-box genes with a conserved domain sequence. A 272-bp fragment of the *CvFUL* coding sequence was amplified using those primers and floral cDNA (Appendix S1). A *CvFUL-CvPDS* construct was also made using modified primers of *CvPDS* with a 5′ BamHI and 3′ XhoI restriction site. This modification enabled the ligation of a *CvPDS* construct to the 3′ end of *CvFUL* (Appendix S1). Restriction-modified fragments of *CvPDS*, *CvFUL*, and *CvFUL-CvPDS* were first cloned into TOPO-TA plasmid, and then excised and ligated into separately digested TRV2 vectors using XbaI, BamHI, and XhoI restriction enzymes and T4 DNA ligase (Promega Corporation, Fitchburg, Wisconsin, USA).

Viral constructs were verified for off-target silencing using siFi21 (Version 1.2.3-0008) (Lück et al., 2019). This software predicts off-targets of RNA interference (RNAi) by comparing user-defined sequences to a local database with Bowtie, a short-read aligner (Langmead et al., 2009; Langmead, 2010). Because Bowtie is purpose built for aligning to a reference, we used the *C. violacea* genome for tracking off-target silencing. One notable limitation is that Bowtie does not distinguish between introns and exons, thus any mRNA sequence that spans two exons may not be recognized. However, because the small interfering RNAs (siRNAs) tested are 21 bp in length, most targets will

![Figure 2](http://www.wileyonlinelibrary.com/journal/AppsPlantSci)
still be identified. For clarification, the average exon length of the closely related _A. thaliana_ is 236.8 bp (Koralowski and Krutovsky, 2011). Assuming _C. violacea_ is similar, 21-bp siRNAs will produce many hits within each exon. We defined on-target sequences by using the BLAST (Altschul et al., 1990) function on https://genomedevolution.org/cgi.e. Coding sequences of _AtPDS_ and _AtFUL_ were matched using BLASTN against the _C. violacea_ genome, and hits with the top E-values/bit-scores were defined as on-target. TRV2-AtPDS, TRV2-CvPDS, and TRV2-CvFUL were also aligned to the _C. violacea_ genome as another way of verifying their similarity to _AtPDS and AtFUL_. The _C. violacea_ genome is divided into scaffolds with sizes that range between 904 bp and 3.8 Mbp, so any sequences aligning to similar positions on the same scaffold are likely related.

TRV2 vectors were then cloned into One Shot TOP10 chemically competent _Escherichia coli_ (Invitrogen) according to the manufacturer instructions. These _E. coli_ were plated onto lysogeny broth (LB) agar plates containing 50 μg/mL kanamycin and grown overnight at 37°C. Colonies were screened via PCR with primers, 156F and 156R (Appendix S1), that span the TRV2 MCS (Gould and Kramer, 2007). Positively screened transformants were grown in LB media with the same antibiotics overnight at 37°C, and their plasmid constructs were purified using a QIAgen Miniprep kit. Construct identity was confirmed using BigDye Terminator sequencing and 156F and 156R primers. All sequences were obtained with an ABI-3730 DNA Analyzer (Applied Biosystems) after being cleaned with a Performa DTR V3 96-well Short Plate Kit (Edge BioSystems, Gaithersburg, Maryland, USA). Nucleotide sequence of the _CvPDS_ construct was deposited under the accession MW505002 in the National Center for Biotechnology Information (NCBI) GenBank, and a partial coding sequence of _CvFUL_ is available under the accession MK584560.

**Agrobacterium tumefaciens** preparation and infiltration

_Agrobacterium tumefaciens_ was prepared for DNA transformation as previously described (Weigel and Glazebrook, 2002). The following vectors were transformed into _A. tumefaciens_ using calcium chloride heat-shock transformation: pTRV1 + pTRV2-AtPDS, pTRV2- CvPDS, pTRV2-CvFUL, pTRV2-CvPDS-CvFUL, pTRV2-MCS, and pTRV1. For each, 100 ng of purified construct was combined with 250 μL of competent _A. tumefaciens_. Transformants were plated on LB media containing 50 μg/mL kanamycin, 50 μg/mL gentamycin, and 25 μg/mL rifampicin; all LB media used to grow _A. tumefaciens_ contained these antibiotics. Transformants were then screened as before using 156F and 156R primers (Appendix S1), and glycerol stocks were made and stored at −80°C (1 : 1 ratio of 50% glycerol and overnight _A. tumefaciens_ culture).

Vacuum infiltration was the chosen method for _C. violacea_ because of its simplicity and efficiency compared with other methods (Wang et al., 2006; Becker and Lange, 2010). Alternative methods, such as agrodrench and booster inoculation (Ryu et al., 2004; Senthil-Kumar and Mysore, 2014), were briefly explored, but were abandoned due to low penetrance (data not shown). The first vacuum infiltration experiments were conducted to examine mortality and penetrance of plants at different developmental stages with heterologous and endogenous constructs. First, plants were categorized based on the number of true leaves: small with 0–3 true leaves, medium with 4–6 true leaves, and large with seven or more true leaves. To prepare for infiltration, _A. tumefaciens_ containing each prepared vector was streaked onto LB agar with antibiotics and incubated for 72 h at room temperature. Individual colonies were used to inoculate 5 mL of LB with antibiotics, which were grown overnight at room temperature with constant shaking of 275 rpm to an OD600 of approximately 1.0. Sequential inoculations using the same media and conditions were then used to scale up growth to a final working volume of 500 mL. That is, 5-mL cultures were used to inoculate 50 mL of LB, which were used as starter cultures for 500 mL. Starter cultures one-tenth of the total volume produce sufficient growth in ~24 h. This process can be scaled up or down depending on the desired number of treated seedlings, and we recommend 500 mL for treatment groups with 50 to 100 seedlings. The final cultures contained: antibiotics, 1 mM MES buffer, and 0.02 mM acetylsyringone. For each TRV2-containing _A. tumefaciens_ culture, one pTRV2-containing _A. tumefaciens_ culture must be grown because a 1 : 1 mixture is required for infiltration. Each culture was then aliquoted into 50-mL conical centrifuge tubes and centrifuged at 3200 g for 20 min at 4°C. The supernatant was decanted from each tube, and cells were resuspended in 20 mL of infiltration buffer (10 mM MES, 10 mM MgCl₂, and 0.2 mM acetylsyringone). Resuspended colonies were recombined in a sterile beaker, and infiltration buffer was added to an OD600 of 2.0 ± 0.1. Colonies were then left at room temperature for a minimum of 4 h to acclimatize. It should be noted that it is also viable to leave cultures overnight if conditions are sterile.

The pTRV2 _A. tumefaciens_ resuspensions were combined 1 : 1 with pTRV1 _A. tumefaciens_ resuspensions. Silwet L-77 surfactant was added to each mixture at 100 μL/L. Groups of seedlings were extracted from the soil, rinsed in distilled water, and briefly air-dried before being submerged in the resuspensions and placed in a vacuum chamber. The vacuum chamber was evacuated for 10 sec, or until the mixture bubbled, and then the vacuum was turned off and seedlings were left for 2 min before pressure was quickly released. The resuspension mixtures were as follows: pTRV1 + pTRV2-CvPDS, pTRV1+ pTRV2-AtPDS, pTRV1 + pTRV2-CvFUL, pTRV1 + pTRV2-CvFUL-CvPDS, and pTRV1 + pTRV2-MCS as a negative control. Ten seedlings were infiltrated each time, and the same mixtures were reused for repeated infiltrations (i.e., 10 more seedlings up to 100 seedlings per 500 mL). Additionally, a group of seedlings were extracted from the soil, rinsed, and air-dried, and then replanted as an untreated control.

Phenotypic scoring varied depending on construct. Phenotypes for both _CvPDS_ and _AtPDS_ downregulated plants became apparent between three and four weeks post-inoculation. Plants treated with pTRV2-CvPDS and pTRV2-AtPDS had their leaves scored using visual approximation of bleaching and were binned into the following categories: pale (<50% of total bleaching), variegated (50–80%), and strong (>80%). Plants treated with pTRV2-CvFUL and pTRV2-CvFUL-CvPDS were scored approximately 30 days after vacuum infiltration. Phenotypes were initially binned into three categories based on the severity of fruit curling and the indenta-

Leaf and fruit tissue from treated and control plants were excised, flash-frozen in liquid nitrogen, and stored at −80°C. Following described protocols (Patchell et al., 2011), scanning electron microscopy (SEM) was used to examine both untreated _C. violacea_ and pTRV2-CvFUL-CvPDS-treated fruits. Leaf and fruit phenotypes
were imaged using a Nikon SMZ 1500 dissecting microscope (Nikon, Tokyo, Japan) with a QImaging Retiga 4000R camera (Teledyne DALSA, Waterloo, Ontario, Canada), and handheld digital Canon DS126181 camera (Canon, Tokyo, Japan). Images were standardized, scaled, color balanced, and assembled into figures using Inkscape version 0.92.5 (https://inkscape.org) and GIMP version 2.10.18 (https://www.gimp.org).

**RT-qPCR expression analysis**

RNA was extracted from approximately 100 mg of treated and control leaf and fruit tissue using an RNeasy Plant Mini Kit (ID:74904) (QIAGEN). The following amendments were made to their Quick-Start protocol: Step 1a, tissue was disrupted with a miniature pestle directly in a 1.5-M L Eppendorf tube. Step 1b was skipped. In Step 2, RLC buffer was used in lieu of RLT buffer for fruit tissue, and tubes were left to incubate at room temperature for 6 min with 1 min of vortexing at 2500 rpm. Step 6 was replaced with steps 4 and 5 from the QIAGEN Micro Kit Quick-Start Protocol to incorporate the column-based DNase I treatment. Step 10 was incubated for 5 min, and eluate was run through the same column twice. All steps with 15 sec centrifuge directions were increased to 30 sec due to centrifuge timer limitations. RNA was quantified and checked for integrity using the NanoDrop ND-1000 (version 3.1; Thermo Fisher Scientific, Waltham, Massachusetts, USA) and Bioanalyzer 2100 (version B.02.09.S1720; Agilent, Santa Clara, California, USA) from the Molecular Biology Service Unit, University of Alberta, Canada. The cDNA for each sample was generated as previously described using 1000 ng of RNA.

Quantitative reverse transcriptase PCR (RT-qPCR) was used to compare relative transcript levels between treated and untreated controls using the delta-delta Ct method. All experiments used ACTIN as a reference gene. Primers were designed using Primer Express 3.0 (Applied Biosystems), ensuring at least one primer was outside of the construct (Appendix S1). Primers were also tested for optimal efficiency using a dilution series. RT-qPCR was run using 10-µL reaction volumes containing 5.0 µL SYBR Green master mix (0.25× SYBR Green, 0.1× ROX, 0.3 units Platinum Taq Polymerase [Invitrogen], and 0.2 mM dNTPs), 2.5 µL of 3.4 µM forward and reverse primer, and 20 ng of cDNA. Assays were run using both the 384-well QuantStudio 6 Real-Time PCR System and the 7500 Fast Real-Time PCR System (both from Applied Biosystems). Three technical replicates per sample were analyzed with a minimum of three biological replicates (range of 3 to 5). One individual plant is equivalent to one biological replicate. VIGS-treated plants will often display varying phenotypes on one plant, and in such cases only one phenotype was used for RT-qPCR.

**RESULTS**

**No off-target silencing was detected for endogenous pTRV2 constructs**

The endogenous constructs had no off-target silencing and were on-target for our genes of interest. The coding sequence of *AtPDS* had top hits to scaffold 38 (650,987 bp), similar to both endogenous and heterologous constructs. The coding sequence of *AtFUL* had a top hit with scaffold 1565 (19,090 bp), similar to TRV2- *CvFUL*, which only had a single hit. No off-targets were found for TRV2- *CvPDS*, TRV2- *CvFUL*, or TRV2- *CvPDS-CvFUL*, and only minimal off-target silencing was detected for TRV2- *AtPDS* (Appendix S2). TRV2- *AtPDS* had 84 total and 36 efficient hits to scaffold 38. TRV2- *AtPDS* also had six off-target hits to scaffold 283 (212,661 bp), although none were efficient. The efficient hits are matched on a more stringent set of parameters and can be considered more representative of true off-targets (Lück et al., 2019). In summary, there were no major off-target sites for any of the constructs, and all constructs appear to target our genes of interest, *PDS* and *FUL*.

**Agrobacterium tumefaciens-mediated pTRV2-AtPDS and pTRV2-CvPDS infection induces silencing of gene targets in Cleome violacea**

An effective VIGS system entails low mortality and high penetrance of transient mutant phenotypes. We explored mortality and penetrance in *C. violacea* using binary vectors pTRV1 + pTRV2- *CvPDS* and pTRV1 + pTRV2- *AtPDS*. Initial experiments tested both heterologous (pTRV2- *AtPDS*) and endogenous (pTRV2- *CvPDS*) constructs to determine how susceptible *C. violacea* is to VIGS (Fig. 3). Treatment with pTRV2- *AtPDS* revealed that *C. violacea* was amenable to VIGS, as most surviving plants exhibited photobleaching (Table 1, Fig. 3A–E). Of the 198 plants treated with pTRV2- *AtPDS*, 119 survived, with roughly half displaying photobleaching. Relative to pTRV2- *AtPDS*, plants treated with pTRV2- *CvPDS* had the highest penetrance (81%) despite a lower survival rate (Table 1, Fig. 3F–J). The greatest penetrance (74%) was in medium plants (Table 1). That is, of the 58 surviving plants that showed altered morphology were medium-sized seedlings (Table 1). Ultimately, medium plants were chosen for all subsequent experiments because of their stronger response to infiltration, i.e., stronger photobleaching. We also tested a species of Brassicaceae, *Erucaria erucarioides* Müll. Berol., with heterologous pTRV2- *AtPDS*, but no further experiments with *E. erucarioides* were completed due to low penetrance (Appendix S3). We mention these preliminary data to show that phylogenetic distance, and similarity of VIGS construct to the target gene, does not necessarily correlate with efficacy of VIGS (Appendix S4).

Follow-up experiments tested whether the observed phenotypes were due to downregulation of *CvPDS*. Here, we only used TRV2- *CvPDS* because it had the highest overall penetrance from earlier trials. The pTRV2- *CvPDS* treatment group had 36 of 86 surviving plants display photobleaching, most with strong photobleaching (Table 2). On average, we observed photobleaching from three to four weeks after inoculation, with the altered phenotypes lasting for several weeks. Leaves showing 50–80% and >80% photobleaching had significant downregulation of *CvPDS* relative to controls and treated plants with unaltered phenotype (Fig. 4A). Although we scored and phenotyped leaves with pale bleaching (1–50%) (Appendix S5A), they were not included in the RT-qPCR analysis because we are primarily interested in strong VIGS penetrance, i.e., our goal was to demonstrate that *CvPDS* is downregulated when we see obvious photobleaching. Leaves with complete photobleaching were also scored (Appendix S5A); however, they were binned with strong photobleaching (>80%) because of their infrequency. These results demonstrate that VIGS functions well in *C. violacea*, is experimentally replicable, and produces strong silencing with endogenous and heterologous constructs.
Another factor in an effective VIGS system is the ability to study genes across developmental stages. As such, we investigated pTRV2-CvFUL treatment on the final stage of reproduction in flowering plants, fruit development. Fruits of *C. violacea* are elongate, bicarpellate capsules, which are characteristic for Cleomeae. Like Brassicaceae, the fruits have valves, which are the portion of the ovary walls that separate at maturity (Fig. 2A). Also, like Brassicaceae, fruits of *C. violacea* have a prominent persistent placenta called the replum. The primary difference between Cleomeae capsule fruits and Brassicaceae siliques is that Cleomeae fruits lack the false septum separating the two locules (Fig. 2B) (Ilis et al., 2011). Like with PDS, initial experiments with pTRV2-CvFUL-CvPDS were completed to determine effectiveness in *C. violacea*. The PDS marker was used to facilitate phenotyping, as we were unsure of the silencing effects of CvFUL alone. Large seedlings were chosen for the initial experiments to account for developmental timing of fruiting. A range of altered fruit morphologies were observed from four to six weeks post-inoculation. Of the 60 large seedlings treated (≥7 true leaves), there was a 95% survival rate, and 36% of survivors displayed some degree of curling and/or indentation (Table 1, Fig. 5A–D).

Later experiments used pTRV2-CvFUL alone, as initial experiments were relatively easy to score. Like with pTRV2-CvFUL-CvPDS, pTRV2-CvFUL-treated plants began displaying altered fruit morphologies by four to six weeks post-inoculation (Table 2, Fig. 5E–H). Most prominently, fruit curling and ovule indentation were observed, i.e., the valve was appressed around the developing seeds (Fig. 5E–H, Appendix S5H, I). In non-control treated plants of *C. violacea*, fruit length was decreased when fruit curling was moderate to heavy (≥90°); however, those with light curling (<90°) and/or indentation did not have a significant difference relative to fruits with non-altered phenotype (Appendix S6). Future studies with CvFUL knockouts would be more informative because variability of PTGS response is likely a major factor in fruit length with VIGS. For seedlings treated with pTRV2-CvFUL, 37 of the 85 surviving plants showed some altered phenotype, mostly mild curvature (Table 2). Incomplete and broad variability of CvFUL silencing made it difficult to accurately bin phenotypes by severity (Fig. 5E–H), despite initial efforts (Appendix S5B–E). Because our primary goal was to assess the general viability of CvFUL silencing using VIGS, and thus the potential for other fruit-patterning genes, we pooled data from all curled fruits for RT-qPCR regardless of severity. This way, ambiguities from incomplete silencing and unknown effects would be reduced while still demonstrating that CvFUL had been successfully downregulated. CvFUL was significantly downregulated relative to untreated plants, pTRV2-MCS-treated plants, and normal-phenotype treated plants (Fig. 4B). It should be noted that pTRV2-MCS had a greater than expected effect on fruit morphology (Appendix SSC, G); this is due to viral vector response and not downregulation of CvFUL (Fig. 4B). It seems likely that *C. violacea* fruits are more sensitive to tobacco rattle virus relative to other structures, which may account for the mild bulging in some fruits (Appendix S5G). It would be informative for future studies to observe the effects of viral response at varying concentrations of *A. tumefaciens* inoculation.

The curling, indentation, and shortening of fruits in pTRV2-CvFUL-CvPDS-treated plants are accompanied by differences in both cell size and shape in the valves and repla of those fruits (Fig. 6).

| Treatment               | s | m | l | Total | % Survival | % Penetration (total) | % Penetration (survivors) |
|-------------------------|---|---|---|-------|------------|-----------------------|--------------------------|
| Untreated               | 23| 30| 18| 71    | 87         | 80                    | 94                       |
| pTRV2-MCS               | 49| 85| 21| 155   | 10         | 38                    | 86                       |
| pTRV2-AtPDS             | 51| 68| 79| 198   | 18         | 78                    | 72                       |
| pTRV2-CvPDS             | 25| 121|77| 223   | 0          | 21                    | 43                       |
| pTRV2-CvFUL-CvPDS      | NA| 2 | 60| 62    | 50         | 95                    | NA                       |

Note: N = number of individuals sampled; NA = not applicable.

DISCUSSION

VIGS is a well-established method for gene knockdown in angiosperms and has provided insight in determining the mechanisms...
underlying differences across diverse lineages such as Ranunculales, asterids, Caryophyllales, and rosids. It remains a powerful tool to address questions about organ identity, sex determination, induction of flowering, and compound leaf development (Hidalgo et al., 2012; Hsieh et al., 2013; Fujita et al., 2019). As such, the major goal of this study was to establish VIGS in Cleomaceae, which is garnering interest as a model family (Bayat et al., 2018). We established that *C. violacea* has high silencing efficiency and moderate mortality with VIGS treatment. Furthermore, *C. violacea* is amenable to treatment with readily available *Arabidopsis* constructs, which implies a potential to explore this method for additional members of Cleomaceae. Finally, we demonstrate that both leaves and fruits are susceptible to treatment, suggesting that other developmental stages and organs could also be studied with this system.

**Arabidopsis thaliana** constructs are valuable for assessing susceptibility to VIGS in Cleomaceae

Initial screens carried out on small, medium, and large seedlings with a heterologous *PDS* construct (pTRV2-AtPDS) were informative about overall susceptibility of *C. violacea* to VIGS (Table 1, Fig. 3A–E). Readily available heterologous constructs are a useful first pass for determining whether select species from Cleomaceae are amenable to VIGS. Survival and penetrance percentages were similar in *C. violacea* between both heterologous and endogenous (pTRV2-CvPDS) *PDS* constructs (Tables 1, 2). Overall, more plants survived after treatment with the *A. thaliana* construct than the endogenous construct, and both constructs had a similar percentage of photobleaching in medium plants. The increased mortality in pTRV2-CvPDS-treated plants was likely due to stronger photobleaching because photobleaching reduces efficiency of photosystem II (Wang et al., 2009). The use likely due to stronger photobleaching because photobleaching in medium plants. The increased mortality in pTRV2-CvPDS-treated plants was likely due to stronger photobleaching because photobleaching reduces efficiency of photosystem II (Wang et al., 2009). The use likely due to stronger photobleaching because photobleaching in medium plants. The increased mortality in pTRV2-CvPDS-treated plants was likely due to stronger photobleaching because photobleaching reduces efficiency of photosystem II (Wang et al., 2009). The use likely due to stronger photobleaching because photobleaching in medium plants. The increased mortality in pTRV2-CvPDS-treated plants was likely due to stronger photobleaching because photobleaching reduces efficiency of photosystem II (Wang et al., 2009). The use likely due to stronger photobleaching because photobleaching in medium plants. The increased mortality in pTRV2-CvPDS-treated plants was likely due to stronger photobleaching because photobleaching reduces efficiency of photosystem II (Wang et al., 2009).

**TABLE 2.** Survival and penetrance data, representing two virus-induced gene silencing trials of medium plants (4–6 true leaves) of *Cleome violacea* grouped by treatment.

| Treatment          | N  | % Survival | % Penetration (total) | % Penetration (survivors) |
|--------------------|----|------------|-----------------------|---------------------------|
| Untreated          | 50 | 86         | NA                    | NA                        |
| Vacuum + surfactant| 50 | 90         | NA                    | NA                        |
| pTRV2-MCS          | 100| 80         | NA                    | NA                        |
| CvPDS              | 100| 86         | NA                    | NA                        |
| CvPDS              | 100| 86         | NA                    | NA                        |
| 0% bleached        | 51 | 44         | 51                    | 44                        |
| <50% bleached      | 7  | 6          | 7                     | 6                         |
| 50–80% bleaching   | 8  | 7          | 8                     | 7                         |
| >80% bleaching     | 34 | 29         | 34                    | 29                        |
| CvFUL              | 100| 85         | NA                    | NA                        |
| Indentation        | 8  | 9          | 8                     | 9                         |
| Mild curvature     | 28 | 33         | 28                    | 33                        |
| Moderate curvature | 8  | 9          | 8                     | 9                         |
| Heavy curvature    | 1  | 1          | 1                     | 1                         |

Note: N = number of individuals sampled; NA = not applicable.

from other species of Solanaceae (Senthil-Kumar et al., 2007); even constructs from *Taxus baccata* (Pinophyta) were successfully used to downregulate *PDS* in *N. benthamiana* (Tafreshi et al., 2012). Close phylogenetic distance between *C. violacea* and *A. thaliana* was not indicative of higher susceptibility to VIGS, as another member of Brassicaceae had lower penetrance despite greater *PDS* sequence similarity (Appendices S4, S7). Different species displaying variable susceptibility to VIGS is unsurprising given that different cultivars of the same species, e.g., Gerbera (Asteraceae), also show different susceptibility to the same *PDS*

**FIGURE 4.** Relative quantification (RQ) vs. control of PHYTOENE DESATURASE (*CvPDS*)–silenced leaves (A) and FRUITFULL (*CvFUL*)–silenced fruits (B) from *Cleome violacea* after virus-induced gene silencing. (A) Untreated (*n* = 4), pTRV2-MCS (*n* = 3), normal phenotype (*n* = 5), >80% photobleaching (*n* = 3), and 50–80% photobleaching (*n* = 3). (B) Untreated (*n* = 1), pTRV2-MCS (*n* = 3), normal phenotype (*n* = 3), and curled (*n* = 6). Error bars indicate RQ maximum and minimum values calculated from standard error. *ACTIN* was used as an endogenous control. Statistical significance was determined using a Welch’s t-test on delta CT means (∞0.05), indicated by an asterisk.

**FIGURE 5.** *Cleome violacea* subjected to virus-induced gene silencing using pTRV2-CvFUL-CvPDS (A–D) and pTRV2-CvFUL (E–H) constructs. (A–D) Fruits displaying photobleaching and atypical development after treatment with pTRV2-CvFUL. (E–H) Fruits displaying atypical development after treatment with pTRV2-CvFUL. pTRV2-CvFUL-CvPDS-treated *C. violacea* with normal development (I) and untreated fruit (J). Scale bar = 2 mm.
vectors (Deng et al., 2012). That is, it appears that susceptibility to a specific vector, and to VIGS itself, is not necessarily predictable by phylogenetic distance.

There are many other factors in determining susceptibility, e.g., the type of viral vector, the length of the insert, and plant growth temperature. Although it varies by study, the effective lower limit for PDS silencing using TRV is as low as ~190 bp in *N. benthamiana* (Liu and Page, 2008), and even a size of 23 bp can produce silencing effects when using a potato virus vector (Thomas et al., 2001). Additionally, VIGS silencing appears to be more efficient at lower temperatures, and inhibited at high temperatures, in some species (e.g., *Lycopersicon esculentum* Mill. [tomato] [Fu et al., 2006], *Catharanthus roseus* (L.) G. Don [periwinkle] [Sun et al., 2014], and *Petunia x hybrida* Hort. ex E. Vilm. [Broderick and Jones, 2014]); nonetheless, temperatures between 22–24°C function well for *C. violacea* and *A. thaliana* (Wang et al., 2006). Our data, in congruence with studies in *N. benthamiana*, strongly suggest that a construct sharing at least partial identity to the gene of interest is capable of knockdown, regardless of species origin. In summary, heterologous constructs are an informative first screen for VIGS in *C. violacea* and should be considered when exploring other members of Cleomaceae.

**VIGS is a practical tool for studying fruit development in the Cleomaceae**

Significant downregulation of the key fruit pattern gene *CvFUL* resulted in altered fruit morphology of *C. violacea* (Figs. 4B, 5). In preliminary treatments, *CvFUL* was downregulated in combination with a *CvPDS* marker (Table 1, Fig. 5A–D). We acknowledge the usefulness of marker genes for identifying difficult-to-spot mutant phenotypes and for quick identification when overall penetrance is low. However, there are limitations to marker genes. For example, in studies of floral symmetry, photobleaching would obscure pigmentation differences between adaxial and abaxial petals of *C. violacea* (Fig. 1). Additionally, there are potentially confounding effects of decreased plant vitality from photobleaching. For these reasons, and because *CvFUL*-silencing phenotypes are relatively easy to score, albeit difficult to bin, additional VIGS *CvFUL* treatments were completed without a marker (Fig. 5E–H).

*FUL* has a critical role in ensuring that the valve-margin genetic pathway is restricted to a thin layer of cells between the valve and replum in *A. thaliana* (Dinneny et al., 2005). Specifically, *FUL* is expressed in the valves and functions in positioning the valve margin in combination with *REPLUMLESS* (*REP*), which has a similar role in the replum. Given the similarities in overall fruit morphology between Brassicaceae and Cleomaceae (Fig. 2B), we predicted a conserved role in the genetic basis of fruit patterning via *FUL*. In *A. thaliana*, *ful-1* mutants have fruits with valve cells that have not properly developed and are much rounder than wild-type plants (Figs. 3, 7 from Gu et al., 1998). Mutant *ful* *A. thaliana* fruits and, to a lesser extent, other fruit-patterning mutants are shorter in length and have alterations in their valve cellular structure and uniformity (Fig. 4 from Liljegren et al., 2004). Like in *A. thaliana*, *CvFUL* influences the uniformity of cell expansion in the valve (Fig. 6D, H). Superficially, treated *C. violacea* fruits (Appendix S5D) appear more like *A. thaliana ful-1 35S::AGL8* rescue mutants (Fig. 7 from Gu et al., 1998), which are less stunted than *ful-1* mutants. Incomplete downregulation of *CvFUL* likely explains the somewhat normal fruit length observed during our trials.

Replum cells in fruits of *C. violacea* (Fig. 6F) are irregularly shaped in *CvFUL-CvPDS*-silenced fruits, a phenotype that is also observed in fruits of *ful-1 A. thaliana* mutants (Fig. 3E from Gu et al., 1998). We propose that the alteration in replum growth is likely due to shearing forces from the offset growth of valve tissue on either side, which is likely due to incomplete silencing of *CvFUL*. When *FUL* is downregulated, it prevents the formation of a dehiscence zone between the replum and the valve (reviewed in Ferrándiz and Fourquin, 2014). Thus, both sides of the valve variably push and pull on the replum, and because it cannot separate at the dehiscence zone, it warps the replum and causes fruit curling. A complete knockout of *CvFUL* should not curl because the replum valve boundary would be disrupted evenly along the length of the fruit, i.e., cells in the valves would exert even pressure on the replum. Altogether, these data exhibit how VIGS can be used to target late developmental stages prior to the initiation of those stages.

Cleomaceae is quickly emerging as a model family to address a range of evolutionary and developmental questions (Bayat et al., 2018). Additional functional tools are invaluable for examining the morphological novelties present in the family. We have demonstrated that mild modifications to established VIGS protocols (Gould and Kramer, 2007; Kramer et al., 2007) are sufficient for high penetration of *PDS* and *FUL* mutant phenotypes in *C. violacea* (Tables 1, 2). We propose that use of *A. thaliana* constructs is a viable first step in future investigations of additional species in the family because of the effectiveness of pTRV2-AtPDS constructs in this study. Furthermore, the published draft genome of *C. violacea* makes this species a prime candidate for further research on a range of traits.

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**AUTHOR CONTRIBUTIONS**

P.M., M.H.D., J.C.H., and S.C. contributed to conception and design of the project. S.C., M.H.D., P.M., and A.R. contributed to data collection and analysis. S.C. and J.C.H. contributed to drafting and revising of the manuscript. All authors provided feedback on drafts and gave consent to publish the material.

**DATA AVAILABILITY**

The *CvPDS* construct sequence (accession no. MW505002) is available at the National Center for Biotechnology Information. Phenotyping and RT-qPCR raw data are available upon request.
**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the supporting information tab for this article.

**APPENDIX S1.** List of primers used for construct design and RT-qPCR in this study. Brackets indicate restriction sites for incorporation into the pTRV2 vector. Extra base pairs upstream of restriction sites are to improve digestion efficiency. EcoRI (G^AATTTC), XbaI (T^CAGA), BamHI (G^GATCC), and XhoI (C^TCGAG).

**APPENDIX S2.** SiFi21 results for pTRV2 constructs used in this study: (A) pTRV2-CvFUL, (B) pTRV2-CvPS, (C) pTRV2-CvFUL-CvPS, (D) pTRV2-ATPDS. Results are compared to the draft genome of *Cleome violacea* (https://genom evolution.org/coge; accession no. 23822) and organized by scaffolds.

**APPENDIX S3.** Hits to the *Cleome violacea* genome (https://genom evolution.org/coge; accession no. 23822) using *Arabidopsis thaliana* coding sequences of *FUL* and *PDS*, and *TRV2-CvFUL* constructs. Default parameters were used for CoGe BLASTN.

**APPENDIX S4.** Percent identity of the pTRV2-ATPDS construct relative to *Arabidopsis thaliana*, *Erucaaria erucarioides*, and *Cleome violacea* (A). Graph of the alignment using Geneious alignment software with 93% similarity cost matrix and default settings (B).

**APPENDIX S5.** (A) *Cleome violacea* leaflets, taken from whole leaves with identical phenotypes, after treatment with pTRV2-CvPS. From left to right: untreated, pale bleaching (0–50%); variegated bleaching (50–80%); strong bleaching (>80%); and complete bleaching. Scale bar = 2 mm. (B–I) *Cleome violacea* fruits after treatment with *TRV2-CvFUL*: untreated control (B, F), pTRV2-MCS (C, G), mild curvature with indentation (D, H), moderate curvature with indentation (E, I). r = replum, v = valve; vm = valve margin; arrows indicate indentation. Scale bars = 0.5 cm (B–E) and 500 μm (F–I).

**APPENDIX S6.** Length of *Cleome violacea* fruits treated with pTRV2-CvFUL. Normal phenotype (n = 24), indentation (n = 8), mild and moderate curvature with indentation (n = 5). Significance was determined using Welch’s t-test (α = 0.01), indicated by an asterisk.

**APPENDIX S7.** Survival and penetrance data combined from multiple *Erucaria erucarioides* virus-induced gene silencing trials. Mortality percentages were taken prior to 30 days post-inoculation. Plants were categorized based on the number of true leaves: small (s) with 0–3 true leaves, medium (m) with 4–6 true leaves, and large (l) with ≥7 true leaves.

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Carey et al.—Virus-induced gene silencing in Cleome violacea

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