PROTOCOL NOTE

Applications in Plant Sciences

Development of a petal protoplast transfection system for *Sinningia speciosa*

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Abstract

Premise: Transient gene expression systems are powerful tools for studying gene interactions in plant species without available or stable genetic transformation protocols. We optimized a petal protoplast transformation protocol for *Sinningia speciosa*, a model plant, to study the development of floral symmetry.

Methods and Results: A high yield of petal protoplasts was obtained using a 6-h enzyme digestion in a solution of 1.5% cellulase and 0.4% macerozyme. Modest transfection efficiency (average 41.4%) was achieved. The viability of the transfected protoplasts remained at more than 90%. A fusion of green fluorescent protein and CYCLOIDEA (SsCYC), the Teosinte branched 1/Cincinnata/Proliferating cell factor transcription factor responsible for floral symmetry, was subcellularly localized inside the nuclei of the protoplasts. Transiently overexpressing *SsCYC* indicates the success of this system, which resulted in the predicted increased (but nonsignificant) expression of its known target *RADIALIS* (*SsRAD1*), consistent with gene network expectations.

Conclusions: The transient transfection system presented herein can be effectively used to study gene-regulatory interactions in Gesneriaceae species.

KEYWORDS

petal, protoplasts, *Sinningia speciosa*, transfection, transient expression

The ornamental plant *Sinningia speciosa* (Lodd.) Hiern, also known as florist's gloxinia, belongs to the Gesneriaceae family. Different cultivars of *S. speciosa* have either mostly zygomorphic (bilaterally symmetrical) or actinomorphic (radially symmetrical) flowers, with the developmental transition of zygomorphy to actinomorphy being attributed to the mutation of a single gene, *CYCLOIDEA* (*SsCYC*), a Teosinte branched 1/Cincinnata/Proliferating (TCP) cell factor transcription factor that controls floral symmetry (Hsu et al., 2017; Dong et al., 2018). Genes downstream of *SsCYC* are therefore responsible for the flower shape changes between zygomorphic and actinomorphic *S. speciosa* accessions. Zygomorphic and actinomorphic *S. speciosa* can be easily crossed to produce recombinant inbred lines, enabling researchers to use *S. speciosa* as a

model plant to study the floral traits associated with various floral developmental genes (Wang et al., 2015; Hsu et al., 2017). Furthermore, the small nuclear genome (approximately 300 Mbp in the diploid *S. speciosa* 'Espírito Santo'), chromosome uniformity ($2n = 26$), and short generation time (approximately 3–6 months) of *S. speciosa* make it an excellent model for scientific research (Skog, 1984; Zaitlin and Pierce, 2010). To study the roles of genes downstream of *SsCYC* in flower development, a stable or transient gene transformation system is required. The *Agrobacterium*-mediated stable genetic transformation of *S. speciosa* has been reported (Xu et al., 2009; Kuo et al., 2018); however, obtaining transgenic plants using this method is a laborious undertaking because of the low transfection efficiency of *Agrobacterium* in *S. speciosa*.

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Developing an *Agrobacterium*-free approach in which DNA may be delivered directly into cells is therefore desirable.

Plant protoplasts are cells in which the cell walls have been digested by enzymes. Studying protoplasts can be an effective approach for observing and analyzing cellular processes involved in various biological functions for two reasons (Marx, 2016). First, protoplasts can be isolated from distinct types of plant tissues to explore cell type–specific and tissue-specific gene expression or function. Second, isolated protoplasts maintain their cellular identity for a certain period of time in vivo (Marx, 2016). Plant protoplast systems therefore facilitate the study of transient gene functions, especially in plant species without well-established stable genetic transformation systems. Protoplast isolation and transfection systems have been established and optimized for numerous plant species, even non-model plant species, since their development from the isolation of bacterial and fungal protoplasts and initial use in plant cells in 1960 (Cocking, 1960); however, most efficient protoplast isolation protocols have been developed using leaves or seedlings from model plants and various crops, including *Arabidopsis thaliana* (L.) Heynh., chickpea (*Cicer arietinum* L.), *Lolium* L., *Liriodendron* L. hybrids, and *Phaseolus vulgaris* L. (Yoo et al., 2007; Nanjareddy et al., 2016; Huo et al., 2017; Cheng and Nakata, 2020; Davis et al., 2020).

Our goal was to study gene regulation in flowers (i.e., genes downstream of *SsCYC*), and existing leaf protoplast systems may not be suitable for studying tissue-specific gene interactions in flowers. It was therefore necessary to establish a petal protoplast isolation and transfection system for *S. speciosa*. Although protoplast isolation protocols for Gesneriaceae species (e.g., *Streptocarpus* Lindl. and *Saintpaulia ionantha* H. Wendl.) are available, the protocols were developed based on small shoot primordia regenerated from cultured leaf blade explants (Afkhami-Sarvestani et al., 2012) or young shootlets regenerated from leaf explants (Hoshino et al., 1995; Winkelmann and Grunewaldt, 1995a, 1995b). Published protoplast isolation protocols for flower tissues remain scarce, and none are available for Gesneriaceae species. A few reports have investigated the use of petal protoplasts in other ornamental plants, such as *Rosa rugosa* Thunb. (Borochov et al., 1976), *Tulipa* L. (Wagner, 1979), and *Dendrobium* Sw. (Hu et al., 1998), and more recently, petal protoplasts have been used as transient systems to study various genetic pathways in flowers, such as *Petunia* Juss. hybrids (Faraco et al., 2011) and *Phalaenopsis* Blume and *Cymbidium* Sw., two species in the orchid (Orchidaceae) family (Lin et al., 2018; Ren et al., 2020). However, directly adapting the petal protoplast isolation protocols from orchids to *S. speciosa* may be difficult due to the differences in texture and cell wall properties between the species. To account for these differences, distinct enzyme digestion and gene transfection treatments must be employed in studies of distinct species.

Herein, we describe an efficient protocol for isolating *Sinningia* Nees petal protoplasts and transforming them with exogenous DNA to examine the transient expression levels of the gene *CYCLOIDEA* (*SsCYC*) and determine the subcellular location of its protein. Using petals from *Sinningia* floral buds, protoplasts with consistent quality (viability) and quantity (yield) can be obtained. In addition, we optimized a large-scale protoplast transfection procedure to allow for the simultaneous examination of the transient upstream and downstream regulation of multiple genes. These contributions will facilitate future studies of gene interactions in floral tissues of *S. speciosa*.

METHODS AND RESULTS

Plant growth conditions

Wild-type *S. speciosa* 'Espírito Santo' ('ES') plants with zygomorphic flowers were cultivated in a walk-in greenhouse at National Taiwan University (Taipei City, Taiwan) under long-day conditions (16 h light/8 h dark) and an LED-produced light intensity of $200\,\mu mol\cdot m^{-2}\cdot s^{-1}$ at 70–80% relative humidity and 22°C to 25°C. Under these conditions, *S. speciosa* 'ES' plants can flower twice a year (flowering periods: February to April and August to October). Fertilizer (2000× diluted water-soluble 25N–5P–20 K) was applied every 1–2 weeks during the nonflowering period. During the flowering period, we reduced the fertilization frequency to once every 3–4 weeks because regular fertilization can drastically reduce the transfection efficiency (to <10%). Protoplasts were not isolated from individuals near the beginning or end of the flowering period.

Petal protoplast isolation and protoplast yield

All the chemicals, solutions, and equipment used in this study are listed in Appendix 1. The proposed small-scale procedures were mainly adopted from optimized protocols for the model plants *Arabidopsis* and poplar (*Populus* L.) (Yoo et al., 2007; Lin et al., 2014). We developed the large-scale procedures to reduce the processing time and labor required in situations where large quantities of RNA must be extracted from transfected protoplasts (e.g., for the analysis of the expression of numerous candidate genes or in transcriptome sequencing). For the small-scale procedure, 1.5 g of fresh petals from 5–6 pre-bloom flowers at floral bud stages 10–12 (FB10–12, at which the length of the corolla tube is 21–30 mm; Pan et al., 2022) were collected (Figure 1A). For the large-scale procedure, 4.5 g of fresh petals from 15 FB10–12 floral buds were used. All the sepals, stamen/staminodes, and carpels were carefully removed and discarded (see Note 1 in
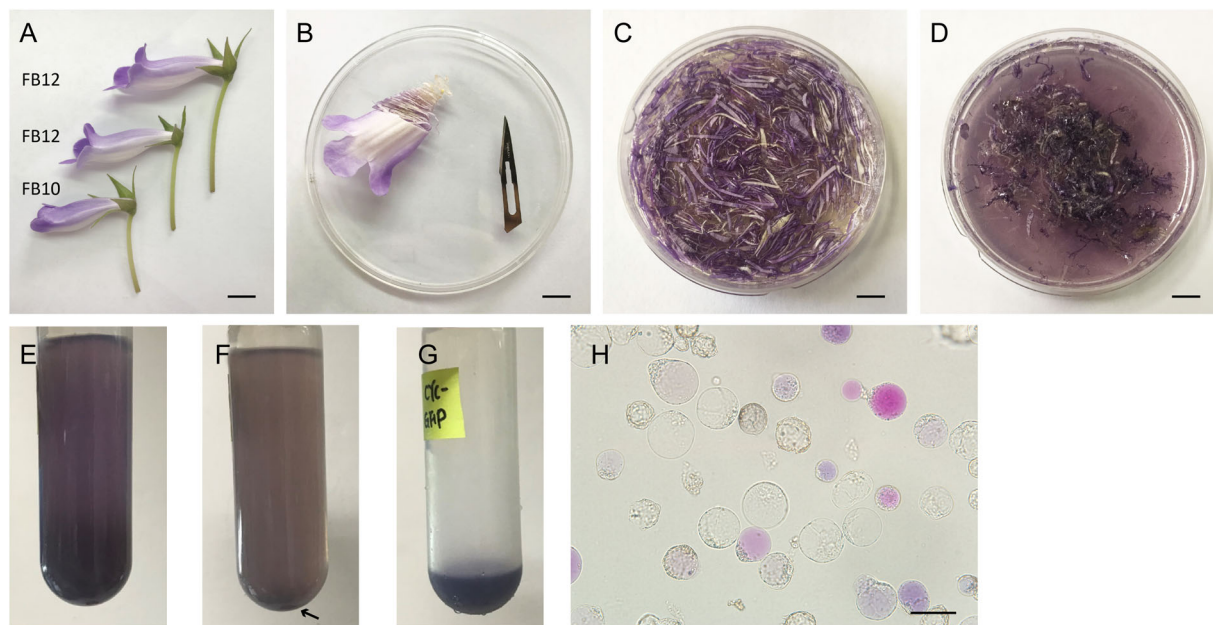
FIGURE 1 Overview of procedures for isolating petal protoplasts of *Sinningia speciosa* 'Espírito Santo' ('ES'). (A) Floral buds harvested at floral development stages FB10–FB12. (B) Petals were cut into strips with widths of 0.5–1.0 mm using a scalpel blade. (C) Petal strips submerged in enzyme solution (after 0 h of digestion). (D) Release of protoplasts from petal strips after 6 h of enzyme digestion. (E–G) Steps of the large-scale protoplast isolation protocol, performed in a 30-mL tube. (E) Protoplasts washed with MMG solution. (F) Protoplast pellet (arrow) visualized after centrifugation. (G) Protoplast pellet resuspended in MMG solution. (H) Isolated petal protoplasts. Scale bars = 1 cm for A–D; 50 μm for H.

Appendix 1). Each petal was cut into 0.5–1-mm strips using a new sharp scalpel blade, without crushing the petal tissue at the cutting site (Figure 1B, Note 2 in Appendix 1). The petal strips were immediately fully submerged in a freshly prepared enzyme solution (Figure 1C, Note 3 in Appendix 1), before being vacuum-infiltrated (30–40 mm Hg) at room temperature (23°C to 26°C) in the dark for 30 min. The enzyme digestion was performed for 5–6 h at room temperature in the dark without shaking (Notes 4 and 5 in Appendix 1). After digestion, the enzyme solution gradually turned purple due to the release of purple petal protoplasts (Figure 1D). The shapes of the released protoplasts in the enzyme solution were examined using a microscope, and were confirmed to be intact and round, and approximately 30–40 μm in diameter. To remove the petal debris, the protoplast solution was filtered through a double layer of 75-μm nylon mesh, which had been cleaned with ddH$_2$O to remove excess water and wetted with MMG solution before filtration. The filtered protoplasts were collected in a 30-mL round-bottomed tube (Figure 1E, total volume 20–30 mL, Note 6 in Appendix 1). The tube was centrifuged for 1–2 min with a swinging-bucket rotor at 200 × g to pellet the protoplasts; the protoplast pellet was visible at the bottom of the tube (Figure 1F, arrow). To harvest the protoplasts, the supernatant was removed as fully as possible, without disturbing the pellet, using a pipette (Note 7 in Appendix 1). After resuspending the protoplast pellet in a small volume of MMG solution by

gentle swirling, the cells were counted using a hemacytometer, and then the final concentration of protoplasts was adjusted with MMG solution (small-scale procedure: 2×10^5/mL, large-scale procedure: 4.16×10^6/mL) (Figure 1G, Note 8 in Appendix 1). The tube with the resuspended protoplasts was placed on ice for at least 30 min. The isolated petal protoplasts remained intact and round, and some of them contained large purple vacuoles at their centers (Figure 1H), indicating that the protoplasts were viable and healthy.

Enzyme concentration and digestion time are key to obtaining a high yield of petal protoplasts (Huang et al., 2013; Wu et al., 2017; Li et al., 2018). We thus compared the yield of protoplasts obtained using an enzyme concentration of 1× (1.5% cellulase and 0.4% macerozyme) and 2×, with digestion times ranging from 3 h to 6 h at room temperature in the dark without shaking. The use of the small-scale procedure with a 6-h digestion time and 1× enzyme concentration produced a yield of 2.84×10^6 protoplasts/g fresh weight (FW; Appendix 2A) or more than 1×10^6 protoplasts/g FW from each isolation procedure. When the large-scale procedure was conducted using the same enzyme treatment, the average yield was approximately 2.95×10^6 protoplasts/g FW. Studies have reported that the protoplast yield from petals (10^5–10^6/g FW) is usually lower than that from leaves (approximately 3.0×10^7/g FW; Wu et al., 2009). Furthermore, most studies have reported low to moderate yields of petal protoplasts, such as 9.5×10^5/g FW from *Phalaenopsis* orchids (Lin et al., 2018), 2×10^5/g FW from *Phaseolus vulgaris*

(Nanjareddy et al., 2016), and $1.5-8 \times 10^6$/g FW from *Petunia* hybrids (Oh and Kim, 1994). One exception was a study of *Cymbidium* orchid petal protoplasts, which reported a yield of 3.5×10^7/g FW (Ren et al., 2020). Because our protocol helped achieve moderate yields of *S. speciosa* petal protoplasts ($1-2.95 \times 10^6$/g FW), we determined that the 6-h digestion time and 1× enzyme concentration were the optimal conditions for protoplast isolation and continued to use them in our subsequent small- and large-scale tests.

DNA–polyethylene glycol–calcium transfection

We transfected the pUC19 plasmid with green fluorescent protein (GFP) driven by the *35S* promoter (pUC19-*35S:GFP*) to evaluate the transfection efficiency of the protoplasts. We calculated the transfection efficiency by dividing the number of protoplast cells displaying GFP fluorescence under a dark-field UV microscope by the total number of protoplast cells observed under a bright-field microscope. To conduct small-scale transfections, 100 µL of protoplasts (2×10^4 protoplasts) were aliquoted into a 2-mL round-bottomed tube and gently mixed with 10 µL (1 µg/µL) of plasmid DNA. To conduct large-scale transfections, 1.6 mL of protoplasts (approximately 6.65×10^6 protoplasts) were added to a 30-mL tube and mixed gently with 200 µL (1 µg/µL) of plasmid DNA. Freshly prepared polyethylene glycol (PEG)-$CaCl_2$ transfection solution (110 µL and 1.76 mL in the small- and large-scale procedures, respectively) was added to the protoplasts and immediately mixed by gently tapping the tube. The transfection mixture was incubated at room temperature for 10–15 min; transfection was stopped by diluting the mixture with W5 solution (440 µL and 7.04 mL in the small- and large-scale procedures, respectively) at room temperature and gently inverting the tube to mix. Equal volumes of the solution were dispensed into 16 new 2-mL round-bottomed tubes. In both the small- and large-scale protoplast transfection procedures, the tubes were centrifuged at $200 \times g$ for 2 min at room temperature, after which the supernatant was removed. To produce a plate-coating buffer, 5 mL of 5% BSA was dispensed on the plastic surface of a 12-well culture plate for a short time (1–2 s). This buffer prevented the protoplasts from sticking to the plate. The transfected protoplasts were resuspended in 0.5 mL of WI solution,

transferred into the wells of the coated culture plate, and incubated at room temperature under light (daylight fluorescent lightbulb) for 16–18 h. The transfection efficiency was then calculated.

Optimizing the PEG concentration and amount of exogenous DNA is crucial for successful transfection (Huang et al., 2013; Nanjareddy et al., 2016; Wu et al., 2017; Li et al., 2018; Page et al., 2019); PEG facilitates the introduction of exogenous DNA (i.e., plasmids) into the targeted cell by adjusting the osmotic pressure of the cell membrane, thereby increasing its permeability. To determine the effect of PEG 4000 concentrations on transfection efficiency, the protoplasts were transfected with 40% or 50% PEG 4000 in combination with digestive enzymes (1× or 2× concentration; Appendix 2B). After a 16-h incubation, the transfection efficiencies of the four combined conditions were between 44.3% and 56.1%. Under the combination of 50% PEG 4000 and 2× concentration conditions, the highest transfection efficiency (56.1%) was achieved. However, the digestive enzymes with 1× and 2× concentration under 40% PEG 4000 resulted in a similar high transfection efficiency (49.9% and 51.2%, respectively). Hence, 40% PEG 4000 and 1× digestive enzyme concentration was therefore used in subsequent tests.

To assess the effect of the amount of plasmid DNA on transfection, the protoplasts were transfected with different amounts of plasmid DNA. The transfection efficiencies obtained using 10, 15, and 25 µg were 49.9%, 35.9%, and 51.1%, respectively (Appendix 2C). A transfection efficiency of nearly 50% was achieved using 10 µg of plasmid DNA; therefore, we used 10 µg of plasmid DNA in our subsequent tests for cost-efficiency. Accordingly, the key factors required to achieve optimal transfection in *S. speciosa* petal protoplasts can be summarized as follows: a 1× concentration of digestive enzymes, 10 µg of exogenous DNA, and 40% PEG 4000.

Moderately high transfection efficiency for petal protoplasts

The high petal protoplast yield of this protocol means that a moderate transfection efficiency (maximum 50% and 58% using the small- and large-scale procedures, respectively) can be achieved (Table 1, Figure 2A, B). The average efficiency (41.40% ± 7%, range: 30–50%, *35S:GFP*) obtained

TABLE 1 Yield, cell viability, and transfection efficiency of the petal protoplasts isolated using our small-scale and large-scale procedures.

Transfection scale	Protoplast yield[a]	Cell viability[b]	Construct	Transfection efficiency
Small scale	1.21×10^6 ($n = 2$)	83.15 ± 7% ($n = 2$, 197/246; 256/298)	*35S:GFP*	41.40 ± 7% (30–50%, $n = 6$)
Large scale	2.95×10^6 ($n = 22$)	75.13 ± 7.2% ($n = 6$, 124/168; 187/232; 113/147; 119/162; 114/166; 159/205)	*35S:GFP*	30.55 ± 9% (20–58%, $n = 18$)
			35S:SsCYC-GFP	30.30 ± 6% (19–42%, $n = 17$)

[a]Protoplasts/g fresh weight.

[b]Cell viability = the number of cells stained with fluorescein diacetate divided by the total number of cells.
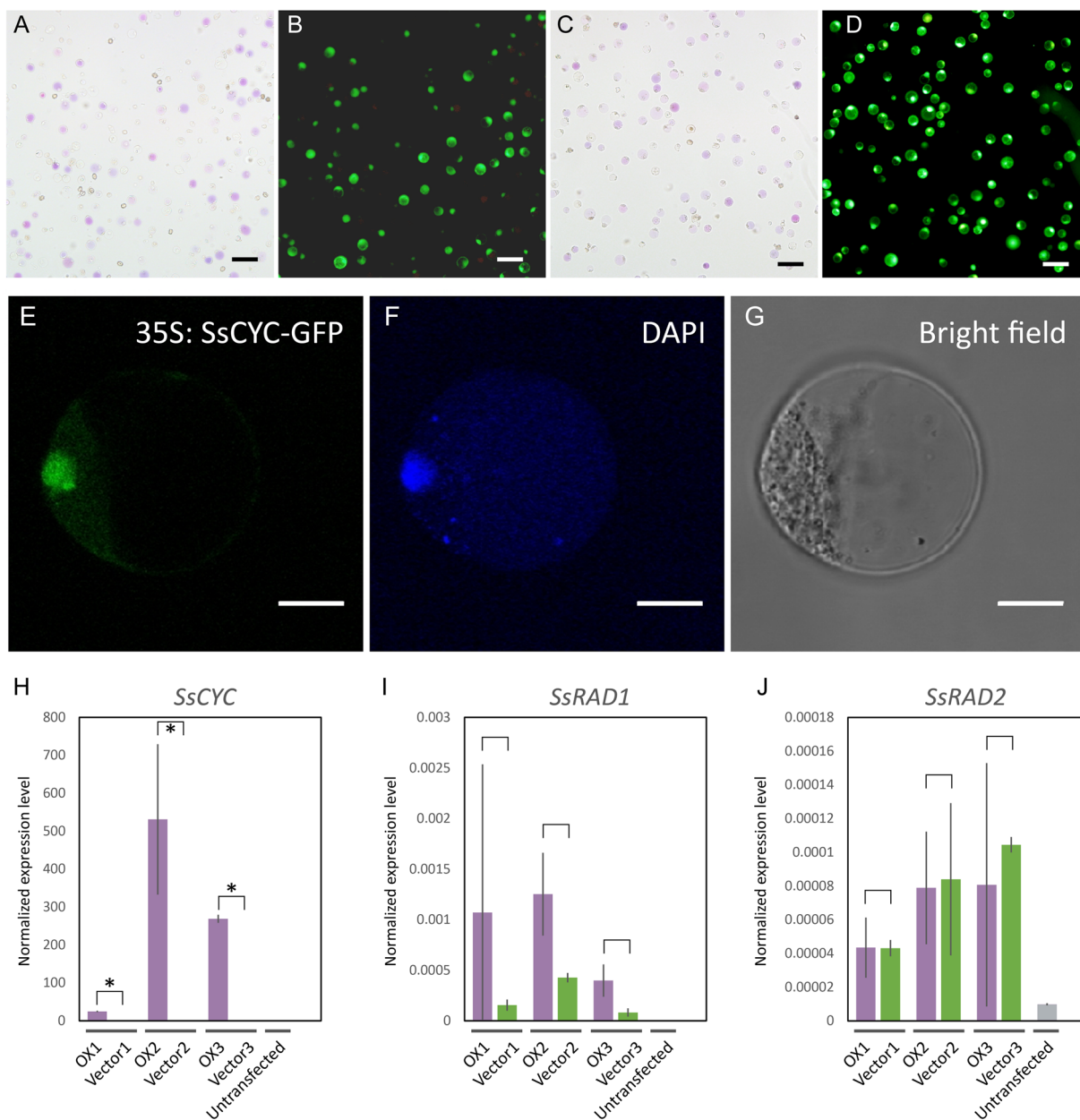
FIGURE 2 Transfection efficiency, viability, and gene regulation analysis of transfected *Sinningia speciosa* 'ES' petal protoplasts. (A, B) Images of GFP-transfected petal protoplasts taken under (A) bright light and (B) ultraviolet light to quantify transfection efficiency. (C, D) Images of transfected protoplasts stained with fluorescein diacetate under (C) bright light and (D) ultraviolet light to determine cell viability. (E–G) Subcellular localization of SsCYC–GFP protein in a transfected petal protoplast. (F) Fluorescence of SsCYC-GFP protein under dark-field microscopy. (F) Fluorescence of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining under dark-field microscopy. (G) Transfected petal protoplast under bright-field microscopy. Scale bars = 10 μm. (H) Validation of *SsCYC* overexpression in three paired petal protoplast replicates transfected with *pUC19-35S:SsCYC-35S:GFP* (OX1–OX3) compared with *SsCYC* expression in protoplasts transformed with vector-only *pUC19-35S:GFP* constructs (Vector1–Vector3) and untransfected protoplasts (Untransfected). (I–J) Expression of *SsRAD1* (I) and *SsRAD2* (J), putative targets of *SsCYC*, in petal protoplast lines overexpressing *SsCYC* (OX1–OX3). Normalized mRNA expression levels were calculated as $2^{-\Delta Ct}$. The mean values ± SD are from technical repeats. Statistical analyses were conducted using Student's *t* tests. *$P \leq 0.05$.

in the small-scale transfections was higher than that obtained through large-scale transfections, the efficiencies of which were similar to the small-scale protocol but considerably more variable. In the large-scale procedures, the transfection efficiencies achieved using *35S:GFP* and *35S:SsCYC-GFP* were 20–58% and 19–42%, respectively (Table 1). Fluorescein diacetate staining revealed that the average viabilities of the petal protoplasts transfected using the small- and large-scale procedures were 83.15% and 75.13%, respectively (Table 1, Figure 2C, D). The transfection efficiency for *S. speciosa* petal protoplasts in our study is almost equivalent to that reported for *Arabidopsis* leaf protoplasts (50%) in previous studies (Yoo et al., 2007; Wu et al., 2009); however, recent studies have reported

exceptionally high transfection efficiencies (approximately 80%) for petal protoplasts of *Cymbidium* (Ren et al., 2020) and *Phalaenopsis* (Lin et al., 2018). One possible explanation for this discrepancy may be differences in the extracellular matrices or the cell wall residues in the petal protoplasts of orchids and *S. speciosa*.

Use of the petal protoplast transient expression system to examine gene regulation and protein subcellular localization

To test the usability of this system, we transfected the petal protoplasts with a gene encoding a floral symmetry transcription factor (*SsCYC*) fused with GFP and observed the subcellular localization of the fused protein. If the SsCYC–GFP signals can be observed entering the protoplast nuclei, the petal protoplast system is viable and can be used to study transient gene expression. We therefore introduced a *pUC19-35S:SsCYC-GFP* construct into petal protoplasts of *S. speciosa* using our protocol. After an overnight incubation for 16 h, GFP fluorescence was detected in the nucleus of the petal protoplasts, which were stained using 4′,6-diamidino-2-phenylindole dihydrochloride, indicating their successful transformation with the ectopic *SsCYC* gene (Figure 2E, F). Our protocol is therefore useful for the detection of the expression of transiently transfected genes.

The *CYC* genes are master regulators of floral symmetry and have been reported to both autoregulate and to activate *RADIALIS* expression in the dorsal petals of zygomorphic flowers in *S. speciosa* and other Gesneriaceae species, as well as in *Antirrhinum* L. (Costa et al., 2005; Yang et al., 2012; Dong et al., 2018). To further verify that this system can be used to study well-characterized upstream–downstream genetic regulatory relationships, such as the ones between *CYC* and *RAD* genes, we investigated whether the overexpression of *SsCYC* in petal protoplasts could alter the expression of *SsRAD*s. To this end, we transfected the effector construct, *pUC19-35S:SsCYC-35S:GFP* (*SsCYC* OX), into the *S. speciosa* petal protoplasts and quantified the transcriptional levels of the *SsRAD* genes *SsRAD1* and *SsRAD2*, both of which have TCP binding sites on their 5′ regulatory regions, using real-time quantitative PCR (Figure 2H–L). We first performed three independent *SsCYC*-overexpression experiments in petal protoplasts (OX1–OX3), in which the *SsCYC* expression level in the transfected protoplasts was significantly higher than those in the empty vector controls (vectors 1–3) and in the untransfected protoplasts (negative control; Figure 2H). The expression of *SsRAD1* was higher, but not significantly upregulated when *SsCYC* was overexpressed, whereas the expression of *SsRAD2* did not differ between the three *SsCYC*-overexpression lines and the controls (Figure 2I–J). The significant overexpression of *SsCYC* after transfection indicated the success of our petal protoplast transient expression system. In addition, the induced upregulation of its known target *SsRAD1*,

while not significant, is consistent with the expectations in the *SsCYC*-overexpressing lines, which demonstrates the utility of this system in studying the corresponding gene regulatory pathway.

CONCLUSIONS

We optimized a petal protoplast transformation protocol for *S. speciosa* 'ES' that was developed for both small-scale and large-scale procedures. The quality and quantity of the isolated petal protoplasts of *S. speciosa* are sufficient for DNA transfection and subsequent examination of protein subcellular localization and regulation of SsCYC transcription factor to *SsRAD* gene expression. Other recently reported methods have used similar modifications of existing protocols from the model plant *Arabidopsis* to optimize protoplast isolation and transfection from various plants, but our protocol implements attributes of the protoplast isolation of *S. speciosa*; for example, the 1× concentration of the digestion enzyme is cost-effective and successfully releases abundant petal protoplasts, and at least 5 to 8 h of incubation time is needed for enzyme digestion to ensure the high yield of protoplasts. The protocol can be easily scaled up to fit the experimental needs of researchers to study a wide range of cellular processes, such as protein–protein and protein–DNA interactions, transcriptional activity, and signal transduction. We expect that our method will be of broad utility in diverse non-model plant species, particularly in plant species that have previously presented challenges in stable gene transformation.

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Appendix 1. Detailed protocol for the isolation and transfection of petal protoplasts of *Sinningia speciosa*.

Plant materials

Plants of *Sinningia speciosa* 'ES' were cultivated in a walk-in greenhouse under long-day conditions (16 h light/8 h dark) and an LED-produced light intensity of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at 70–80% relative humidity and 22°C to 25°C. Protoplasts were not isolated from plants near the beginning or end of the flowering period. Petals of prebloom flowers at floral bud stages 10–12 (FB10–12) were freshly collected (Pan et al., 2022).

Chemicals/solutions

- 0.2 M 4-Morpholineethanesulfonic acid (MES): adjust to pH 5.7 and sterilize using a 0.45-μm filter (MES monohydrate; MW 231.2; no. E169-250G, Amresco, Solon, Ohio, USA)
- 0.8 M Mannitol: sterilize using a 0.45-μm filter (mannitol; MW 182.17; no. M4125-500G, MilliporeSigma, Burlington, Massachusetts, USA)
- 1 M $CaCl_2$: sterilize using a 0.45-μm filter ($CaCl_2$; MW 110.98; Shimakyu's Pure Chemicals, Osaka, Japan)
- 2 M KCl: sterilize using a 0.45-μm filter (KCl; MW 74.55; no. 20598, Affymetrix/USB, Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- 2 M $MgCl_2$: sterilize using a 0.45-μm filter ($MgCl_2.6H_2O$; MW 203.3; no. 18461-500 G, Affymetrix/USB, Thermo Fisher Scientific)
- 1% Bovine serum albumin (BSA) (w/v): sterilize using a 0.45-μm filter (BSA; MW 66 KDa; no. AAJ1086722, Affymetrix/USB, Thermo Fisher Scientific)
- 5 M NaCl: sterilize using a 0.45-μm filter (NaCl; MW 58.44; no. 0241-1KG, Amresco)
- 0.1 M Glucose: sterilize using a 0.45-μm filter (glucose; MW 180.156; no. G-7520, MilliporeSigma)
- Cellulase R10 (Yakult Pharmaceutical Industry, Nishinomiya, Japan)
- Macerozyme R10 (Yakult Pharmaceutical Industry)
- Polyethylene glycol (PEG) 4000 (PEG 4000, no. 81240-1KG, MilliporeSigma)
- β-Mercaptoethanol (no. M6250, MilliporeSigma)
- Enzyme solution: The enzyme solution should be freshly prepared. Prepare 20 mM MES (pH 5.7) containing 1.5% (w/v) cellulase R10, 0.4% (w/v) macerozyme R10, 0.4 M mannitol, and 20 mM KCl. Heat the solution at 55°C for 10 min and cool to room temperature. Add 10 mM $CaCl_2$, 5 mM β-mercaptoethanol, and 0.1% BSA. Filter the enzyme solution using a 0.45-μm syringe filter.
- MMG solution: prepare 4 mM MES (pH 5.7) containing 0.4 M mannitol and 15 mM $MgCl_2$.
- PEG-$CaCl_2$ transfection solution: freshly prepare using 40% (w/v) PEG 4000 containing 0.2 M mannitol and 100 mM $CaCl_2$.
- W5 solution: prepare 2 mM MES (pH 5.7) containing 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, and 0.1 M glucose.
- WI solution: prepare 4 mM MES (pH 5.7) containing 0.5 M mannitol and 20 mM KCl.
- Maxi Plus Ultrapure Plasmid Extraction System (no. GMV2002, Viogene, Sunnyvale, California, USA)
- Trizol reagent (no. 15596018, Thermo Fisher Scientific)
- KAPA SYBR FAST qPCR Master Mix (2X) Kit (no. KK4600, MilliporeSigma)

Equipment

- Olympus DP72 fluorescence microscope (Olympus Corp., Tokyo, Japan)
- Zeiss LSM 780 inverted confocal microscope (Carl Zeiss, Jena, Germany)
- Sorvall ST 8 small benchtop centrifuge (Thermo Fisher Scientific)
- 0.45-μm sterile membrane syringe filter
- Nylon mesh (75 mm)
- Neubauer improved counting chamber 0.1 mm (no. 0650030, Marienfeld Superior, Lauda-Königshofen, Germany)
- 30-mL round-bottomed tube
- 2-mL round-bottomed microcentrifuge tube
- 12-well culture dish (no. 3046, Falcon)
- CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, California, USA)

Procedure

Protoplast isolation (steps 1–10)

1. Around 5–15 prebloom flowers from healthy plants were harvested, and their sepals, stamens, and carpels were removed (see Note 1).
2. All the petals were cut into 0.5–1.0-mm strips using a sharp scalpel blade, without crushing the petals (see Note 2).
3. The petal strips were quickly and gently transferred into the freshly prepared enzyme solution (see Note 3).
4. The petal strips were vacuum-infiltrated for 30 min in the dark.
5. Enzyme digestion was conducted for 5–6 h in the dark without shaking (see Notes 4 and 5).
6. The morphology of the released protoplasts was observed under a light microscope. The protoplasts should remain intact and round in shape.
7. The protoplast solution was filtered in a 30-mL round-bottomed tube through a double layer of 75-μm nylon mesh to remove plant debris (see Note 6).
8. The protoplast solution was centrifuged at $200 \times g$ for 1–2 min in a swinging-bucket rotor (see Note 7).
9. The supernatant was removed without disrupting the pellet to harvest the protoplasts.

10. The protoplast pellet was resuspended to a certain concentration in MMG solution after counting the cell numbers using a hemacytometer (see Note 8).

DNA–PEG–calcium transfection (steps 11–20)

11. The resuspended protoplast solution was placed on ice for at least 30 min.
12. 10 µL (1 µg/µL) plasmid DNA and 100 µL (2×10^4 cells) protoplast solution were gently mixed for a small-scale transfection, while 200 µL (1 µg/µL) plasmid DNA and 1.6 mL (6.65×10^6 cells) protoplast solution was used for a large-scale transfection.
13. Freshly prepared PEG solution was added (110 µL and 1.76 mL in the small- and large-scale procedures, respectively), and then immediately mixed by gently tapping the tube.
14. The transfection mixture was incubated at room temperature for 10 min.
15. The transfection procedure was stopped by adding W5 solution (two times the volume of the transfection mixture). The tube was gently inverted to mix. The tubes should each contain 440 µL and 7.04 mL in the small- and large-scale procedures, respectively.
16. The mixture was centrifuged at $200 \times g$ for 2 min at room temperature, and the supernatant was subsequently removed.
17. The transfected protoplasts were resuspended in 0.5 mL of WI solution, transferred into the 12-well culture plate coated with 5% BSA, and incubated at room temperature under light (daylight fluorescent lightbulb) for 16–18 h.
18. The transfected protoplasts were transferred to new tubes and harvested by centrifugation at $200 \times g$ for 1 min.
19. The supernatant was removed, after which the protoplasts were resuspended.
20. The GFP or GFP-fusion protein was observed under a fluorescence microscope.

Notes on protoplast isolation and transfection

NOTE 1: Pollen grains must not mix with the petals in the enzyme digestion process, as they exhibit autofluorescence under the microscope and can be mistaken for GFP signals from the reporter constructs of the transfected protoplasts. The flowering status is sensitive to growing conditions (i.e., sufficient humidity without drought and sufficient light intensity and quantity).

NOTE 2: We switched to a new blade after cutting 5–6 flowers to avoid the risk of tissue crushing by a dull blade.

NOTE 3: We used 10 mL of enzyme solution to digest 5–8 flowers and 15 mL to digest 9–15 flowers.

NOTE 4: Shaking should be avoided during enzyme digestion because it reduces the yield of protoplasts.

NOTE 5: To achieve a transfection efficiency of up to 40%, the total enzyme digestion time should be at least 5 h but should not exceed 8 h.

NOTE 6: The nylon mesh was placed against the inner wall of the 30-mL round-bottomed tube during the filtering process to ensure that the solution would slowly flow through rather than directly dropping against the tube wall and breaking the protoplasts.

NOTE 7: Centrifuging at a higher speed ($\geq 300 \times g$) may crush the protoplasts and cause the aggregation of a cell pellet.

NOTE 8: The protoplast pellet was resuspended in MMG solution (5–8 flowers: 1–3 mL, 9–15 flowers: 3–5 mL).

Appendix 2. Optimization of conditions for high petal protoplast yield and transfection efficiency. (A) Effect of enzyme concentration and digestion time on petal protoplast yield. The highest protoplast yield is achieved in a 6-h digestion with 1× enzyme mix (1.5% cellulase and 0.4% macerozyme). (B) Effect of different enzyme concentrations in combination with different PEG 4000 amounts on transfection efficiency. A 40% PEG concentration with 1× enzyme mixed was found to be a cost-effective optimal condition. (C) Effect of the amount of plasmid DNA on transfection efficiency. A 10-µg DNA aliquot was sufficient to reach high efficiency.