

Establishment of an *Agrobacterium*-mediated genetic transformation system for *Cymbidium faberi* Rolfe

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Abstract

Background: *Cymbidium faberi* Rolfe is an ornamental orchid of high economic value, however, an efficient genetic transformation system has not yet been reported for this species.

Aims: This study aimed to establish an *Agrobacterium tumefaciens*-mediated genetic transformation system for *C. faberi* and to optimize the key factors affecting transformation performance.

Methods: Rhizomes, calli, and inflorescences were used as explants. The pCAMBIA3301-EGFP vector was introduced using *Agrobacterium* strain GV3101. Explant type, pre-culture duration, bacterial density (OD₆₀₀), infection time, co-cultivation period, and antibiotic selection were systematically optimized. Putative transformants were screened by green fluorescence and verified by polymerase chain reaction (PCR).

Results: Calli pre-cultured for 3 d were the most suitable recipient material. The optimal transformation conditions were an *Agrobacterium* suspension of OD₆₀₀ = 0.2, infection for 5 min, and co-cultivation for 2 d; under these conditions, explant survival reached 72.22%. Two independent transgenic plantlets with stable integration of the enhanced green fluorescent protein gene were obtained and confirmed by PCR.

Conclusions: This study provides an *Agrobacterium*-mediated transformation protocol for *C. faberi* and offers a technical basis for molecular breeding and gene function studies in this orchid.

Keywords: *Agrobacterium* GV3101, *Agrobacterium*-mediated transformation, callus, *Cymbidium faberi* Rolfe, genetic transformation, inflorescence, pCAMBIA3301-EGFP, rhizome.

Introduction

Orchids (*Orchidaceae*) comprise a large group of ornamental plants valued for both foliage and flowers. Important Chinese orchid species include *Cymbidium goeringii*, *C. ensifolium*, *C. faberi*, *C. kanran*, and *C. sinense*. Their arching leaves, subtle flower colors, and distinctive fragrance have made them highly appreciated throughout their long cultivation history. In addition to their ornamental and economic value, orchids have become useful systems for studying plant development, evolution, and adaptation (Ji, 2022; Luo et al., 2023; Yu et al., 2023).

Cymbidium faberi Rolfe is a traditional Chinese orchid with an upright growth habit and delicately fragrant flowers. Because commercial production still relies heavily on wild resources, excessive collection has seriously threatened natural germplasm. Efficient tissue culture and rapid propagation systems are therefore essential for germplasm conservation. At the same time, the generation of novel variants with diverse morphological traits is important for increasing the commercial value of this species. However, the acquisition of mutants in *C. faberi* still depends mainly on spontaneous mutation (Wu et al., 2024). Conventional breeding approaches, including

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; BA - 6-benzylaminopurine; AC - activated carbon; Cef - cefotaxime; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; Kan - kanamycin; KT - kinetin; MS - Murashige and Skoog; NAA - 1-naphthaleneacetic acid; PCR - polymerase chain reaction; PVP - polyvinylpyrrolidone; Rif - rifampicin; TDZ - thidiazuron; Tim - timentin.

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Conflict of interest: The authors declare that they have no conflict of interest.

hybridization and mutagenesis, remain important but are limited by reproductive barriers among orchid taxa, which restrict interspecific trait transfer. Moreover, genetic engineering in orchids has developed more slowly than functional genomic studies of plant architecture, floral morphology, pigmentation, and fragrance (Thiruvengadam and Yang, 2009; Xiang *et al.*, 2011; Zou *et al.*, 2015; Fei *et al.*, 2019; Tian *et al.*, 2020). The establishment of reliable transformation systems would provide powerful tools for dissecting the molecular mechanisms underlying flower development, stress tolerance, and mycorrhizal symbiosis. Such systems would also facilitate targeted trait improvement, germplasm innovation, and the acceleration of breeding cycles (Peng, 2015).

Genetic transformation of *C. faberi* is more difficult than that of many economic crops and model plants. Low transformation efficiency and unstable inheritance have limited its use in Chinese orchid breeding. These difficulties are associated with several factors. First, *C. faberi* lacks highly regenerable explants, and the transition from single cells to complete plants is slow and unstable, weakening the basis for stable transformation. Second, both *Agrobacterium*-mediated transformation and biolistic methods have low efficiency in delivering foreign genes into cells and achieving stable integration (Hsing *et al.*, 2016). Third, transformed cells are difficult to select because *C. faberi* grows slowly and is sensitive to selective agents. Its large and complex genome may also affect transgene expression (Sun *et al.*, 2021; Yang *et al.*, 2021). In addition, validation of traits after flowering often requires 3 - 5 years, greatly increasing the time and cost of research. Therefore, an effective transformation system is urgently needed to support functional genomics and molecular breeding of this species.

Agrobacterium-mediated transformation has been successfully applied to several orchids and has considerable potential for *Orchidaceae* genetic engineering (Mii and Chin, 2018). Belarmino and Mii first reported *Agrobacterium*-mediated transformation in *Phalaenopsis*, and Yu *et al.* subsequently achieved transformation in *Dendrobium* (Belarmino and Mii, 2000; Yu *et al.*, 2001). More recently, the AP3 gene from *Cymbidium sinense* was introduced into *Dendrobium officinale* using this approach (He *et al.*, 2023). Transgenic studies have mainly focused on commercial orchids such as *Oncidium* (Thiruvengadam *et al.*, 2011), *Phalaenopsis* (Hao *et al.*, 2010; Zhang *et al.*, 2016; Meng *et al.*, 2018), *Dendrobium* (Suwanaketchanatit *et al.*, 2007; Wu *et al.*, 2015), and *Cymbidium* hybrids (Li, 2005; Zhang *et al.*, 2008). In contrast, molecular breeding of traditional Chinese orchids remains constrained by the lack of efficient transformation systems, partly because many orchids are relatively insensitive to *Agrobacterium* and because suitable expression vectors are limited (Ding and Fu, 2000). Although Kong preliminarily established a transformation protocol for *Cymbidium goeringii* and confirmed GUS gene integration, a functional genetic transformation system has not yet been reported for *C. faberi* (Kong, 2008).

In the previous work, we established an aseptic rapid propagation system and achieved *in vitro* floral

induction in *C. faberi*. In the present study, we developed an *Agrobacterium*-mediated genetic transformation system for *C. faberi* using strain GV3101 carrying the pCambia3301-EGFP plasmid. Three explant types, namely rhizomes, calli, and inflorescences, were evaluated. We further optimized key parameters, including recipient material, pre-culture duration (0 - 7 d), bacterial suspension density ($OD_{600} = 0.2 - 0.8$), infection time (10 - 40 min), co-cultivation period (2 - 5 d), and antibiotic combinations for bacterial suppression. The effects of these factors on explant survival and transformation response were systematically analyzed. This work provides methodological support and baseline data for establishing a stable transformation platform in *C. faberi* and offers a technical reference for future gene function studies and molecular breeding.

Materials and methods

Plant material: Surface-sterilized *C. faberi* seeds were inoculated on asymbiotic germination medium containing MS basal salts, 10 g/L sucrose, 10 g/L glucose, 3 g/L peptone, 2.0 mg/L 6-benzylaminopurine (6-BA), and 2.0 mg/L alpha-naphthalenacetic acid (NAA). Rhizomes developed after 120 d of culture and were subcultured at 8-week intervals.

For callus induction, rhizomes were transferred to half-strength MS medium supplemented with 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 2 mg/L indole-3-acetic acid (IAA), 2 mg/L indole-3-butyric acid (IBA), 0.5 mg/L 6-BA, 0.5 mg/L kinetin (KT), 1 g/L activated carbon (AC), and 1 g/L polyvinylpyrrolidone (PVP). Callus formation occurred within 30 d, and the calli were then subcultured every 8 weeks.

Inflorescences were induced by culturing rhizomes on half-strength MS medium supplemented with 1 mg/L NAA, 0.1 mg/L 2,4-D, 1 mg/L 6-BA, 1 mg/L KT, 1 mg/L thidiazuron (TDZ), 3 g/L peptone, 100 mL/L coconut water, 1 g/L AC, 1 g/L PVP, 1.5 g/L sodium azide (NaN_3), 7 g/L agar, 10 g/L sucrose, and 10 g/L glucose. Floral structures emerged after 120 d and were maintained by subculturing every 12 weeks.

Bacterial strain and vector: *Agrobacterium tumefaciens* strain GV3101, preserved in the Laboratory of Horticulture and Plant Protection, Henan University of Science and Technology, was used for transformation. GV3101 is a disarmed C58-derived strain widely used for plant transformation. It carries a rifampicin resistance marker in its chromosomal DNA and harbors the non-transmissible Ti plasmid pMP90 (pTiC58D T-DNA), which retains functional *vir* genes required for T-DNA transfer but lacks autonomous T-DNA mobilization. The pMP90 backbone enables efficient T-DNA processing from co-resident binary vectors through *vir*-gene complementation.

The binary vector pCambia3301-EGFP, constructed and maintained in our laboratory, was derived from the pCambia3301 backbone (11 319 bp) and is compatible with *Agrobacterium*-mediated transformation.

The vector contains an enhanced green fluorescent protein (EGFP) reporter gene (Fig. 1A) driven by a constitutive promoter, allowing rapid visual screening of transgenic tissues.

Bacteriostatic agent screening: Kanamycin (Kan; 25, 50, 75, 100, and 125 mg/L), cefotaxime (Cef; 100, 200, 300, 400, and 500 mg/L), and timentin (Tim; 100, 200, 300, 400, and 500 mg/L) were added separately to callus redifferentiation medium containing half-strength MS salts, 7 g/L agar, 10 g/L sucrose, 10 g/L glucose, 0.3 mg/L TDZ, and 0.1 mg/L IBA. For each treatment, 18 uniform and healthy calli were cultured, with three biological replicates. After 30 d, antibiotic responses were evaluated by calculating redifferentiation and mortality rates:

Redifferentiation rate (%) = (number of redifferentiated calli/total number of surviving calli) × 100

Mortality rate (%) = (number of necrotic calli/total number of inoculated calli) × 100

The optimal bacteriostatic concentration for *C. faberi* transformation was determined based on high redifferentiation and low mortality.

Pre-culture of explants: Uniform rhizomes, inflorescences, and calli of *C. faberi* (Fig. 1B-D) were selected as recipient materials. The explants were aseptically wounded with scalpel blades to promote *Agrobacterium* attachment and then transferred to pre-culture medium containing half-strength MS salts, 7 g/L agar, 10 g/L sucrose, 10 g/L glucose, 1 mg/L NAA, 0.1 mg/L 2,4-D, 1 mg/L 6-BA, 1 mg/L KT, and 1 mg/L TDZ (pH 5.4). Pre-culture was conducted at 25 ± 2°C under a photosynthetic photon flux

density of 33 μmol/m²s provided by Toshiba FLR40S/W/M/36 fluorescent lamps (Philips, Foshan, China; model: T8 DoubleEnded HO LEDtube) for 3 - 7 d to activate cell division before transformation.

Agrobacterium culture: YEB liquid medium (50 mL; 0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5% sucrose, and 0.2% MgSO₄·7H₂O) was autoclaved at 121°C for 20 min and supplemented with 50 mg/L Kan and 50 mg/L Rif. A 50-μL aliquot of glycerol-preserved *Agrobacterium tumefaciens* GV3101 harboring pCambia3301-EGFP was inoculated into the medium and cultured at 28°C with shaking at 200 rpm for 12 h until the mid-log phase (OD₆₀₀ approximately 0.6 - 0.8). The bacterial suspension was centrifuged at 4 000 × g for 10 min at 25°C and resuspended in fresh infection medium before co-cultivation.

Agrobacterium-mediated transformation of *C. faberi*

Preparation of infection suspension: Bacterial cultures were centrifuged at 12 000 × g for 5 min at 4°C in 10-mL tubes. The *Agrobacterium* pellet was resuspended in a solution containing 50 g/L sucrose and 100 mg/L acetosyringone (AS) to the required OD₆₀₀ of 0.2 - 0.6.

Infection and transformation: Pre-cultured calli without visible bud initiation were immersed in the infection suspension for 5 - 20 min. Excess suspension was removed by blotting the explants on sterile filter paper. The infected explants were placed on redifferentiation medium and co-cultivated in the dark at 25°C for 2 - 5 d.

Bacteriostatic culture: After co-cultivation, the calli were washed three times with sterile water, agitated for 30 min in sterile water containing 300 mg/L Tim, and

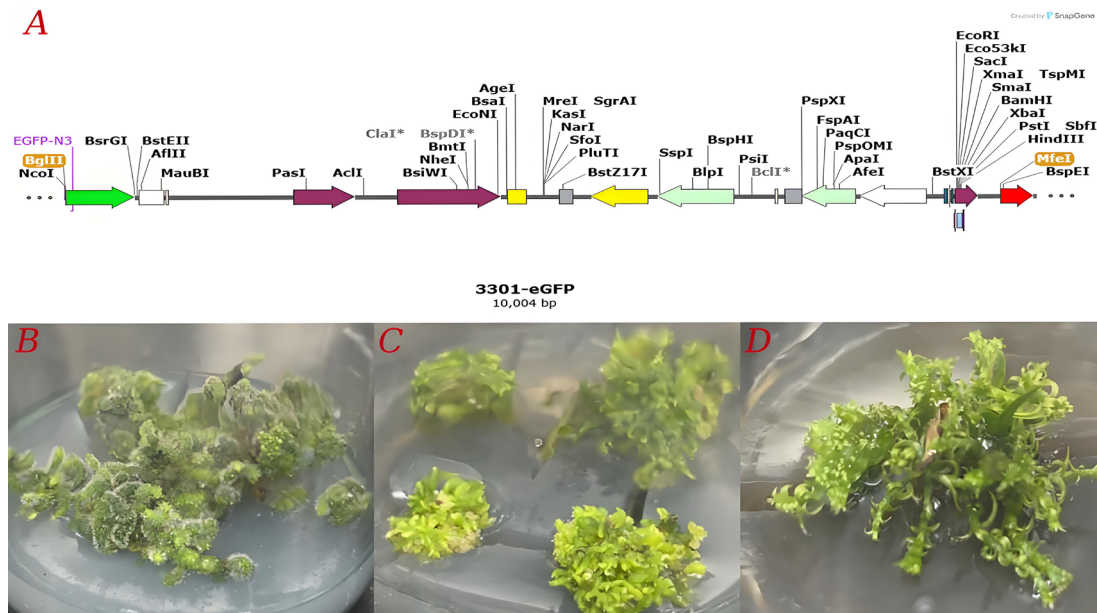


Fig. 1. A: Expression vector pCambia3301-EGFP. The arrows represent the direction of transcription, and the colored boxes indicate different functional elements of the vector, including regulatory sequences, reporter genes, and selection markers. Restriction sites are shown for cloning and vector construction purposes. B - D: Transformation recipients of *Cymbidium faberi*: B: Rhizomes subcultured for 8 weeks; C: Callus subcultured for 8 weeks; D: Inflorescence from test-tube flowering plants subcultured for 12 weeks.

then rinsed three additional times with sterile water. The calli were blotted dry and transferred to bacteriostatic medium containing half-strength MS salts, 7 g/L agar, 10 g/L sucrose, 10 g/L glucose, 0.3 mg/L TDZ, 0.1 mg/L IBA, 3 g/L peptone, 100 mL/L coconut water, 1 g/L AC, 1 g/L PVP, and 300 mg/L Tim. Untransformed controls were treated identically except that they were not exposed to *Agrobacterium*.

Selection of transgenic material: After 30 d of bacteriostatic culture, the calli were transferred to selection medium supplemented with 125 mg/L Kan. Necrotic tissues were removed during biweekly medium renewal. Putative transformants were screened for EGFP fluorescence under UV excitation at 365 nm.

PCR verification of transgenic plants: Fluorescence-positive regenerants were subjected to molecular verification. Tissue samples (30 - 50 mg) were homogenized in 300 - 500 μ L of 0.5 M NaOH and centrifuged at $10\,000 \times g$ for 3 min at 4°C. The supernatant (5 - 10 μ L) was neutralized with 50 - 100 μ L of 100 mM Tris-HCl (pH 8.0). PCR amplification was performed in 10- μ L reaction mixtures using vector-specific primers (Kim, 2023).

The PCR reaction was performed in a total volume of 10 μ L, which contained 5 μ L of *2X San Taq PCR Mix* (with blue dye; Sangon Biotech Co., Ltd., Shanghai, China), 1 ng to 1 μ g of template DNA, 0.2 μ L each of the forward and reverse primers (10 μ M each), with the final volume adjusted to 10 μ L using sterile ddH₂O.

The PCR reaction mixture was thoroughly vortexed and subjected to thermal cycling under the following programmed conditions: initial denaturation at 94°C for 5 min; 30 cycles of denaturation (94°C for 30 s), annealing (56°C for 30 s), and extension (72°C for 40 s); final extension at 72°C for 10 min; followed by indefinite storage at 4°C.

For electrophoretic analysis, 6 μ L of PCR product and 3 μ L of *DL2000 DNA Marker* (Sangon Biotech Co., Ltd., Shanghai, China) were loaded onto a 1% (w/v) agarose gel prepared in 1 $\times$ TAE buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.3). Electrophoresis was performed at 120 V for 20 min. DNA bands were visualized under UV illumination at 302 nm using a *GelDoc XR+* imaging system (Bio-Rad Laboratories, Hercules, CA, USA). Images were archived and analyzed using *ImageLab 6.1* software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis: All experiments were conducted in triplicate. Data were organized using *Excel 2021* and visualized with *GraphPad Prism 8.0* (GraphPad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) was performed using *SPSS 26.0* (IBM Corp., Armonk, NY, USA), and Duncan's multiple range test was used to compare differences among means. Statistical significance was set at $P < 0.05$. Results are presented as means $\pm$ standard deviation.

Results

Bacteriostatic agent screening: To suppress *Agrobacterium* overgrowth and reduce bacterial cytotoxicity

during transformation, bacteriostatic antibiotics were incorporated into the post-transformation medium (Xie *et al.*, 2015). Observations after 30 d showed that timentin affected *C. faberi* callus development in a concentration-dependent manner (Fig. 2A-F). Calli remained viable at all tested concentrations (100 - 500 mg/L), but bud formation was significantly inhibited as the Tim concentration increased. The bud initiation rate decreased from 82.3% at 100 mg/L to 40.74% at 500 mg/L, representing a 2.02-fold reduction at the highest concentration. Comparison of the three bacteriostatic agents indicated that 500 mg/L timentin was suitable for the *C. faberi* transformation protocol because it effectively suppressed *Agrobacterium* while maintaining high explant viability (Fig. 2G,H).

Selection of explant material and optimization of pre-culture duration: Three explant types, namely rhizomes, calli, and inflorescences, were evaluated, and pre-culture duration was optimized to reduce transformation-induced oxidative stress. As shown in Table 1, rhizomes had the highest post-transformation survival rate but did not produce fluorescent transformants. Inflorescences also showed no detectable EGFP expression. Calli showed transformation competence only under specific pre-culture conditions. When pre-cultured for 3 d, calli maintained moderate survival and produced detectable EGFP fluorescence. In contrast, 2 d of pre-culture caused severe browning despite transient fluorescence. These results indicate that calli pre-cultured for 3 d are the most suitable recipient material for *C. faberi* transformation, providing a balance between explant survival and detectable transgene integration.

Optimization of *Agrobacterium* suspension density and infection duration: Calli pre-cultured for 3 d responded differently to the transformation parameters (Table 2). High bacterial density ($OD_{600} = 0.6$) caused complete explant necrosis because of bacterial overgrowth, whereas $OD_{600} = 0.2$ maintained 72.22% survival and yielded one explant with detectable EGFP fluorescence. Infection duration also affected the transformation response. Prolonged infection increased the number of fluorescent explants but severely reduced subsequent organogenesis. Only calli infected for 5 min developed normal shoots, whereas longer exposure arrested development at intermediate callus-rhizome stages (Tables 2 and 3; Fig. 3). Therefore, $OD_{600} = 0.2$ combined with a 5-min infection period provided the best balance between transformation response and post-transformation viability of *C. faberi* calli.

Optimization of co-cultivation duration: In *C. faberi* callus transformation trials, extended co-cultivation for 4 - 5 d resulted in complete explant mortality because of uncontrolled bacterial overgrowth that persisted during bacteriostatic culture (Table 4). Co-cultivation for 2 - 3 d produced fluorescent explants; however, only the 2-d treatment supported subsequent shoot organogenesis. Although transient fluorescence was detected after 3 d of

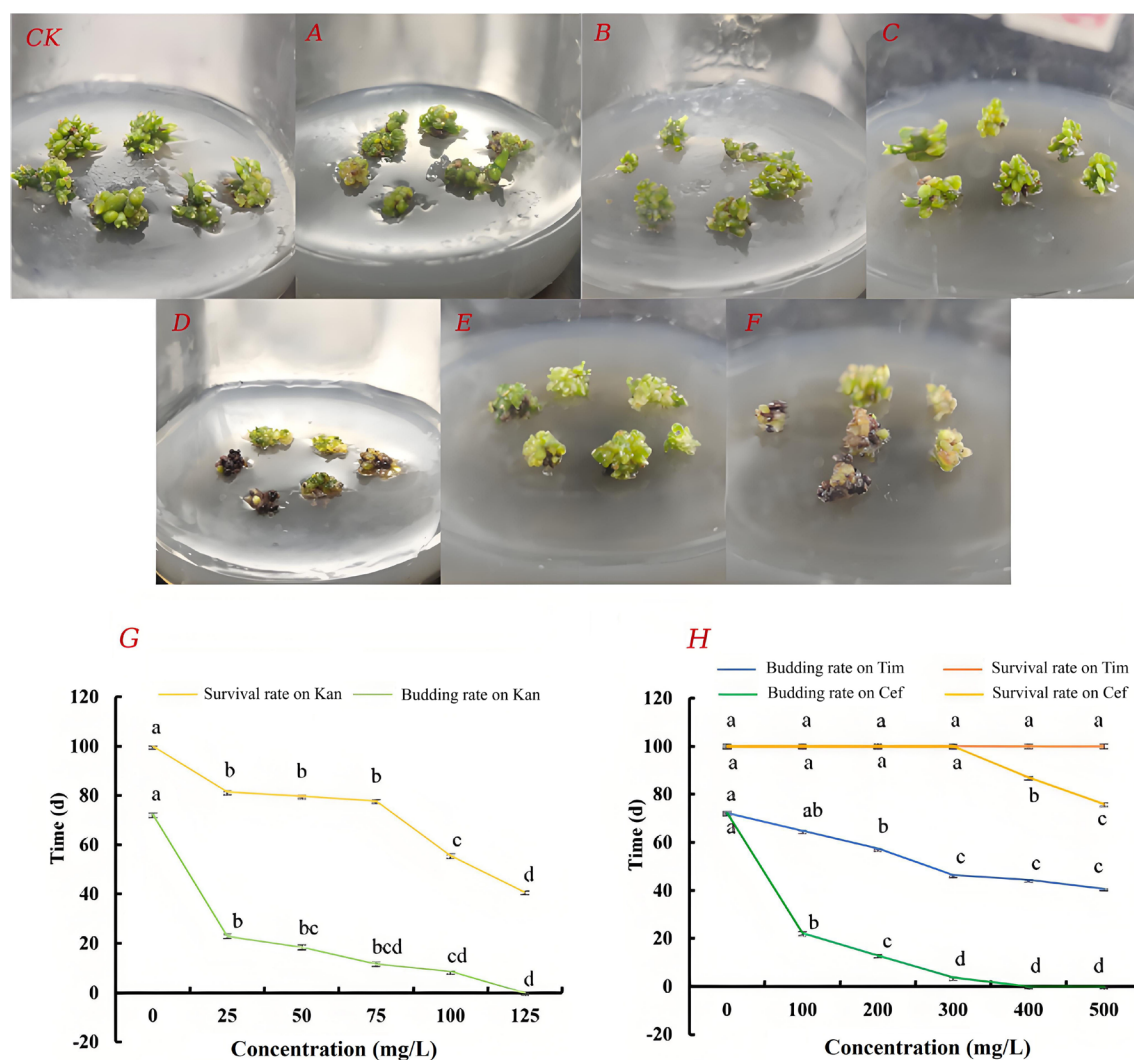


Fig. 2. A - F: Effect of bacteriostatic agents on growth of *Cymbidium faberi* Rolfe callus tissues. CK – control without added bacteriostatic agents. A: kanamycin 25 mg/L; B: timentin 100 mg/L; C: cefotaxime 100 mg/L; D: kanamycin 125 mg/L; E: timentin 500 mg/L; F: cefotaxime 500 mg/L. G: Effect of kanamycin on *C. faberi* callus tissues. H: Effect of timentin and cefotaxime on *C. faberi* callus tissues. Data are means $\pm$ SD ($n = 3$). Means with different letters differ significantly according to Duncan's multiple range test ($P < 0.05$).

Table 1. Effect of receptor material and pre-culture time on the genetic transformation of *Cymbidium faberi* Rolfe. Inoculation number = 36 per treatment. Survival rate = (surviving explants/inoculated explants) $\times$ 100. Transgenic explants were confirmed by EGFP fluorescence and PCR.

Receptor material	Pre-culture time (d)	Inoculation time (min)	Bacterial suspension concentration (OD ₆₀₀)	Co-culture time (d)	Number of explants inoculated	Number of surviving explants after recovery	Survival rate (%)	Number of fluorescent explants
Rhizome	1	5	0.2	2	36	35	97.22	0
	2	5	0.2	2	36	36	100.00	0
	3	5	0.2	2	36	36	100.00	0
Callus tissue	1	5	0.2	2	36	16	44.44	0
	2	5	0.2	2	36	19	52.78	1
	3	5	0.2	2	36	27	75.00	1
Inflorescence	1	5	0.2	2	36	20	55.56	0
	2	5	0.2	2	36	21	58.33	0
	3	5	0.2	2	36	28	77.78	0

Table 2. Effect of bacterial solution concentration on genetic transformation of *Cymbidium faberi* Rolfe callus tissues. Values are means $\pm$ SD ($n = 3$). Survival rate after recovery (%) = (number of surviving explants after recovery/number of explants inoculated) $\times$ 100. Fluorescent transgenic explants were confirmed by EGFP fluorescence and PCR.

Bacterial suspension concentration (OD ₆₀₀)	Pre-culture time (d)	Inoculation time (min)	Co-culture time (d)	Number of explants inoculated	Number of surviving explants after recovery	Survival rate after recovery (%)	Number of fluorescent transgenic explants
0.2	3	5	2	36	26	72.22	1
0.4	3	5	2	36	13	36.11	0
0.6	3	5	2	36	0	0	0

Table 3. Effect of inoculation time on genetic transformation of *Cymbidium faberi* Rolfe callus tissues. Each treatment used 36 explants. Survival rate after recovery (%) = (number of surviving explants after recovery/36) $\times$ 100. Fluorescent transgenic explants were confirmed by EGFP fluorescence and PCR.

Inoculation time (min)	Bacterial suspension concentration (OD ₆₀₀)	Number of explants inoculated	Number of surviving explants after recovery	Survival rate after recovery (%)	Number of fluorescent transgenic explants
5	0.2	36	26	72.22	1
7.5	0.2	36	25	69.44	1
10	0.2	36	25	69.44	2
15	0.2	36	24	66.67	2
20	0.2	36	20	55.56	1

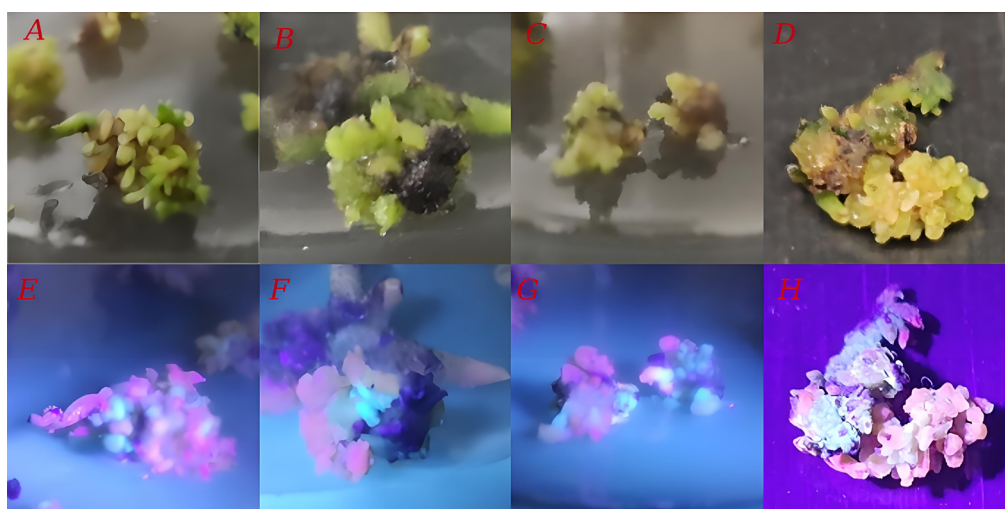


Fig. 3. Partially fluorescent exosomes. *A - D*: Morphological characteristics of regenerated explants under bright-field illumination. *E - H*: Corresponding EGFP fluorescence signals detected under blue-light excitation. Fluorescent regions indicate the expression of the introduced EGFP reporter gene in transformed explants.

co-cultivation, the explants failed to initiate shoots. Thus, 2 d was the optimal co-cultivation duration for balancing transformation response and post-transformation viability in the *C. faberi* callus system.

Identification of genetically transformed plants:

The EGFP reporter gene was introduced into *C. faberi* calli via *Agrobacterium*-mediated transformation, yielding a total of five regenerated plantlets. Fluorescence screening revealed that two of these plantlets (Fig. 4A,B) emitted clear green fluorescence under UV excitation, indicating successful EGFP expression, whereas the

other three plantlets showed no detectable fluorescence. Control plants (CK-A and CK-B) were untransformed plants photographed under natural light. To verify the fluorescence results, genomic DNA was extracted from leaves of fluorescence-positive transgenic plantlets (lanes 1 and 2), fluorescence-negative putative transformants (lanes 3 - 5), and wild-type plants (WT), and PCR amplification was performed. As shown in Fig. 4C, the expected PCR product was detected in the plasmid-positive control (CK+) and in the fluorescence-positive lines (1 and 2), but no amplification was observed in the fluorescence-negative transformants (3 - 5) or in

Table 4. Effect of different co-culture times on genetic transformation of *Cymbidium faberi* Rolfe callus tissues. Each treatment used 36 explants. Survival after recovery was recorded before selection. Fluorescent transgenic explants were confirmed by EGFP fluorescence and PCR. Co-culture beyond 3 days resulted in complete explant mortality (browning and necrosis).

Co-culture time (d)	Inoculation time (min)	Bacterial suspension concentration (OD ₆₀₀)	Pre-culture time (d)	Number of inoculations	Number of transgenic explants	Number of surviving explants after recovery
2	5	0.2	3	36	25	1
3	5	0.2	3	36	25	1
4	5	0.2	3	36	0	0
5	5	0.2	3	36	0	0

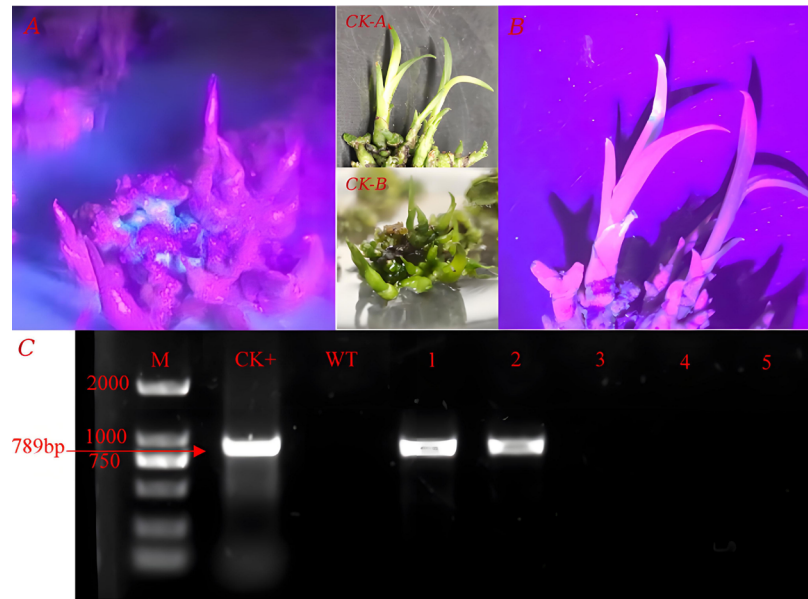


Fig. 4. *A,B*: Fluorescence verification of *Cymbidium faberi* Rolfe transgenic plants; *CK-A* and *CK-B*: untransformed plants photographed under natural light; *C*: PCR validation of transgenic plants. M: Marker (Sangon Biotech Co., Ltd., Shanghai, China); CK+: positive control plasmid; WT: non-transgenic *Cymbidium faberi* Rolfe plant; 1,2: EGFP transgenic positive plants that exhibited fluorescence; 3 - 5: plants that underwent genetic transformation but did not show fluorescence.

wild-type plants (WT). Taken together, a total of five regenerated plantlets were obtained, of which two were both EGFP-fluorescence-positive and PCR-positive, yielding a transformation efficiency of 40%.

Discussion

Agrobacterium-mediated transformation is one of the most widely used methods in plant genetic engineering and has been successfully applied to many ornamental plants, including *Tagetes erecta* (Yu et al., 2023), *Lilium* spp. (Chen et al., 2023), *Narcissus* (Hu et al., 2023), *Phalaenopsis* (Tong et al., 2020), and *Oncidium* (Liu et al., 2019). However, species-specific biological barriers require careful optimization, particularly in taxa such as *C. faberi* for which no transformation system has previously been established. In this study, we developed an *Agrobacterium*-mediated transformation protocol for *C. faberi* using calli as recipient material and verified EGFP integration by fluorescence screening and PCR. A major difficulty was the inhibitory effect of conventional antibiotics, especially cefotaxime and kanamycin, on bud

regeneration. Timentin was therefore selected because of its comparatively lower phytotoxicity. Transformation performance was affected by multiple factors. Calli were more suitable than rhizomes and inflorescences, probably because their less differentiated tissues and thinner primary cell walls facilitated bacterial infection. The optimized conditions, namely OD₆₀₀ = 0.2, infection for 5 min, and co-cultivation for 2 d, balanced bacterial control with explant survival and transgene recovery. Under these conditions, two confirmed transgenic lines were obtained.

Kanamycin, cefotaxime, and timentin are commonly used in plant genetic transformation. Previous studies of *Agrobacterium*-mediated transformation in orchids have shown that kanamycin sensitivity differs markedly among species and explant types. Hsieh et al. (unpublished) selected transgenic *Phalaenopsis* calli using 100 mg/L Kan for 30 d, whereas Zheng et al. used 200 mg/L Kan for *Dendrobium* transformation (Zheng et al., 2012). In contrast, Peng reported successful selection in *Cymbidium* at 25 mg/L Kan (Peng, 2015), while Hao et al. used 500 mg/L Kan for *Phalaenopsis* (Hao et al.,

2010). This variation highlights the divergent kanamycin tolerance of different orchid taxa. In the present study, 125 mg/L Kan strongly inhibited bud formation in *C. faberi* calli, completely suppressing organogenesis although some tissues survived (Fig. 2G).

Although cephalosporins showed limited *Agrobacterium* suppression in our system (Fig. 2F), they remain widely used in orchid transformation protocols. Liu identified 150 mg/L Cef as the minimum inhibitory concentration for *Cymbidium* Junhonglan and reported complete bacterial suppression at 200 mg/L (Liu, 2017). Lv used a stepwise reduction strategy beginning with 200 mg/L Cef and removing antibiotics after two months (Lv, 2004). Yao showed that 100 - 300 mg/L Cef effectively inhibited *Agrobacterium* EHA101 in *Phalaenopsis* (Yao, 2005). In the present study, *C. faberi* calli maintained moderate survival under cephalosporin treatment, but bud formation decreased progressively from 67.2% at 100 mg/L to complete suppression at 400 mg/L. These results indicate that antibiotic screening should be performed specifically for each orchid species and explant type.

The choice of recipient explant is a key determinant of genetic transformation success. Protocorms are commonly used in *Orchidaceae* transformation because of their high regeneration capacity and rapid organogenesis (Yu, 2018), but they may generate chimeric plantlets, complicating the selection of stable transformants. Callus-based systems can reduce chimerism, although survival rates may be lower (Man *et al.*, 2007). Previous studies have reported different outcomes among orchid genera. Raffener *et al.* achieved ETR1 integration in *Oncidium* and *Rhynchostylis* protocorms (Raffener *et al.*, 2009), while Tee *et al.* reported stronger transgene expression in inflorescence-derived *Dendrobium* callus than in other morphotypes (Tee *et al.*, 2003). Suspension cells derived from *Phalaenopsis* callus have also been shown to be competent for transformation (Belarmino and Mii, 2000). In our system, calli were the only explant type that produced stable EGFP-positive plantlets, supporting their use as recipient material for *C. faberi* transformation.

Precise adjustment of *Agrobacterium* suspension density and infection duration is critical for successful transformation. If bacterial density or infection time is too low, bacterium-explant interaction and T-DNA delivery may be insufficient; if they are too high, bacterial cytotoxicity can cause irreversible tissue damage (Cao, 2020). In orchid biotechnology, effective OD₆₀₀ values generally range from 0.1 to 1.0, but species-specific optimization is required. Belarmino and Mii (2000) transformed *Phalaenopsis* using OD₆₀₀ = 0.1 with 10 h of co-cultivation, whereas Cui *et al.* optimized *Phalaenopsis* protocorm transformation at OD₆₀₀ = 0.2 - 0.3 with 120 min of infection (Cui *et al.*, 2012). Zhang *et al.* reported better results in *Phalaenopsis* at OD₆₀₀ = 0.4 - 0.6 with a short 10 - 15 min infection, and Xie *et al.* observed peak GUS expression in *Cymbidium sinense* rhizomes at OD₆₀₀ = 0.9 (Xie *et al.*, 2015; Zhang *et al.*, 2016). These findings show that bacterial density and infection time must be optimized together according to the target species and explant type.

Co-cultivation duration also strongly affects transformation efficiency in orchids. This period allows bacterial attachment and T-DNA transfer, but insufficient co-cultivation may prevent complete T-DNA delivery, whereas excessive co-cultivation can lead to *Agrobacterium* overgrowth and explant necrosis (Belarmino and Mii, 2000; Hao *et al.*, 2010; Phlaetita *et al.*, 2015; Hsing *et al.*, 2016; Yu, 2016). Most orchid transformation protocols use 2 - 3 d of co-cultivation. Pan obtained seven resistant *Phalaenopsis* regenerants using OD₆₀₀ = 0.3, 20 min infection, and 4 d co-cultivation (Pan, 2007). Wu optimized a two-step *Cymbidium* transformation protocol using OD₆₀₀ = 0.2, 20 min infection, and 6 - 9 d co-cultivation (Wu, 2008). Liu reported the highest transformation efficiency in *Cymbidium* rhizomes after 10 d of co-cultivation, indicating strong explant-specific requirements (Liu, 2017). In the present study, 2 d was optimal for *C. faberi* calli; longer co-cultivation caused bacterial overgrowth and reduced regenerative capacity.

Orchidaceae species are valued for their diverse flower colors, complex floral forms, fragrance, and high commercial value, but modern orchid production is constrained by unpredictable flowering, disease and pest susceptibility, limited abiotic stress tolerance, and demanding cultivation requirements. Conventional hybridization has contributed greatly to orchid improvement, yet its outcomes are often unpredictable and are limited by biological incompatibility and long juvenile phases (Cao, 2020). Interspecific hybridization barriers further restrict the transfer of desirable traits among ecologically distinct orchids. For example, fragrant terrestrial *Cymbidium* species such as *C. faberi* and *C. sinense* generally produce compact flowers with relatively short flowering periods, whereas epiphytic genera such as *Phalaenopsis* and *Cattleya* often produce large, long-lasting, but scentless flowers. Understanding the genetic networks controlling fragrance biosynthesis, floral architecture, and flowering time would facilitate the precise improvement of commercially important traits through transgenic approaches (Kong, 2008; Cui *et al.*, 2012).

In conclusion, this study established an *Agrobacterium*-mediated transformation protocol for *C. faberi*. Calli pre-cultured for 3 d were identified as the most suitable recipient material. The optimal transformation conditions were OD₆₀₀ = 0.2, infection for 5 min, and co-cultivation for 2 d. Under these conditions, two independent EGFP-positive transgenic plantlets were obtained and confirmed by PCR. Although further improvement is still needed to increase transformation frequency and regeneration stability, the protocol provides a useful methodological framework for *C. faberi* genetic transformation and supports future research on gene function, molecular breeding, and germplasm innovation in traditional Chinese orchids.

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