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Highly Efficient *Agrobacterium*-mediated Stable Transformation of *Cymbidium goeringii*

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Keywords. *Cymbidium ensifolium*, *Cymbidium faberi*, *Cymbidium goeringii*, Oriental orchids, stable transformation

Abstract. **Oriental orchids possess great potential in the consumer market because of their diverse leaf and flower patterns and enchanting fragrance. Nevertheless, their long growth period, stringent growth environment requirements, low germination rates, and limited transformation methods pose significant obstacles to genetic improvement. To improve the regeneration efficiency of oriental orchids and enhance their transformation efficiency, the solid–liquid oscillation–solid alternating cultivation mode was used to efficiently obtain stable transformants of *Cymbidium* orchids in this study. The results showed that within approximately 2 months, the regeneration rate of *C. goeringii* derived from protocorm-like bodies reached a remarkable 77.30% with this method. The transformation efficiency of *C. goeringii* achieved through polymerase chain reaction amplification was 18.66% when inoculated with *Agrobacterium* LBA4404, and the positive transformation rate reached 75.03%. This experimental result was further validated by β-glucuronidase staining and green fluorescent protein fluorescence detection. This methodology significantly increased the positive transformation rate and shortened the regeneration period of *C. goeringii*. Furthermore, this cultivation mode was successfully implemented for *C. faberi* and *C. ensifolium*. Therefore, the solid–liquid oscillation–solid alternating cultivation mode provides a technical basis for efficient transformation, which will promote the rapid development of genetically improved oriental orchids.**

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As the second largest family in the plant kingdom, there are more than 900 genera and 27,000 species of orchids, some of which have great economic value (Li et al. 2022). For example, numerous species of orchids are widely used for cut flowers or cultivated as potted plants. After the COVID-19 pandemic, the scale of the orchid industry in China slightly increased, reaching 30.73 billion RMB in 2023. However, because of the overexploitation of wild orchid resources, many species have become endangered and listed in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES); therefore, modern breeding technologies are necessary to develop new cultivars (Guo et al. 2016).

Oriental orchids belong to the genus *Cymbidium* in the subfamily Epidendroideae, with notable species including *Cymbidium ensifolium*, *C. sinense*, *C. goeringii*, *C. faberi*, *C. kanran*, *C. tortisepalum*, and *C. longibracteatum*. The relatively slow growth of orchids and the long vegetative phase hindered the conventional breeding processes based on artificial pollination. The naturally obtained seeds of orchids are generally microscopically small and embryonically immature, thus making them unable to germinate without symbiosis with suitable mycorrhizal fungi (Hsiao et al. 2011). Therefore, modern propagation technology is needed to rapidly breed new oriental orchid cultivars.

Tissue culture technology is a preferred alternative to sexual reproduction because it can overcome the limits of slow plant growth and offers many advantages. First, it greatly shortens the breeding period, especially for the slow-growing species. Second, it helps maintain consistent plant materials, avoiding character segregation in the progeny. Third, it permits the large-scale propagation of endangered plants or desirable cultivars in a greenhouse.

The explant type and regeneration mode are crucial for in vitro plant growth. In orchids, seeds develop into a spherical embryonic cell mass called protocorm, which differentiates into completely new plants. Protocorm-like bodies (PLBs) were developed by optimizing the tissue culture process. Although similar to protocorms, they are derived from somatic tissues and not seeds (Cardoso et al. 2020). Because of their powerful and quick regeneration capacity, PLBs are the main type of explant used for transgenic transformation, especially for *Oncidium*, *Cattleya*, and *Phalaenopsis* orchids (Chai et al. 2002; Li et al. 2005; Zhang et al. 2010). In some *Cymbidium* orchids, seeds can be germinated into rhizomes and horizontal rhizomorphous stems. They can elongate to develop into shoots and roots, enabling rapid vegetative propagation. In this study, PLBs of *C. goeringii* as well as rhizomes of *C. faberi* and *C. ensifolium*, respectively, were used as explants for transgenic technology.

Many transient transformation technologies have been widely used in orchid research, including virus-induced gene silencing as well as protoplast transformation in *Phalaenopsis* and *Cymbidium* orchids (Hsieh et al. 2013; Ren et al. 2020). Novel transgenic technologies such as transgene-free coediting (Huang et al. 2023) or the cut-dip-budding delivery system mediated by *Agrobacterium rhizogenes* can be used to modify explants (Cao et al. 2022). They can facilitate the functional identification of novel genes in original plants within a short period of time; furthermore, the operation procedures are simple and can be conducted under nonsterile conditions. However, one obvious shortcoming is that these technologies cannot yield stably modified plants with inheritable novel characteristics, which impedes the breeding of new orchid cultivars. *Agrobacterium tumefaciens* is used for stable transformation in both dicotyledonous and monocotyledonous plants, including orchids such as *Phalaenopsis* (Belarmino and Mii 2000), *Oncidium* (Liau et al. 2003), and

Table 1. Comparison of regeneration rates of *Cymbidium goeringii* in different solid culture media.

Group	NAA (mg/L)	6-BA (mg/L)	TDZ (mg/L)	Regeneration rate
1	1	2	0	7.7 ± 1.5/50 (15.33%) d
2	1	5	0	11.3 ± 1.2/50 (22.67%) d
3	0.5	8	0	21.3 ± 1.2/50 (42.67%) c
4	0.5	8	0.3	26.0 ± 2.6/50 (52.00%) b
5	0.1	8	0.3	38.7 ± 1.2/50 (77.30%) a

There were 50 explants per group, and three replications of each group were performed. Different superscript letters indicate significant differences among each group as determined by Duncan's multiple range test followed by a one-way analysis of variance with $P < 0.05$ considered significant.
6-BA = benzyladenine; NAA = naphthaleneacetic acid; TDZ = thidiazuron.

Dendrobium (Utami et al. 2018). In addition, biolistic bombardment was successfully used for *Phalaenopsis* (Su and Hsu 2003), *Oncidium* (Liu et al. 2012), and *Dendrobium* (Men et al. 2003). However, the transformation efficiency is low and the cultivation period is long because of the intrinsically slow growth of orchids.

To improve the transformation efficiency and shorten the cultivation period, an *Agrobacterium tumefaciens*-mediated method was applied to obtain stable transgenic individuals of three oriental orchids, *C. faberi*, *C. goeringii*, and *C. ensifolium*. The choice of explants and the cultivation mode were the most important factors that determine the efficacy of orchid transgene technology. This study offers a powerful technical basis for accelerating the process of orchid breeding, which will accelerate the mining of new orchid genes for molecular genetic breeding.

Materials and Methods

Cymbidium plant materials. The tissue culture plantlets of *C. goeringii* and *C. ensifolium* were kindly provided by Professor Bo Yang of Wuhan Botanical Garden, Chinese Academy of Sciences, in 2011. The PLBs of *C. goeringii* and rhizomes of *C. ensifolium* were induced from sterilized plantlets and allowed to proliferate in clustered formations to preserve the explants for subsequent transgenic operations. The rhizomes of *C. faberi* were obtained via seed germination of wild plants that have been collected and preserved since 2011 (Xu et al. 2019). The proliferation condition of PLBs and rhizomes of *Cymbidium* was 1/2 Murashige and Skoog (MS) medium (2.47 g/L) supplemented with 1.2 mg/L naphthaleneacetic acid (NAA), 0.4 mg/L 6-benzyl adenine (6-BA), 35.0 g/L sucrose, 1.5 g/L activated charcoal, and 10.0 g/L agar powder (pH = 5.8). The plants and explants were grown in a greenhouse under a 16-h photoperiod (illuminance intensity of 2000–3000 Lx) at 22 ± 2 °C and 60% humidity.

Regeneration of cymbidium faberi, C. goeringii, and C. ensifolium by tissue culture. The PLBs of *C. goeringii* were directly immersed in liquid culture medium (4.74 g/L MS with 0.1 mg/L NAA, 8.0 mg/L 6-BA, 0.3 mg/L thidiazuron (TDZ), 35.0 g/L sucrose, and 0.4 g/L activated charcoal; pH = 5.8) with continuous oscillation at 80 rpm under a 16-h light/8-h dark cycle. One month later, PLBs were regenerated into young shoots with original leaves and elongated stems. Then, they were transferred to solid culture medium (4.74 g/L MS with NAA, 6-BA, TDZ, 35.0 g/L sucrose, 1.5 g/L activated charcoal, and 10.0 g/L agar powder; pH = 5.8) with the addition of 80.0 g/L banana extract. The combinations and concentrations of plant growth regulators added in the solid culture medium are listed in Table 1. During this period, many regenerated young shoots with a complete organ structure appeared.

The rhizomes of *C. faberi* and *C. ensifolium*, respectively, were cultured in liquid 1/2 MS basic medium (2.47 g/L) comprising 1.2 mg/L NAA, 0.4 mg/L 6-BA, 35.0 g/L sucrose, and 0.4 g/L activated charcoal (pH = 5.8) under continuous shaking at 80 rpm under a 16-h light/8-h dark cycle until white bulbs appeared on the surface of rhizomes, which required approximately 30 d. Then, the rhizomes were transferred to solid culture medium (as described previously). After 1 week, the rhizomes were transferred again to the liquid culture medium for approximately 1 month until many white bulbs became green. The liquid culture medium was the same as that of the solid culture, except the amount of activated charcoal was decreased to 0.4 g/L and no agar was added. Then, the regenerated rhizomes were placed on solid culture medium again. During this period, many regenerated young shoots appeared from the end of the rhizomes.

The cultivation mode was classified into the following five methods: constant solid (S) culture; constant liquid oscillation (L) culture; solid–liquid oscillation (S–L) culture; liquid oscillation–solid (L–S) culture; and solid–liquid oscillation–solid alternating (S–L–S) culture. In the alternating culture mode, the solid culture generally lasted 1 week, whereas the liquid oscillation culture spanned 1 month. The number of explants per group was 50, with three replicates per group. The reagents for tissue culture were purchased from Sangon Biotech (Shanghai, China).

Stable transformation of C. faberi, C. ensifolium, and C. goeringii. The precultured rhizomes of *C. faberi* and *C. ensifolium* or PLBs of *C. goeringii* were immersed into a logarithmic culture of *Agrobacterium tumefaciens* (OD_{600} = 0.8 to 1.0) with 100 μM acetosyringone for 30 min under constant oscillation at 80 rpm. Then, the explants were blotted with sterilized filtered paper to remove excess moisture, transferred to solid coculture medium (MS medium with 0.1 mg/L NAA, 8.0 mg/L 6-BA, 0.3 mg/L TDZ, 35.0 g/L sucrose, 1.5 g/L activated charcoal, and 10.0 g/L agar powder; pH = 5.8), and grown for 4 d. Then, the cultured tissues were transferred to solid selection medium (SC with 350.0 mg/L cefotaxime sodium and 5.0 mg/L hygromycin) to inhibit the growth of *Agrobacterium*. One week later, the explants were transferred to liquid selection medium (MS with 0.1 mg/L NAA, 8.0 mg/L 6-BA, 0.3 mg/L TDZ, 35.0 g/L sucrose, 0.4 g/L activated charcoal, 350.0 mg/L cefotaxime sodium, and 5.0 mg/L hygromycin; pH = 5.8) and cultured under continuous oscillation at 80 rpm under a 16-h light/8-h dark cycle for 1 month. Finally, the cultured tissue pieces were transferred to solid rooting culture medium (SS medium with banana extract). Analogous to the regeneration protocol, the explants regenerated numerous young shoots from the end of the rhizome and the PLBs of *C. goeringii* that developed into complete plantlets.

To detect the influence of *Agrobacterium* strains on the transformation efficiency, GV3101, LBA4404, and EHA105, respectively, were used to transform the orchid explants. The commercial vectors pCAMBIA 1301 harboring a β-glucuronidase (GUS) expression cassette and hygromycin resistance and pCAMBIA1302 harboring a green fluorescent protein (GFP) expression cassette and hygromycin resistance, respectively, were used to transform explants of *Cymbidium* using *Agrobacterium* strains.

Polymerase chain reaction detection. The genomic DNA of transgenic plants was isolated using a CTAB kit (Takara Biotechnology Co. Ltd., Dalian, China) according to the manufacturer's instructions. The diluted gDNAs were used as templates to amplify the specific gene fragments of *gus* and *gfp*. The sequences of primer pairs were as follows: GUS-F1,

Table 2. Comparison of regeneration rates of *Cymbidium goeringii* in different culture modes.

Group	Culture mode	Regeneration period	Regeneration rate
1	S	90–102 d	(31.33 ± 7.37)/50 (62.67%) b
2	L	≈50 d	(4.33 ± 2.08)/50 (8.67%) d
3	S–L culture	≈50 d	(5.33 ± 1.15)/50 (10.67%) d
4	L–S culture	≈80 d	(18.33 ± 3.51)/50 (36.67%) c
5	S–L–S culture	50–60 d	(38.67 ± 1.15)/50 (77.30%) a

There were 50 explants per group, and three replications of each group were performed. Different superscript letters indicate significant differences among each group as determined by Duncan's multiple range test followed by one-way analysis of variance with $P < 0.05$ considered significant.
L = constant liquid oscillation culture; L–S culture = liquid oscillation–solid culture; S = constant solid culture; S–L culture = solid–liquid oscillation culture; S–L–S culture = alternating solid–liquid oscillation–solid culture.

Table 3. Components of culture media used in different stages of the solid–liquid oscillation–solid alternating cultivation mode.

Culture media	Components
Preculture (oscillation)	1/2 MS (2.47 g/L) + sucrose (35.0 g/L) + 6-BA (0.4 mg/L) + NAA (1.2 mg/L) + AC (0.4 g/L)
Coculture (CC)	MS (4.74 g/L) + sucrose (35.0 g/L) + 6-BA (8.0 mg/L) + NAA (0.1 mg/L) + TDZ (0.3 mg/L) + AC (1.5 g/L) + agar (10.0 g/L)
Solid selection culture (SS)	MS (4.74 g/L) + sucrose (35.0 g/L) + 6-BA (8.0 mg/L) + NAA (0.1 mg/L) + TDZ (0.3 mg/L) + Cef (350.0 mg/L) + hygromycin (5.0 mg/L) + AC (1.5 g/L) + agar (10.0 g/L)
Liquid selection culture (LS) (oscillation)	MS (4.74 g/L) + sucrose (35.0 g/L) + 6-BA (8.0 mg/L) + NAA (0.1 mg/L) + TDZ (0.3 mg/L) + Cef (350.0 mg/L) + hygromycin (5.0 mg/L) + AC (0.4 g/L)
Rooting culture (RC)	MS (4.74 g/L) + sucrose (35.0 g/L) + 6-BA (8.0 mg/L) + NAA (0.1 mg/L) + banana extract (80.0 g/L) + Cef (350.0 mg/L) + hygromycin (10.0 mg/L) + AC (1.5 g/L) + agar (10.0 g/L)

6-BA = benzyladenine; AC = activated charcoal; Cef = cefotaxime sodium; MS = Murashige and Skoog; NAA = napthaleneacetic acid; TDZ = thidiazuron.
All of the culture media were adjusted to pH = 5.8.

ATGGTAGATCTGACTAGTAAAG; GUS-R1, TCACACGTGGTGGTGGTGGTGG (Tm = 55 °C); GFP-F1, CAGTGGAGAGGGTGAAGGTGAT; and GFP-R1: TG AAGTTGGCTTTGATGCCG (Tm = 55 °C). The polymerase chain reaction (PCR) contained 50 to 80 ng of template DNA, 2.0 μL of 2.5 mM dNTPs, 2.0 μL of 10× Ex Taq DNA polymerase buffer, 0.1 μL of Ex Taq DNA polymerase, 0.5 μL each of 10 mM forward and reverse primers, and ddH_2O to a total volume of 20.0 μL. The PCR temperature program was as follows: 94 °C, 5 min; 94 °C, 30 s; 55 °C, 30 s; 72 °C, 1 min/kb (35 cycles); 72 °C, 8 min; and hold at 16 °C. The PCR products were detected by agarose gel electrophoresis. The PCR reagents were purchased from Takara Biotechnology Co. Ltd.. The specific fragments were retrieved from the gel for Sagner sequencing by Sangon Biotech Co. Ltd.

GUS staining. The young leaves, roots, and explants were immersed in GUS staining solution (5.0 mg 5-bromo-4-chloro-3-indolyl β-D-glucuronide, X-Gluc in 1.0 mL dimethyl formamide, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 1.0 mM EDTA, 10% Triton X-100) and placed under a vacuum for 3 min at 30-s intervals. Then, the tissues of plants were stained in a dark incubator at 37 °C for 7 d. Thereafter, the tissues were destained in 95% ethanol to remove the chlorophyll and recorded using a scanner (ScanMaker i800; Microtek, Zhongjing, China). All reagents for GUS staining were bought from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China).

GFP detection. The young leaves and explants were cut into slices with a scalpel and placed on the glass slide with a cover slide; thereafter, they were photographed under a fluorescence microscope (BX53/Flex; Olympus, Tokyo, Japan). Fluorescence emission spectra were measured following excitation at 460 to 550 nm together with a 500- to 550-nm beam splitter.

Statistical analysis. The experimental data were expressed as means ± standard error (*SE*) (n = number of explants) and processed using Excel (Microsoft, Redmond, WA, USA) and SPSS 19.0 software (IBM, Chicago, IL, USA) to calculate the average value, *SE*, and analysis of variance (ANOVA) results. The regeneration rate was calculated as the number of differentiated plantlets divided by the total number of explants (50) × 100%. The transformation efficiency was calculated as the number of all plantlets obtained with *Agrobacterium* inoculation divided by the total number of inoculated explants (50) × 100%. The positive transformation rate was calculated as the number of transgenic plantlets containing the target fragments according to PCR detection divided by the number of all plantlets obtained with *Agrobacterium* inoculation × 100%.

Results

Optimal regeneration culture media and culture mode for explants of cymbidium. To investigate how various plant growth regulators and their concentrations impact the regeneration of *C. goeringii*, five distinct plant growth regulator combinations, which partially referenced a previous report of flowering initiation directly from rhizomes, were tested (Ahmad et al. 2022). The regeneration rates of *C. goeringii* are summarized in Table 1. The optimal regeneration culture medium was basic MS medium with 0.1 mg/L NAA, 8.0 mg/L 6-BA, and 0.3 mg/L TDZ, which resulted in the highest regeneration rate of 77.30%. Compared with the groups treated with two plant growth regulators of NAA and 6-BA, the regeneration rates ranged from 15.33% to 42.67%. The incorporation of TDZ significantly improved the regeneration capacity of the explants and ranged from 52.00% to 77.30%, confirming earlier research findings (Park et al. 2018). Therefore, the subsequent transformation and induction experiments used this plant growth regulator combination and concentration.

The following five culture modes were applied to compare the regeneration rates of *C. goeringii*: S culture; L culture; S–L culture; L–S culture; and S–L–S culture. The results showed that the optimum culture mode for *C. goeringii* explants was S–L–S cultivation, reaching a regeneration rate of 77.30%. Although the S culture method also yielded a satisfactory regeneration rate of 62.67%, this process could last up to 100 d. In contrast, the alternating cultivation mode significantly shortened the culture period to approximately 60 d. In contrast to S culture, the L culture method often yielded albino or vitrified shoots and obtained the regeneration rate of 8.67%, which was attributed to the high moisture in the culture process. Both the S–L culture mode and the L–S culture mode are capable of producing regenerated shoots, with regeneration rates of 10.67% and 36.67%, respectively. The regeneration periods for these two modes are approximately 50 d and 80 d, respectively (Table 2). The optimal induction conditions and cultivation methods originally developed for *C. goeringii* were applied to the regeneration processes of *C. faberi* and *C. ensifolium*. However, the regeneration rates for these two species were notably lower, reaching only 30.03% and 22.36%, respectively. Additionally, the regeneration period extended beyond 9 months; this process was not only time-intensive but also heightened the risk of contamination. This may be attributed to the possibility that the rhizomes of *C. faberi* and *C. ensifolium* possess a lower differentiation ability compared with that of the PLBs of *C. goeringii*.

Optimal agrobacterium strains for stable transformation of cymbidium. The components of different culture media used in stable

Table 4. Transformation efficiency and positive transformation rates of *Cymbidium goeringii* using different *Agrobacterium* strains.

Group	*Agrobacterium* strain	Explants	Transformants	Transformation efficiency	Positive transformants	Positive transgenic rates
1	LBA4404	50	9.33 ± 1.53	18.66% a	7.00 ± 2.00	75.03%
2	GV3101	50	1.0 ± 1.0	2.00% b	n.d.	n.d.
3	EHA105	50	0.33 ± 0.58	0.67% b	n.d.	n.d.

There were 50 explants per group, and three replications of each group were performed. Different superscript letters indicate significant differences among each group as determined by Duncan's multiple range test followed by a one-way analysis of variance with $P < 0.01$ considered significant.
n.d. = not detected.

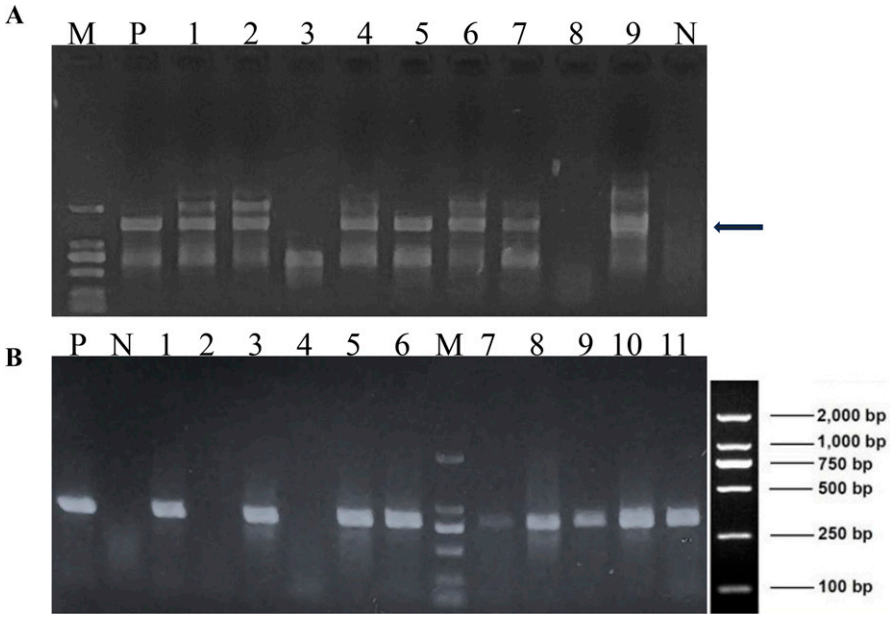

Fig. 1. Agarose gel electrophoresis of polymerase chain reaction products from the transgenic plantlets of *Cymbidium goeringii*. (**A**) Transgenic plantlets harboring pCAMBIA1301. (**B**) Transgenic plantlets harboring pCAMBIA1302. M = DL2000 DNA marker; N = negative control; P = positive control.

transformation of *C. goeringii* are listed in Table 3, including the cocultured medium, solid selection medium, liquid selection medium, and solid rooting culture medium. The basic culture media included the plant growth regulator combination and concentration used during the regeneration process. During the selection culture process, cefotaxime sodium and hygromycin were incorporated to suppress the proliferation of *A. tumefaciens* and facilitate the selection of positive transformants. In the rooting culture medium, organic additives comprising 80.0 g/L banana extract were added to supplement the nutrients necessary for root growth.

Three distinct *Agrobacterium tumefaciens* strains (LBA4404, GV3101, and EHA105) were compared to identify the most suitable strain for the stable transformation of *C. goeringii*. The results showed that strain LBA4404 had slower growth and could efficiently transform the explants of *C. goeringii* with exogenous DNA fragments. As shown in Table 4, The transformation efficiency of LBA4404 strain harboring pCAMBIA1301 and pCAMBIA1302 reached 18.66%, markedly surpassing that of GV3101 and EHA105 strains. In contrast, the GV3101 and EHA105 strains grew vigorously and disrupted the development of *Cymbidium* explants, which could not be alleviated even with the application of high concentrations of cefotaxime sodium.

Rapid detection of the transformation efficiency by the PCR analysis. The gDNA of transformants was extracted and used as a template for the PCR analysis. The PCR products were detected by agarose gel electrophoresis (Fig. 1). The expected lengths of the *gus* and *gfp* fragments were 1500 bp and 750 bp, respectively, for pCAMBIA1301 and pCAMBIA1302 plasmids and their corresponding transformants. Amplified fragments with the same molecular weight as that of the positive control (corresponding plasmids as the templates) indicated positive transformants, and samples that did not yield the expected fragments were marked as false-positive transformants. The negative control did not yield any PCR bands. In addition, the recycled fragments were sequenced to confirm the identity of the PCR products. Among nine transgenic plants with pCAMBIA1301 vectors, two plants of line 3 and line 8 were false-positive plants, and the positive transformation efficiency was 77.78%. Two of 11 transgenic plants with pCAMBIA1302 vectors were false-positive plants, with positive transformation efficiency of 81.82%. The overall average positive transformation rate of *C. goeringii* was 75.03% (Table 4).

β-glucuronidase staining of cymbidium goeringii transformants. The young transgenic plantlets transformed with pCAMBIA1301 vector were stained with GUS solution. The leaf veins and bracts of the pseudobulbs of a representative *C. goeringii* shoot were stained blue after ethanol decolorization, suggesting that the *gus* expression cassette had been stably inserted into *Cymbidium* and was expressed normally (Fig. 2).

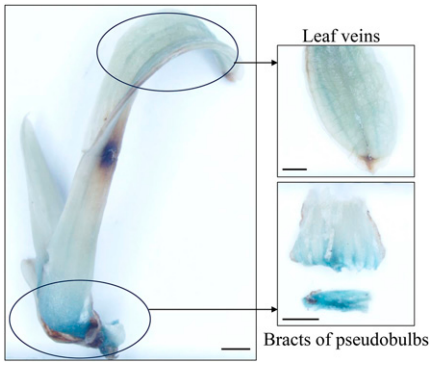

Fig. 2. β-glucuronidase staining of transgenic plantlet of *Cymbidium goeringii*. The obviously stained blue regions, such as leaf veins and bracts of pseudobulbs, were partially enlarged. Bar = 1 mm.

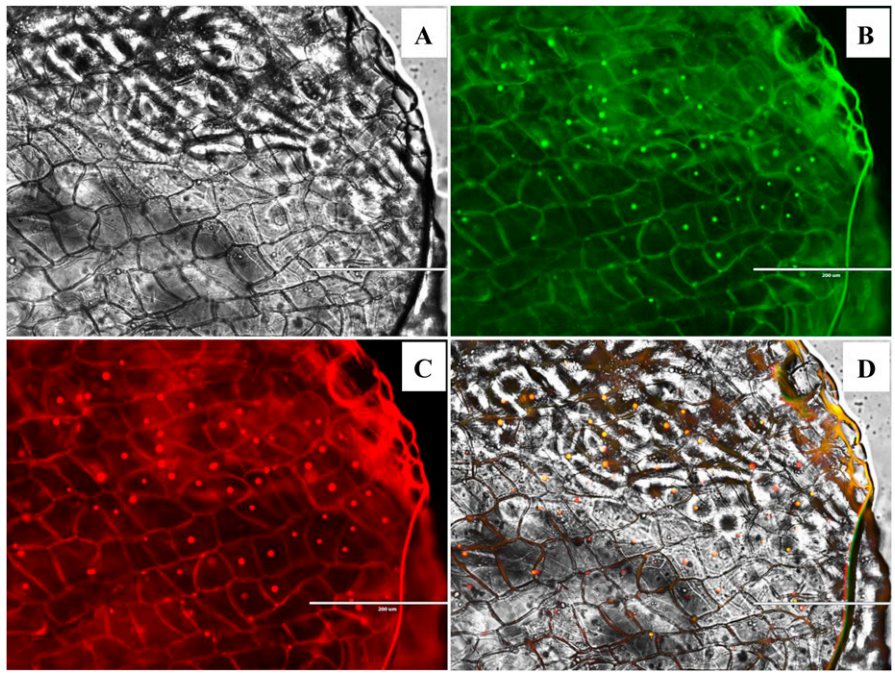

Fig. 3. Green fluorescent protein detection in a transgenic plantlet of *Cymbidium goeringii*. (**A**) Bright light. (**B**) Green fluorescence. (**C**) Red fluorescence. (**D**) Merged. Bar = 200 μm.

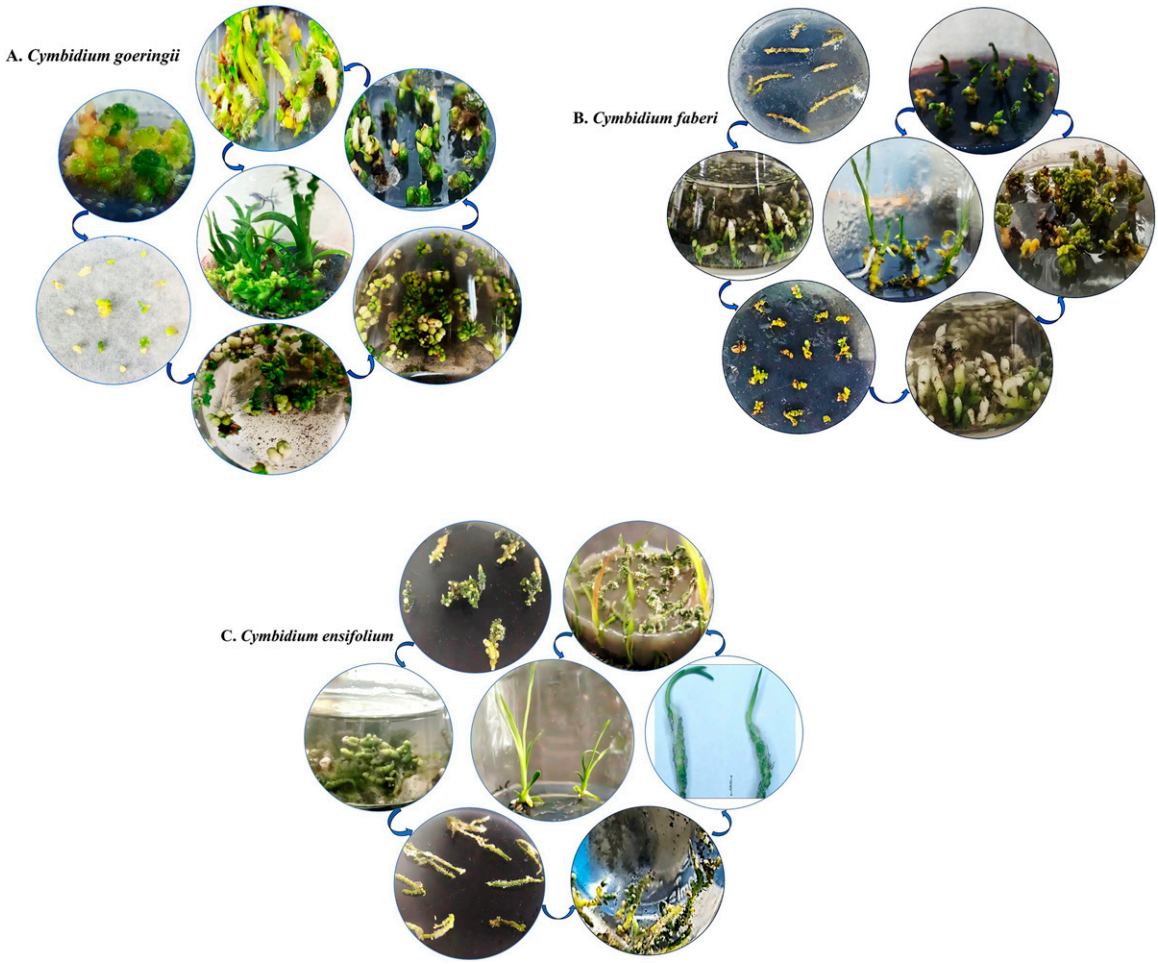

Fig. 4. Flowchart of the genetic modification procedure of three oriental orchids. (**A**) *Cymbidium goeringii*. Initially, the protocorm–like bodies (PLBs) underwent preculturing on solid culture media. Following inoculation into *Agrobacterium* suspension, the PLBs were subsequently transferred to solid coculture media. Subsequently, a solid–liquid oscillation–solid alternating culture mode was used for a single cycle to facilitate the development of young transgenic plantlets. Finally, robust transgenic plantlets were harvested from the solid rooting culture media. (**B**) *Cymbidium faberi*. The rhizomes were initially precultured in liquid culture media using a flask-shaking culture mode. Subsequently, they were subjected to *Agrobacterium* inoculation. Following this, an alternating culture method involving two to three cycles of solid–liquid oscillation–solid culture was applied to promote the growth and development of young transgenic plantlets. Ultimately, a small number of transgenic plantlets were successfully harvested from the solid rooting culture media. C = *Cymbidium ensifolium*. The operational procedure closely resembles that used for *C. faberi*.

GFP detection of cymbidium goeringii transformants. The young transgenic plantlets transformed with pCAMBIA1302 vector were observed to detect the localization of GFP. We found that the *gfp* expression cassette was active, with green fluorescence observable in the nucleus and cell membranes of mesophyll cells of a representative *C. goeringii* shoot (Fig. 3). Thus, the *gfp* expression cassette was stably inserted in the genome of *Cymbidium* and was expressing normally.

Discussion

The so-called oriental orchids, including cultivars of *Cymbidium ensifolium*, *C. goeringii*, and *C. faberi*, have a long history of cultivation in China, with great ornamental and economic value. However, their broader popularization and worldwide commercialization are hindered by their slow growth rate and sensitivity to culture conditions. To break the limitations of traditional propagation methods, tissue culture and stable transformation technology were applied to three species of oriental orchids in this study.

In tissue culture, exogenous and endogenous plant growth regulators are crucial for the regeneration and induction of the explants. Both 6-BA and NAA are often used to induce callus formation and shoot regeneration. In *Phalaenopsis* orchids, 0.1 mg/L NAA and 1.0 mg/L 6-BA successfully induced cell clumps to develop into transformed plantlets, which required approximately 7 months (Belarmino and Mii, 2000), achieving the highest transformation efficiencies of 1.9% (Mishiba et al. 2005) and 14.6% (Chai et al. 2002). Explants of PLBs were induced to generate transgenic *Dendrobium* orchid plants using 0.5 mg/L NAA, 0.5 mg/L 6-BA, and 0.5 mg/L thidiazuron (TDZ), and this protocol required approximately 9 months, with the highest transformation efficiency of 70% in *Dendrobium lasianthera* J.J.Sm (Utami et al. 2018). The PLBs of *Cymbidium* were applied to regenerate transgenic plantlets with *Agrobacterium* under induction conditions of 2.5 g/L gellan gum-solidified NDM containing 10 g/L sucrose, 20 mg/L hygromycin, and 40 mg/L meropenem. The positive transgenic rate reached as high as 83%, although the transformation efficiency was not documented (Chin et al. 2007). Protocorms of *C. ensifolium*, *C. sinense*, and *C. goeringii* cultured with 0.5 mg/L NAA and 8.0 mg/L 6-BA developed into abnormal flowering structures without leaves and roots, which required 90 to 180 d (Ahmad et al. 2022). Based on previously reported protocols and studies, the combination of three plant growth regulators achieved the highest regeneration rate in this study, particularly when combined with the S–L–S culture mode.

Most tissue culture methods involve the use of solid culture media for the entire process. Shake–flask culture methods are generally used to culture aerobic microorganisms, as well as animal or plant cells. This culture mode can significantly accelerate the reproduction of tissue clumps and shorten the culture period, but it was rarely used for plant tissue culture because it limits the autotrophic capacity of explants. During the growth and

differentiation period of plant tissue culture, metabolism is robust and requires highly efficient gas exchange along with sufficient illumination, rendering liquid culture unsuitable for cultivating plant tissues. Therefore, an S–L–S alternating cultivation mode was applied in this study. Despite having a relatively complex operation process and increasing the risk of contamination, this mode could significantly shorten the cultivation period and enhance the transformation efficiency. Rhizomes of *C. ensifolium* rapidly generated complete plantlets in liquid shake–flask cultivation with an oscillating speed of 100 rpm (Liu et al. 2012). In a study of in vitro propagation of *C. goeringii*, rhizomes not only had a high reproductive rate but also exhibited rapid growth in the alternating conditions of L culture and S culture, with an oscillation speed of 60 rpm (Shi et al. 2013). Furthermore, the effect of L culture is superior to that of L static culture because the former can also minimize browning damage to the rhizomes. However, the authors did not discuss the oscillation cultivation period or the overall generation period. Most related studies focused on the regeneration of explants and did not detail the transformation processes.

In this study, the implementation of the alternating S–L–S culture methodology markedly enhanced the regeneration rate, achieving a level of 77.30%, while concurrently reducing the regeneration timeline. This advancement created a robust foundation for the genetic transformation of oriental orchids. Furthermore, a comparison was conducted among three distinct strains of *Agrobacterium tumefaciens* (LBA4404, GV3101, and EHA105) to ascertain the most appropriate strain for the experiment. The most suitable *Agrobacterium* strain was LBA4404, which could achieve transformation efficiency of 18.66% and a positive transformation rate of 75.03%. Additionally, the time required to obtain the transformed plantlets could be significantly shortened to 50 to 60 d. The growth of orchids is relatively slow, and some studies have reported that the WOX12 transcription factor is related to highly efficient pluripotency acquisition, which could be relevant to monocot plant transformation (Tian et al. 2022).

In fact, this cultivation mode and genetic transformation method have been implemented for *C. faberi* and *C. ensifolium*. A flowchart of the transformation steps for three oriental orchids is displayed in Fig. 4. However, the regeneration and positive transformation rates of *C. faberi* and *C. ensifolium* were lower than those of *C. goeringii*. This genetic transformation method has yielded promising outcomes for all three oriental orchids and could potentially be applied to other nonmodel plants, particularly those with slow growth rates.

Conclusions

To improve the ornamental and economic value of oriental orchids, offer more abundant high-quality plant resources, and popularize them in the global market, it is necessary to rapidly develop novel cultivars. The S-L-S alternating cultivation mode offers encouraging results for the rapid cultivation of new cultivars of oriental orchids. This mode simultaneously improved both the regeneration and transformation efficiency within approximately 2 months, thereby providing a crucial technical basis for the advancement of the orchid industry.

References Cited

Ahmad S, Chen J, Chen G, Huang J, Hao Y, Shi X, Liu Y, Tu S, Zhou Y, Zhao K, Lan S, Liu Z, Peng D. 2022. Transcriptional proposition for uniquely developed protocorm flowering in three orchid species: Resources for innovative breeding. Front Plant Sci. 13:942591. https://doi.org/10.3389/fpls.2022.942591

Belarmino MM, Mii M. 2000. *Agrobacterium*-mediated genetic transformation of a *Phalaenopsis* orchid. Plant Cell Rep. 19(5):435–442. https://doi.org/10.1007/s002990050752.

Cao X, Xie H, Song M, Lu J, Ma P, Huang B, Wang M, Tian Y, Chen F, Peng J, Lang Z, Li G, Zhu JK. 2022. Cut-dip-budding delivery system enables genetic modifications in plants without tissue culture. Innovation (Camb). 4(1):100345. https://doi.org/10.1016/j.xinn.2022.100345.

Cardoso JC, Zanello CA, Chen JT. 2020. An overview of orchid protocorm-like bodies: Mass propagation, biotechnology, molecular aspects, and breeding. Int J Mol Sci. 21(3):985. https://doi.org/10.3390/ijms21030985.

Chai ML, Xu CJ, Senthil KK, Kim JY, Kim DH. 2002. Stable transformation of protocorm-like bodies in *Phalaenopsis* orchid mediated by *Agrobacterium tumefaciens*. Sci Hortic. 96(1–4):213–224. https://doi.org/10.1016/S0304-4238(02)00084-5.

Chin DP, Mishiba K, Mii M. 2007. *Agrobacterium*-mediated transformation of protocorm-like bodies in *Cymbidium*. Plant Cell Rep. 26(6):735–743. https://doi.org/10.1007/s00299-006-0284-5.

Guo YY, Huang LQ, Liu ZJ, Wang XQ. 2016. Promise and challenge of DNA barcoding in *Venus slipper* (*Paphiopedilum*). PLoS One. 11(1):e0146880. https://doi.org/10.1371/journal.pone.0146880.

Hsiao YY, Pan ZJ, Hsu CC, Yang YP, Hsu YC, Chuang YC, Shih HH, Chen WH, Tsai WC, Chen HH. 2011. Research on orchid biology and biotechnology. Plant Cell Physiol. 52(9):1467–1486. https://doi.org/10.1093/pcp/pcr100.

Hsieh MH, Lu HC, Pan ZJ, Yeh HH, Wang SS, Chen WH, Chen HH. 2013. Optimizing virus-induced gene silencing efficiency with *Cymbidium* mosaic virus in *Phalaenopsis* flower. Plant Sci. 201–202:25–41. https://doi.org/10.1016/j.plantsci.2012.11.003.

Huang X, Jia H, Xu J, Wang Y, Wen J, Wang N. 2023. Transgene-free genome editing of vegetatively propagated and perennial plant species in the T_0 generation via a co-editing strategy. Nat Plants. 9(10):1591–1597. https://doi.org/10.1038/s41477-023-01520-y.

Li SH, Kuoh CS, Chen YH, Chen HH, Chen WH. 2005. Osmotic sucrose enhancement of single-cell embryogenesis and transformation efficiency in *Oncidium*. Plant Cell Tiss Organ Cult. 81(2):183–192. https://doi.org/10.1007/s11240-004-4955-z.

Li Y, Zhang B, Yu H. 2022. Molecular genetic insights into orchid reproductive development. J Exp Bot. 73(7):1841–1852. https://doi.org/10.1093/jxb/erac016.

Liau CH, You SJ, Prasad V, Hsiao HH, Lu JC, Yang NS, Chan MT. 2003. *Agrobacterium tumefaciens*-mediated transformation of an *Oncidium* orchid. Plant Cell Rep. 21(10):993–998. https://doi.org/10.1007/s00299-003-0614-9.

Liu C, Meng Y, Wang C, Zhang X, Yang B. 2012. Kinetic study on tissue culture and rhizome multiplication of *Cymbidium ensifolium* (Linn.) Sw (in Chinese). J Nanchang Univer (Nat Sci). 36:264–267, 272.

Liu XJ, Chuang YN, Chiou CY, Chin DC, Shen FQ, Yeh KW. 2012. Methylation effect on chalcone synthase gene expression determines anthocyanin pigmentation in floral tissues of two *Oncidium* orchid cultivars. Planta. 236(2):401–409. https://doi.org/10.1007/s00425-012-1616-z.

Mishiba K, Chin DP, Mii M. 2005. *Agrobacterium*-mediated transformation of *Phalaenopsis* by targeting protocorms at an early stage after germination. Plant Cell Rep. 24(5):297–303. https://doi.org/10.1007/s00299-005-0938-8.

Men S, Ming X, Wang Y, Liu R, Wei C, Li Y. 2003. Genetic transformation of two species of orchid by biolistic bombardment. Plant Cell Rep. 21(6):592–598. https://doi.org/10.1007/s00299-002-0559-4.

Park HY, Kang KW, Kim DH, Sivanesan I. 2018. *In vitro* propagation of *Cymbidium goeringii* Reichenbach fil. through direct adventitious shoot regeneration. Physiol Mol Biol Plants. 24(2):307–313. https://doi.org/10.1007/s12298-017-0503-2.

Ren R, Gao J, Lu C, Wei Y, Jin J, Wong SM, Zhu G, Yang F. 2020. Highly efficient protoplast isolation and transient expression system for functional characterization of flowering related genes in *Cymbidium* orchids. Int J Mol Sci. 21(7):2264. https://doi.org/10.3390/ijms21072264.

Shi L, Peng J, Li P. 2013. A study on *in vitro* culture of rhizomes of *Cymbidium goeringii* (in Chinese). Jiangsu Agr Sci. 41:51–53.

Su V, Hsu BD. 2003. Cloning and expression of a putative cytochrome P450 gene that influences the colour of *Phalaenopsis* flowers. Biotechnol Lett. 25(22):1933–1939. https://doi.org/10.1023/b:bile.0000003989.19657.53.

Tian J, Jiang W, Si J, Han Z, Li C, Chen D. 2022. Developmental characteristics and auxin response of epiphytic root in *Dendrobium catenatum*. Front Plant Sci. 13:935540. https://doi.org/10.3389/fpls.2022.935540.

Utami ESW, Hariyanto S, Manuhara YSW. 2018. *Agrobacterium tumefaciens*-mediated transformation of *Dendrobium lasianthera* J.J.Sm: An important medicinal orchid. J Genet Eng Biotechnol. 16(2):703–709. https://doi.org/10.1016/j.jgeb.2018.02.002.

Xu Q, Wang S, Hong H, Zhou Y. 2019. Transcriptomic profiling of the flower scent biosynthesis pathway of *Cymbidium faberi* Rolfe and functional characterization of its jasmonic acid carboxyl methyltransferase gene. BMC Genomics. 20(1):125. https://doi.org/10.1186/s12864-019-5501-z.

Zhang L, Chin DP, Mii M. 2010. *Agrobacterium*-mediated transformation of protocorm-like bodies in *Cattleya*. Plant Cell Tiss Organ Cult. 103(1):41–47. https://doi.org/10.1007/s11240-010-9751-3.