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Preliminary Establishment of an Efficient Regeneration and Genetic Transformation System for *Hemerocallis middendorffii* Trautv. & C. A. Mey.

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Abstract: *Hemerocallis middendorffii* is widely used in the landscaping of Northern China for its exceptional ornamental and ecological attributes. It is also the focus of a substantial body of germplasm development and stress tolerance research. However, the absence of an efficient regeneration and genetic transformation system has been a critical barrier to conducting gene function studies on this species. In this research, the aerial parts of seed-derived *H. middendorffii* plantlets were used as explants, and the callus induction, proliferation, subculture, differentiation, and rooting conditions in the in vitro regeneration process were optimized. A callus induction rate of 95.6% was achieved, with a regeneration rate of 84.4%. Based on this procedure, a simple and effective *Agrobacterium*-mediated genetic transformation system was preliminarily developed using a hygromycin-based selection system. The system comprised an *Agrobacterium tumefaciens* culture solution optical density at 600 nm (OD₆₀₀) of 0.6, an acetosyringone concentration of 100 µmol·L⁻¹ in both the *A. tumefaciens* infection solution and the co-cultivation medium, a sterilization culture with Timentin at 300 mg·L⁻¹, and a selection culture with hygromycin at 9 mg·L⁻¹. Transgenic *H. middendorffii* T0 rooted plants were produced within a 5-month period, with a transformation rate of 11.9% and positive rate of 32.8%. The regeneration and genetic transformation system established in this study should help advance functional gene research and genetic improvement in *H. middendorffii*. However, the genetic transformation was only validated in the T0 plants. To confirm stable integration and long-term transgene stability, future research on the phenotypic and molecular characterization of T1 progeny, including segregation analysis and Southern blot verification, will be conducted.



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Keywords: *Hemerocallis middendorffii*; regeneration system; genetic transformation; callus; *Agrobacterium tumefaciens*

1. Introduction

Hemerocallis middendorffii Trautv. & C. A. Mey., also known as Amur daylily, belongs to the *Hemerocallis* genus in the Asphodelaceae family. It is a herbaceous perennial that inhabits meadows, wet grasslands, forest margins, and rocky cliffs of East Asia from temperate to alpine zones [1,2]. It has linear arching leaves with golden yellow or orange flowers that are slightly fragrant. Additionally, *H. middendorffii* exhibits strong tolerance to drought, freezing, and saline–alkali stresses [3–5]. Hence, it is highly suitable for landscaping in gardens and lands with poor ecological conditions due to its high ornamental value, early flowering period, and superior abiotic stress tolerance [6,7]. Despite the above-mentioned

advantages, the cultivation of *H. middendorffii* in China is impeded by key obstacles, such as its long growth cycles and short flowering phase, all of which limit its wider usage [8]. To address these challenges, altering these traits through genetic improvement is likely a useful approach.

Tissue culture and genetic transformation have emerged as vital tools for gene function research and the improvement of specific individual traits without altering the existing characteristics [9]. Although attempts at using explants from species in the *Hemerocallis* genus for proliferation cultivation date back to the 1950s, early efforts were hindered by the low proliferation rate and slow growth of the genus [10–13]. In recent decades, research on regeneration systems for *H. middendorffii* has made some progress. An in vitro callus induction and plant regeneration system was recently established utilizing various organs of *H. middendorffii* including tender scapes, leaves, and receptacles [3]. However, owing to the seasonal limitation and difficulties in maintaining sterile cultures, these explants have not been widely used in *H. middendorffii* tissue culture systems.

Hemerocallis is a genus of perennial monocotyledons, with a complex genetic background and heterozygosity arising from interspecific hybridization [14]. The few successful reports of genetic transformation of *Hemerocallis* spp. have included the use of gene guns [15], the pollen tube pathway [16], and *Agrobacterium* [17]. A genetic transformation system for *H. middendorffii* was preliminarily established by Liu [3]. However, the explants used in this system were derived from the field and were difficult to thoroughly disinfect, resulting in a contamination rate of up to 20%. In addition, genetic transformation in this system was only detected by PCR amplification, which not only had a low amplification rate (7.6%), but also could not accurately demonstrate the actual genetic transformation efficiency. At present, the lack of a reliable and efficient genetic transformation system has restricted the wider application of genetic engineering in developing new *H. middendorffii* varieties. Therefore, improving the genetic transformation of *H. middendorffii* is of great importance.

To validate the effectiveness of the developed transformation system, the *FLOWERING LOCUS T (FT)* gene was selected as a functional marker, which is a key regulator of flowering time and plant development [18–20]. Although the functional role of *HmFT* in *H. middendorffii* remains uncharacterized, its homologs in model plants were found to be pivotal regulators of flowering and stress adaptation [18–22]. While reporter genes like *GUS* could confirm transformation success, *HmFT* provides a functional marker that directly links genetic modification to agronomic improvement, aligning with our goal of advancing molecular breeding in *H. middendorffii*.

Based on the above rationale, the objective of this study was to preliminarily develop an efficient and stable regeneration and genetic transformation system for *H. middendorffii* using aerial parts of seed-derived plantlets as explants. First, various critical factors in the in vitro regeneration of *H. middendorffii*, including the selection of explants and the concentration of plant growth regulators (PGRs) were optimized. Next, factors influencing *Agrobacterium*-mediated transformation, including the concentrations of hygromycin, Timentin, and acetosyringone, as well as *A. tumefaciens* density, were explored, utilizing transformation with *HmFT*. The results of this research should help enable future functional genomic research and the molecular enhancement of traits in *H. middendorffii*. In future research, Western blot analysis of *H. middendorffii* T0 plants as well as segregation analysis and Southern blot verification of T1 transgene plants will be conducted to further validate the effectiveness and stability of the developed genetic transformation protocol.

2. Materials and Methods

2.1. Preparation of Media and Acquisition of Explants

Murashige and Skoog (MS) basal medium (HB8469-5, Qingdao Hope Bio-Technology, Qingdao, China) contained both inorganic salts and organic components (vitamins, myo-inositol, and glycine) as originally formulated by Murashige and Skoog (1962) [23]. The medium was supplemented with 30 g·L⁻¹ sucrose, 7.6 g·L⁻¹ agarose (A8190, Solarbio, Beijing, China), and various PGRs at specific concentrations. The pH value of the medium was adjusted to 5.8, followed by autoclaving at 121 °C for 20 min prior to the tissue culture experiments. Acetosyringone (AS), hygromycin (Hyg), and Timentin (TMT) were filter-sterilized (0.22 µm membrane) and added after the medium had cooled to 50–60 °C. Media compositions for the regeneration and genetic transformation procedures are presented in Tables 1 and 2, respectively. Basal Luria–Bertani (LB) liquid medium was used for bacterial culture [24].

Table 1. Media compositions for the regeneration of *H. middendorffii*.

Procedure	Medium Composition
Seed germination	MS + 1.0 mg·L ⁻¹ 6-BA + 1.0 mg·L ⁻¹ KT + 0.5 mg·L ⁻¹ IBA + 30 g·L ⁻¹ sucrose + 7.6 g·L ⁻¹ agarose
Callus induction	MS + (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0) mg·L ⁻¹ 6-BA + (0.1, 0.2, 0.3) mg·L ⁻¹ NAA + 30 g·L ⁻¹ sucrose + 7.6 g·L ⁻¹ agarose
Callus proliferation	MS + (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0) mg·L ⁻¹ 6-BA + (0.1, 0.2, 0.3) mg·L ⁻¹ NAA + 0.1 mg·L ⁻¹ 2,4-D + 30 g·L ⁻¹ sucrose + 7.6 g·L ⁻¹ agarose
Callus differentiation	MS + (0, 0.5, 1.0, 1.5, 2.0) mg·L ⁻¹ 6-BA + 0.2 mg·L ⁻¹ NAA + 30 g·L ⁻¹ sucrose + 7.6 g·L ⁻¹ agarose
Rooting	1/2MS + 0.2 mg·L ⁻¹ NAA + 30 g·L ⁻¹ sucrose + 7.6 g·L ⁻¹ agarose

MS: Murashige and Skoog; 6-BA: 6-benzylaminopurine; KT: kinetin; IBA: 3-indolebutyric acid; NAA: naphthaleneacetic acid; 2,4-D: 2,4-dichlorophenoxyacetic acid.

Table 2. Media compositions for the genetic transformation of *H. middendorffii*.

Procedure	Medium Composition
Pre-cultivation	MS + 1.0 mg·L ⁻¹ 6-BA + 0.2 mg·L ⁻¹ NAA + 30 g·L ⁻¹ sucrose + 7.6 g·L ⁻¹ agarose
<i>Agrobacterium</i> infection	MS + (0, 50, 100, 150, 200) µmol·L ⁻¹ AS + 30 g·L ⁻¹ sucrose
Co-cultivation	MS + 1.0 mg·L ⁻¹ 6-BA + 0.2 mg·L ⁻¹ NAA + (0, 50, 100, 150, 200) µmol·L ⁻¹ AS + 30 g·L ⁻¹ sucrose + 7.6 g·L ⁻¹ agarose
Resting	MS + 1.0 mg·L ⁻¹ 6-BA + 0.2 mg·L ⁻¹ NAA + (0, 100, 200, 300, 400) mg·L ⁻¹ TMT + 20 g·L ⁻¹ sucrose + 7.6 g·L ⁻¹ agarose
Selection	MS + 1.0 mg·L ⁻¹ 6-BA + 0.2 mg·L ⁻¹ NAA + (0, 3, 6, 9, 12) mg·L ⁻¹ Hyg + (0, 100, 200, 300, 400) mg·L ⁻¹ TMT + 30 g·L ⁻¹ sucrose + 7.6 g·L ⁻¹ agarose
Rooting	1/2MS + 0.2 mg·L ⁻¹ NAA + 30 g·L ⁻¹ sucrose + 7.6 g·L ⁻¹ agarose

MS: Murashige and Skoog; 6-BA: 6-benzylaminopurine; NAA: naphthaleneacetic acid; Hyg: hygromycin; TMT: Timentin; AS: acetosyringone.

Seeds of *H. middendorffii* were collected from the Horticulture Experimental Station of Northeast Agricultural University (Harbin, China; 126.7° E, 45.7° N). Plump and uniform *H. middendorffii* seeds without physical damage were selected and soaked in distilled water for 36–48 h at room temperature (25 ± 2 °C). The seeds were then categorized into three groups based on the seed coat treatment using flame-sterilized forceps: M1, seed coat left intact; M2, black leathery outer seed coat removed; and M3, both the brown villous inner seed coat and the black leathery outer seed coat removed. Following this, all seeds were surface-sterilized by immersion in a 3% (*v/v*) sodium hypochlorite solution for 20 min and subsequently rinsed thoroughly with sterile water five to six times (2 min per rinse). Ten seeds were

placed in each glass culture dish (90 mm × 15 mm), with 15 culture dishes constituting each group. Each dish contained 30 mL of MS seed germination medium (Table 1). The dishes were then incubated at 22 ± 2 °C under a 16 h/8 h (light/dark) photoperiod with a photosynthetic photon flux density of $50 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ to promote germination.

2.2. Optimization of Plant Regeneration Conditions

2.2.1. Callus Induction, Proliferation, and Subculture

The aerial parts of seed-derived plantlets were cultured on callus induction medium supplemented with varying concentrations of 6-benzylaminopurine (6-BA 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and $4.0 \text{ mg}\cdot\text{L}^{-1}$) and naphthaleneacetic acid (NAA 0.1, 0.2, and $0.3 \text{ mg}\cdot\text{L}^{-1}$). The medium compositions are summarized in Table 1. After the formation of calli around the explants, vigorously growing calli segments were selected and transferred to the callus proliferation medium (Table 1). The proliferation medium was formulated based on the optimized induction medium, and further supplemented with $0.1 \text{ mg}\cdot\text{L}^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D). All cultures were maintained in darkness at a steady temperature of 22 ± 2 °C, with subculturing performed every 15 days. For each treatment, there were 30 explants, with the experiment replicated in triplicate. Subsequent experiments adhered to the same experimental setup.

2.2.2. Differentiation and Rooting

Calli obtained from proliferation culture were divided into fragments measuring approximately 0.5 cm^3 and then placed in the callus differentiation medium. The medium was enriched with 6-BA at various concentrations (0, 0.5, 1.0, 1.5, and $2.0 \text{ mg}\cdot\text{L}^{-1}$) and $0.2 \text{ mg}\cdot\text{L}^{-1}$ NAA (Table 1). After two cycles of subculture, with each subculture lasting 18–20 days, the cluster buds (the structure formed by the dense growth of multiple adventitious buds on the surface of calli) reached a height of 3–5 cm, at which point individual plants were isolated and transferred into the rooting medium (Table 1). After the roots reached a length of 5–10 cm, the tissue culture bottles were unsealed for a 3-day acclimatization period. Subsequently, the rooted seedlings were removed, and the agarose surrounding the roots was rinsed away with warm water. The seedlings were then transplanted into a substrate mixture composed of vermiculite, peat soil, and garden soil at a 1:1:1 ratio ($v/v/v$). After transplantation, the seedlings were thoroughly watered.

2.3. Construction of Overexpression Vector and *A. tumefaciens* Strain for Transformation

Based on the transcriptomic data of *H. middendorffii* in our previous study, the *FLOWERING LOCUS T* (*FT*) gene was selected as the target for vector construction. Specific primers for *FT* amplification were designed using Primer Premier 5.0 (Premier Biosoft, Palo Alto, CA, USA), and the sequences are presented in Supplementary Table S1. The binary vector pCambia1300 (Cambia, Canberra, Australia) was modified by inserting the cauliflower mosaic virus (CaMV) 35S promoter into the *EcoRI* and *SacI* restriction sites and replacing the native CaMV polyA terminator with the nopaline synthase (NOS) terminator (Figure 1). The cloned *FT* gene from *H. middendorffii*, designated *HmFT* (GenBank No. KU821026, full sequence in Supplementary Figure S1), was ligated into the *KpnI* and *PstI* sites of the modified vector (pCambia1300-35S) using T4 DNA ligase (Tsingke Biotechnology, Beijing, China). The resulting recombinant plasmid, pCambia1300-35S-*HmFT*, was introduced into *A. tumefaciens* strain EHA105 (Tsingke Biotechnology, Beijing, China) via the freeze–thaw method [25].

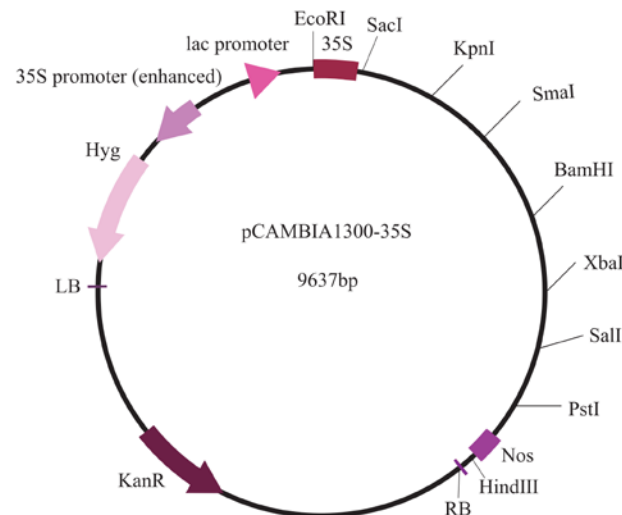


Figure 1. The pCambia1300-35S vector.

2.4. Evaluation of the Factors Affecting Transformation Rate

2.4.1. Determination of the Tolerance of Calli to Hyg

Calli that exhibited healthy growth were carefully chosen and divided into small pieces measuring approximately 0.2 cm^3 . These calli were then inoculated onto the pre-cultivation medium (Table 2) containing Hyg at varying concentrations (0, 3, 6, 9, and $12 \text{ mg} \cdot \text{L}^{-1}$). After 30 days, the browning rate of the calli was recorded.

2.4.2. Determination of the Tolerance of *A. tumefaciens* to TMT

The lethality of TMT at various concentrations to *A. tumefaciens* was investigated to identify the minimum lethal concentration. In the experiment, $20 \mu\text{L}$ of *A. tumefaciens* culture solution at an optical density at 600 nm (OD_{600}) of 1.0 was inoculated into 20 mL of LB liquid medium. This medium was supplemented with TMT at concentrations of 0, 100, 200, 300, and $400 \text{ mg} \cdot \text{L}^{-1}$, respectively. The culture was transferred to a shaker at 28°C and 220 rpm for 48 h. After incubation, the OD_{600} values were measured to assess the impact of TMT concentration on *A. tumefaciens*.

2.4.3. Screening of AS Concentration

AS at different concentrations ($0, 50, 100, 150$, and $200 \mu\text{mol} \cdot \text{L}^{-1}$) was added to both the *A. tumefaciens* infection solution and the co-cultivation medium to examine the effect of AS concentration on callus transformation. The OD_{600} value of the *A. tumefaciens* solution was calibrated to 0.5. The transformation rate utilizing this culture solution was evaluated after 35 days.

2.4.4. Screening of *A. tumefaciens* Optical Density

The pCambia1300-*HmFT* plasmid was transformed into *A. tumefaciens* EHA105 cells and cultured in 3 mL of LB liquid medium containing $50 \text{ mg} \cdot \text{L}^{-1}$ kanamycin and $50 \text{ mg} \cdot \text{L}^{-1}$ rifampicin at 28°C overnight. Positive transformation was confirmed by PCR using *HmFT*-F/R primers. A transformed bacterial strain was inoculated into a LB liquid medium at a 1:20 ratio (*v/v*) and transferred to a shaker at 28°C and 220 rpm for 12 h. Then, the bacterial precipitate was collected by centrifugation at $3438 \times g$ for 10 min. The collected bacterial pellet was resuspended in a sterile resuspension solution of varying volumes, with its pH value being adjusted to 5.7, to prepare the *A. tumefaciens* infection solution. The OD_{600} value of the culture solution was calibrated to one of five different concentrations: 0.3, 0.4, 0.5, 0.6, or 0.7. The transformation rate was evaluated after 35 days.

2.5. Transformation of Calli via *A. tumefaciens* Cells

After 4 weeks of proliferation on medium supplemented with $0.1 \text{ mg} \cdot \text{L}^{-1}$ 2,4-D, differentiated and senescent regions (identified by browning or hardening) were removed. Only the friable, pale-yellow calli from proliferation medium were selected for transformation. These calli were sectioned into 0.3 cm^3 fragments and pre-cultured for 3 days prior to *Agrobacterium* infection. Subsequently, the calli were immersed in *A. tumefaciens* infection solution at varying concentrations for 25 min. During this transformation period, gentle and continuous agitation was employed to ensure the thorough penetration of the transforming agent while avoiding damage caused by excessive agitation. Following *A. tumefaciens* infection, the bacterial suspension was carefully decanted. Three sheets of clean filter paper were placed at the bottom of a sterile culture dish. The calli were evenly distributed on the filter papers using flame-sterilized forceps, followed by overlay with an additional sheet of sterile filter paper. This configuration enabled simultaneous absorption of residual *A. tumefaciens* suspension and controlled desiccation of the transformed recipient calli. After 30 min, the calli (without the filter paper) were transferred to the co-cultivation medium (Table 2) and incubated in darkness at $22 \pm 2^\circ \text{C}$ for 2 days.

After co-cultivation, the transformed recipient calli were transferred to a resting medium and kept in dark conditions at $22 \pm 2^\circ \text{C}$ (Table 2). After a 5-day period, these recipient calli were inoculated onto the selection medium (Table 2). Throughout the selection period, the transformed recipients were subcultured every 15 days. Initially, they were incubated in darkness at $22 \pm 2^\circ \text{C}$. After 15 days, they were kept in conditions of a 16 h/8 h (light/dark) photoperiod to encourage further growth. Preliminarily resistant buds were observed after 45 days.

2.6. Confirmation of Transgenic Plants

During the regeneration period of *H. middendorffii* transformants, numerous regenerative shoots were produced and then separated into individual plantlets for the purpose of subsequent subculturing. To verify the successful transformation of these plantlets, DNA was extracted from the young leaves using the CTAB method [26]. With the genomic DNA serving as the template, a set of 35S sequence-specific primers was designed based on the binary vector pCAMBIA1300-35S-*HmFT*, spanning a total of 801 bp, and the primer sequences are presented in Supplementary Table S1. The objective was to preliminarily evaluate the potential positive transformants among the regenerated plantlets. Non-transgenic wild-type (WT) plantlets and the overexpression plasmid vector were utilized as the negative and positive controls, respectively. PCR amplification was conducted using $E \times \text{Taq}$ enzyme (Takara, Beijing, China) with 0.2 ng of DNA template. The thermal cycling program for PCR consisted of an initial denaturation at 98°C for 10 s, followed by annealing at 57°C for 30 s, and then extension at 72°C for 1 min, repeated for a total of 35 cycles, and concluding with a final extension at 72°C for 2 min. PCR products were then resolved on a 1% agarose gel via electrophoresis and visualized using a gel documentation system (SH-510, Shenhua Science Technology, Hangzhou, China).

Quantitative real-time PCR (qRT-PCR) was further used to verify the positive plants and analyze *HmFT* expression in WT and transgenic *H. middendorffii* plantlets. The total RNA was extracted from the fresh leaves using RNAiso Plus RNA extraction reagent (Takara) according to the manufacturer's instructions. Next, cDNA was synthesized from 1 mg of the extracted RNA after removal of genomic DNA. Then, qRT-PCR was conducted using SYBR Green qPCR Master Mix (Vazyme, Nanjing, China). The Actin gene, amplified using primers based on the *H. fulva* gene sequence, was used as the internal reference gene [27]. All the primer sequences used for qRT-PCR in the present studies are listed in Supplementary Table S1. Both biological and technical replicates were performed in

triplicate. The delta–delta cycle threshold $2^{-\Delta\Delta C_t}$ method was used to calculate the relative gene expression [28].

2.7. Formulas

The various parameters reported in this study were calculated as indicated by the following formulas:

$$\text{Seed germination rate (\%)} = (\text{the number of germinated seeds} / \text{the total number of tested seeds}) \times 100; \quad (1)$$

$$\text{Seed contamination rate (\%)} = (\text{the number of contaminated seeds} / \text{the total number of tested seeds}) \times 100; \quad (2)$$

$$\text{Callus induction rate (\%)} = (\text{the number of explants forming calli} / \text{the total number of explants}) \times 100; \quad (3)$$

$$\text{Regeneration rate (\%)} = (\text{the number of calli forming shoots} / \text{the total number of calli}) \times 100; \quad (4)$$

$$\text{Callus browning rate (\%)} = (\text{the number of calli turning brown} / \text{the total number of calli}) \times 100; \quad (5)$$

$$\text{Transformation rate (\%)} = (\text{the number of hygromycin-resistant plants} / \text{the total number of infectious explants}) \times 100; \quad (6)$$

$$\text{Positive rate (\%)} = (\text{the number of PCR positive plants} / \text{the number of hygromycin-resistant plants}) \times 100 \quad (7)$$

2.8. Statistical Analysis

Data are presented as mean $\pm$ standard deviation (SD) values. SPSS statistical analysis software (Version 26, IBM Corp., Armonk, NY, USA) was utilized for one-way analysis of variance (ANOVA) followed by Duncan's multiple range test, with the significance level determined at a threshold of $p < 0.05$.

3. Results

3.1. Plant Regeneration Conditions

3.1.1. Effect of Seed Coat Treatments on Primary Culture

Significant variance in both contamination and germination rates existed among the three different seed coat treatments (Table 3). Notably, the treatment with both the inner and outer seed coats removed (M3) exhibited an average contamination rate of 6.7% and an impressive average germination rate of 98.0%. In comparison, the other two seed coat treatments (M1 and M2) presented much higher contamination rates and lower germination rates, offering insufficient potential in providing sterile and viable plant materials for subsequent callus induction. Consequently, the aerial parts of plantlets derived from seeds with both the inner and outer seed coats removed were utilized as proper explants (Figure 2a,b). The optimal 6-BA and NAA concentrations during the stages of callus induction and differentiation were further explored.

Table 3. Contamination and germination rates of *H. middendorffii* seeds under different seed coat treatments. The number of seeds used for each treatment was 100.

Experimental Treatment	Seed Contamination Rate (%)	Seed Germination Rate (%)
M1	92.2 $\pm$ 6.9 a	32.6 $\pm$ 4.6 c
M2	68.9 $\pm$ 10.2 b	74.5 $\pm$ 10.7 b
M3	6.7 $\pm$ 6.7 c	98.0 $\pm$ 3.9 a

M1: seed coat left intact, M2: outer seed coat removed, and M3: both inner and outer seed coats removed. Different lowercase letters within the same column indicate significant differences at the 5% level based on Duncan's multiple range test ($p < 0.05$).

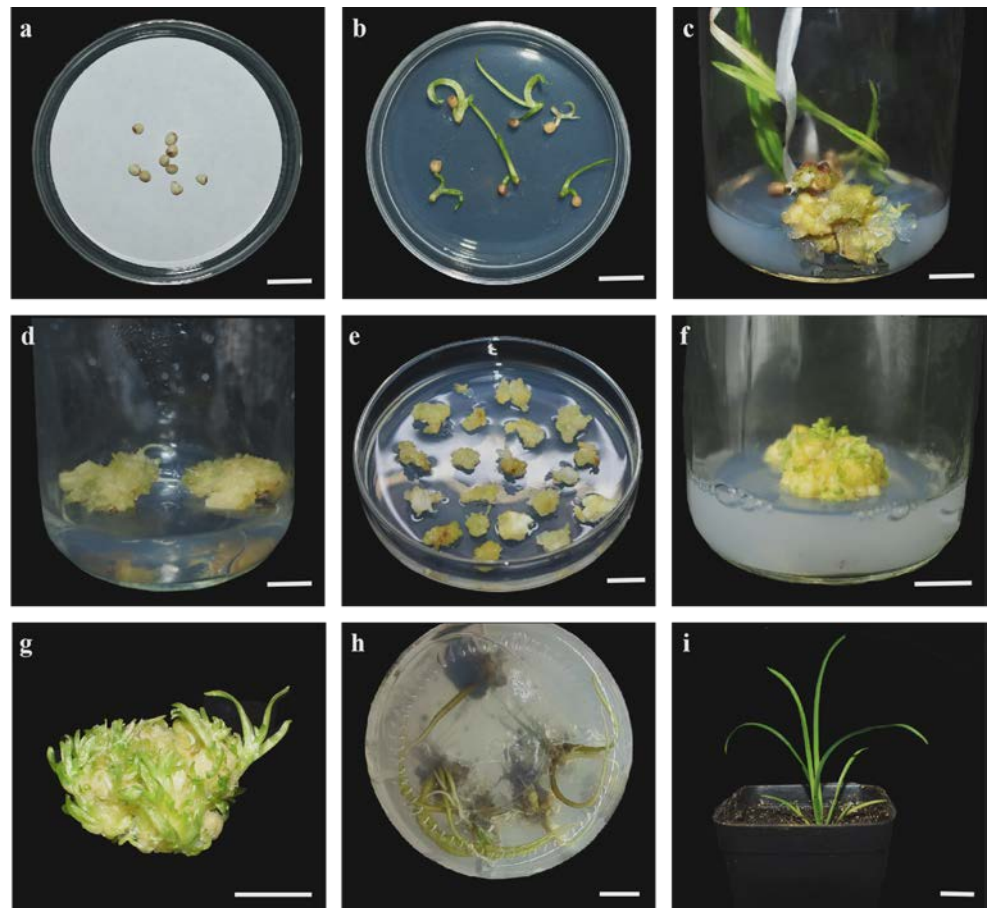


Figure 2. Regeneration process of *H. middendorffii* plantlets from seeds. Seeds removed from both the inner and outer seed coats (a). Germination status of seeds after 14 days (b). Calli induced from seedlings after 6 weeks of cultivation on the callus induction medium (c). Proliferation of calli on the callus proliferation medium (d). Initiation of shoot formation from calli on the differentiation medium (e). Progress of shoot induction after 3 weeks (f). Development of shoots after 5 weeks (g). Root initiation observed in a single shoot on the rooting medium after 1 month (h). Plantlets being acclimatized in pots (i). Scale bar = 1 cm.

3.1.2. Establishment of the Callus Induction and Shoot Induction Protocol

To develop a highly efficient *Agrobacterium*-mediated genetic transformation protocol for *H. middendorffii*, eighteen different medium formulations were created (six 6-BA concentrations and three NAA concentrations) to stimulate callus formation from the aerial parts of seed-derived plantlets. After cultivation for one month, the initiation of calli was noted (Figure 2c). It was observed that 6-BA at lower concentrations slowed down the callus formation process, whereas NAA had a weak effect on the induction of calli, as shown in Table 4. The callus induction rate was 28.9% under the addition of $1.5 \text{ mg} \cdot \text{L}^{-1}$ 6-BA and $0.3 \text{ mg} \cdot \text{L}^{-1}$ NAA, whereas a considerably higher induction rate of 95.6% was attained with $3.5 \text{ mg} \cdot \text{L}^{-1}$ 6-BA and $0.1 \text{ mg} \cdot \text{L}^{-1}$ NAA. These results indicated that a synergistic effect involving higher concentrations of 6-BA and lower concentrations of NAA significantly boosted callus formation in *H. middendorffii*. Then, 2,4-D was incorporated into the subculture medium to promote the proliferation of calli (Figure 2d). Initially, the induced calli exhibited a compact and nodular morphology with a yellow and calloused appearance. As callus proliferation progressed, the texture became soft and friable, and the color transitioned to a pale yellow.

Table 4. Effect of 6-BA and NAA concentrations on the callus induction from *H. middendorffii*. The number of explants used for each treatment was 30.

6-BA (mg·L ⁻¹)	NAA (mg·L ⁻¹)	Number of Calli per Medium	Induction Rate (%)
1.5	0.1	11.3 ± 1.5 efg	37.8 ± 5.1 efg
1.5	0.2	13.7 ± 2.5 ef	45.6 ± 8.4 ef
1.5	0.3	8.7 ± 2.1 g	28.9 ± 6.9 g
2.0	0.1	15.7 ± 1.5 def	52.2 ± 5.1 def
2.0	0.2	15.3 ± 1.5 def	51.1 ± 5.1 def
2.0	0.3	12.7 ± 1.5 ef	42.2 ± 5.1 ef
2.5	0.1	18.3 ± 1.5 cd	61.1 ± 5.1 cd
2.5	0.2	15.7 ± 3.0 de	52.2 ± 1.0 de
2.5	0.3	14.7 ± 2.1 de	48.9 ± 6.9 de
3.0	0.1	26.3 ± 1.5 ef	87.8 ± 5.1 ef
3.0	0.2	24.0 ± 1.7 bc	80.0 ± 5.8 bc
3.0	0.3	21.0 ± 2.7 bc	70.0 ± 8.8 bc
3.5	0.1	28.5 ± 5.8 a	95.6 ± 1.9 a
3.5	0.2	26.7 ± 2.5 b	85.6 ± 5.1 b
3.5	0.3	22.7 ± 2.1 bc	75.6 ± 6.9 bc
4.0	0.1	25.3 ± 1.5 bc	84.4 ± 5.1 bc
4.0	0.2	21.0 ± 2.7 cd	70.0 ± 8.8 cd
4.0	0.3	22.7 ± 2.1 bc	75.6 ± 6.9 bc

6-BA: 6-benzylaminopurine, NAA: naphthaleneacetic acid. Different lowercase letters within the same column indicate significant differences at the 5% level based on Duncan's multiple range test ($p < 0.05$).

The effect of 6-BA at varying concentrations on shoot induction was assessed by altering its concentration in the callus induction medium. When the NAA concentration was 0.2 mg·L⁻¹, the regeneration rate of shoots decreased as the 6-BA concentration increased from 1.0 to 3.0 mg·L⁻¹. Additionally, 0.5 mg·L⁻¹ 6-BA also yielded a relatively high growth rate (Table 5). It was also observed that 6-BA and NAA at low concentrations not only promoted the growth of small shoots but also facilitated their formation and proliferation. Consequently, the optimal medium for shoot regeneration was determined to be MS medium supplemented with 1.0 mg·L⁻¹ 6-BA and 0.2 mg·L⁻¹ NAA (Figure 2e–g).

Table 5. Effect of 6-BA and NAA concentrations on the shoot induction from *H. middendorffii*. The number of calli used for each treatment was 30.

6-BA (mg·L ⁻¹)	NAA (mg·L ⁻¹)	Number of Calli Forming Shoots	Regeneration Rate (%)
0.5	0.2	22.0 ± 2.0 ab	73.3 ± 6.7 ab
1.0	0.2	25.3 ± 3.1 a	84.4 ± 10.2 a
1.5	0.2	18.7 ± 0.6 bc	62.2 ± 1.9 bc
2.0	0.2	15.7 ± 1.5 cd	52.2 ± 5.1 cd
2.5	0.2	12.7 ± 2.5 d	42.2 ± 8.4 d
3.0	0.2	8.3 ± 1.2 e	27.8 ± 3.9 e

6-BA: 6-benzylaminopurine, NAA: naphthaleneacetic acid. Different lowercase letters within the same column indicate significant differences at the 5% level based on Duncan's multiple range test ($p < 0.05$).

After a 5–6-week period of shoot induction culture, the individually separated plantlets were moved to a rooting medium. The initiation of rooting commenced on the 15th day. By the 30th day, the roots had grown to a length of 3–5 cm; then, the plantlets were transplanted into pots.

3.2. Factors Influencing *Agrobacterium*-Mediated Transformation

3.2.1. Hyg Concentration

In order to determine the optimal Hyg concentration, a sensitivity assessment was conducted, as its effectiveness varies among species. Hyg at the appropriate concentration effectively inhibits the growth of non-transformed plants and induces their gradual death, while it only slightly impairs the normal growth of transformed recipients.

The calli were cultured on pre-cultivation medium containing Hyg at varying concentrations. There was significant variation in the browning rate of calli. Specifically, Hyg at concentrations of 9 and 12 mg·L⁻¹ effectively hindered the differentiation of calli, leading to their browning and subsequent death (Figure 3a). Consequently, 9 mg·L⁻¹ Hyg was determined as the threshold concentration for effectively screening calli in the genetic transformation process.

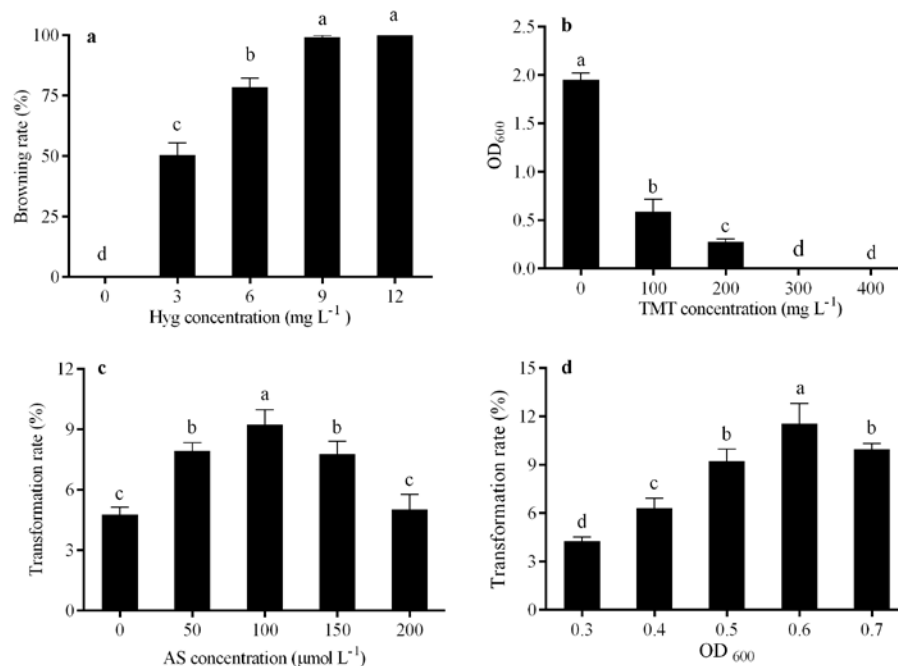


Figure 3. Effect of hygromycin (Hyg) concentration on the callus browning rate (a). Impact of Timentin (TMT) concentration on the *A. tumefaciens* culture solution optical density at 600 nm (OD₆₀₀) (b). Effect of OD₆₀₀ value on the transformation rate (c). Influence of acetosyringone (AS) concentration on the transformation rate (d). Hyg, hygromycin; TMT, Timentin; AS, acetosyringone. Different lowercase letters indicate significant differences at the 5% level based on Duncan's multiple range test ($p < 0.05$).

3.2.2. TMT Concentration

TMT was utilized as the antibiotic to suppress *A. tumefaciens* growth. After propagation culture, the bacteria were introduced into the LB liquid medium with TMT at varying concentrations for further culturing, and the change in bacterial density was monitored.

As shown in Figure 3b, the OD₆₀₀ value rose to 2.0 in the LB liquid medium without TMT supplementation. The increase in TMT concentration significantly inhibited the reproduction of *A. tumefaciens*, resulting in a pronounced decrease in OD₆₀₀ value. *A. tumefaciens* was essentially inactivated at a TMT concentration of 300 mg·L⁻¹, with the OD₆₀₀ value declining to nearly 0. Increasing the TMT concentration to 400 mg·L⁻¹ did not alter the growth dynamic of *A. tumefaciens* as compared with that at 300 mg·L⁻¹, suggesting that 300 mg·L⁻¹ was the empirically supported critical concentration of TMT.

3.2.3. AS Concentration

Numerous studies have demonstrated that AS, a small phenolic compound, can induce the activation of vir genes in *A. tumefaciens*, thereby enhancing the successful transfer of Ti plasmids into host cells [29,30]. Among the tested concentrations of AS, the maximum transformation rate of 9.2% was observed at an AS concentration of $100 \mu\text{mol}\cdot\text{L}^{-1}$. Concentrations above $100 \mu\text{mol}\cdot\text{L}^{-1}$ yielded lower transformation rates (Figure 3c). Furthermore, AS at high levels was found to disrupt the normal growth of the recipients, leading to the excessive growth of *A. tumefaciens* and browning of the recipients. Therefore, $100 \mu\text{mol}\cdot\text{L}^{-1}$ AS was determined to be the most effective concentration for enhancing transformation rate.

3.2.4. Bacterial Cell Density

The effect of *A. tumefaciens* infiltration solution density on the transformation rate was investigated. As shown in Figure 3d, the optimal transformation rate of 11.6% was achieved with recipients exposed to an OD_{600} value of 0.6. A slight decrease in the transformation rate was observed when the *A. tumefaciens* culture OD_{600} was further increased.

3.3. Stability Test of the *H. middendorffii* Transformation System

To assess the stability of the developed *Agrobacterium*-mediated transformation system for *H. middendorffii*, three experimental replicates were conducted using the optimal combination of genetic transformation factors (Figure 4). The results confirmed the system's stability, yielding an average transformation rate of 11.9% (Table 6). In addition, it took approximately 4.5–5.0 months to obtain transgenic *H. middendorffii* T0 rooted plants by using this system.

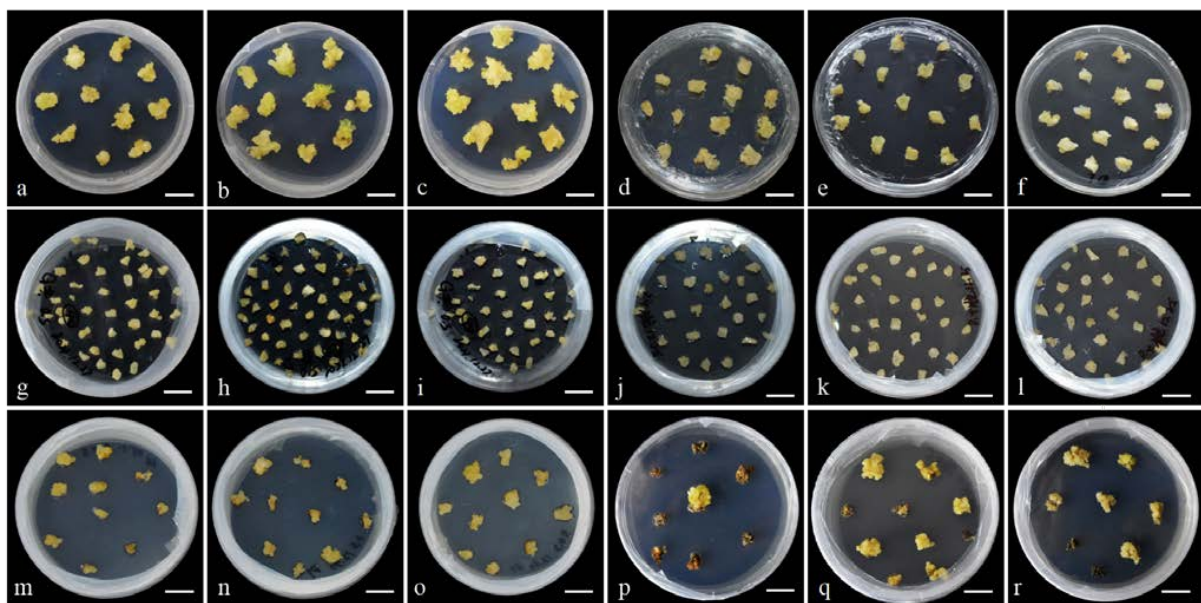


Figure 4. Genetic transformation process of *H. middendorffii*. Healthy and robust transformed recipients (a–c). Pre-cultivation on MS medium supplemented with $1.0 \text{ mg}\cdot\text{L}^{-1}$ 6-BA, $0.2 \text{ mg}\cdot\text{L}^{-1}$ NAA, $30 \text{ g}\cdot\text{L}^{-1}$ sucrose, and $7.6 \text{ g}\cdot\text{L}^{-1}$ agarose for 3 days (d–f). Co-cultivation on MS medium supplemented with $1.0 \text{ mg}\cdot\text{L}^{-1}$ 6-BA, $0.2 \text{ mg}\cdot\text{L}^{-1}$ NAA, $100 \mu\text{mol}\cdot\text{L}^{-1}$ AS, $30 \text{ g}\cdot\text{L}^{-1}$ sucrose, and $7.6 \text{ g}\cdot\text{L}^{-1}$ agarose for 2 days (g–i). Resting on MS medium supplemented with $1.0 \text{ mg}\cdot\text{L}^{-1}$ 6-BA, $0.2 \text{ mg}\cdot\text{L}^{-1}$ NAA, $300 \text{ mg}\cdot\text{L}^{-1}$ TMT, $20 \text{ g}\cdot\text{L}^{-1}$ sucrose, and $7.6 \text{ g}\cdot\text{L}^{-1}$ agarose for 5 days (j–l). Selection 1 phase on MS medium supplemented with $1.0 \text{ mg}\cdot\text{L}^{-1}$ 6-BA, $0.2 \text{ mg}\cdot\text{L}^{-1}$ NAA, $9 \text{ mg}\cdot\text{L}^{-1}$ Hyg, $300 \text{ mg}\cdot\text{L}^{-1}$ TMT, $30 \text{ g}\cdot\text{L}^{-1}$ sucrose, and $7.6 \text{ g}\cdot\text{L}^{-1}$ agarose for 15 days (m–o). Selection 2 phase for 30 days (p–r). Scale bar = 1 cm.

Table 6. Stability test of the *Agrobacterium*-mediated transformation system for *H. middendorffii*.

Replicate	Number of Explants	Transformation Rate (%)	Positive Rate (%)
1	43	11.6	25.0
2	50	12.0	33.3
3	41	12.2	40.0
Average		11.9	32.8

3.4. Validation of Transgenic Plants

The transgenic status of regenerated plants was initially confirmed by PCR amplification using 35S sequence-specific primers. Following hygromycin selection, a total of 16 candidate positive plants were obtained and designated as lines 1–15. After differentiation, each line produced at least two replicates. Through three independent experimental replicates, PCR products corresponding to the 801 bp 35S sequence fragment were successfully amplified (Figure 5a). The PCR products were derived from transgenic lines 1-1, 1-2, 1-3, 7-1, 7-2, 8-1, 8-2, 11-2, and 13-2, resulting in a positive line rate of 32.8%. Lines 2, 3, 4, 5, 6, 9, 10, 12, 14, and 15 were identified as false positives. The Sanger sequencing validation results are presented in Supplementary Figure S2.



Figure 5. PCR analysis and phenotypic status of wild-type (WT) and transgenic *H. middendorffii*. PCR amplification of the 35S sequence (801 bp) in WT plant and five transgenic lines (a). Growth state of WT and pCAMBIA1300-35S-*HmFT* transgenic plants (b). Lane M, DNA maker; Lanes 1-1, 1-2, 1-3, 7-1, 7-2, 8-1, 8-2, 11-2, and 13-2, transgenic lines; Lane P, plasmid control, Lane WT, wild-type plant.

The growth status of the *H. middendorffii* plantlets indicated that the length and the number of lateral roots in WT plants were significantly lower than those in pCAMBIA1300-35S-*HmFT* transgenic plants under the same growth conditions (Figure 5b). Moreover, the root growth ability of the transgenic pCAMBIA1300-35S-*HmFT* plants was enhanced (Supplementary Table S2).

The qRT-PCR results indicated that the expression levels of *HmFT* in transgenic plants were significantly upregulated compared with WT plants (Figure 6). The relative expression level in the 13-2 transgenic line was 100 times higher than that in WT plants. These results demonstrated that pCAMBIA1300-35S-*HmFT* expression vector plasmid was successfully introduced into the *H. middendorffii* genome and expressed.

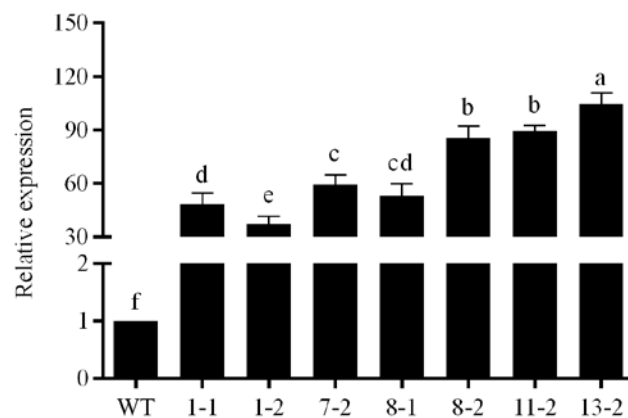


Figure 6. Expression levels of *HmFT* in leaves of wild-type (WT) and transgenic *H. middendorffii* plants by qRT-PCR. The expression level of *HmFT* in WT plants used as a control. Transgenic lines: 1-1, 1-2, 7-2, 8-1, 8-2, 11-2, and 13-2. The *Actin* gene, amplified using primers based on the *H. fulva* gene sequence, was used as the internal reference gene. The expression level of *HmFT* gene in WT plant was used as a control. Different lowercase letters indicate significant differences at the 5% level based on Duncan's multiple range test ($p < 0.05$).

4. Discussion

Plant tissue culture is widely employed in large-scale plant reproduction, virus elimination, secondary metabolite production, and in vitro cloning [31,32]. Extensive studies have shown that the healing rates of *Hemerocallis* flower organs, including flower stems, pedicels, and petals, were superior to those of other parts in tissue culture [17]. However, the act of cutting for explant collection can activate polyphenol oxidases in these tissues, which oxidize phenolic substances into brownish quinone compounds, leading to the browning at the cut sites of the explants. This phenomenon may inhibit normal explant development or even lead to explant death [33,34]. In this study, the aerial parts of seed-derived *H. middendorffii* plantlets were utilized as explants, which acted as excellent hosts for *Agrobacterium*-mediated genetic transformation. Furthermore, the removal of both the inner and outer seed coats reduced the contamination rate. Meanwhile, this process eliminated the potential influence of trace phenolic compounds within the seed coats, rendering it the optimal source for *H. middendorffii* tissue culture materials. Moreover, before the seed coats were removed, the seeds were soaked in distilled water for 36–48 h. When the soaking time was shorter than 36 h, the seed coats were insufficiently softened, which hindered seed coat removal. When the soaking time was longer than 48 h, the seeds showed signs of germination and were susceptible to physical damage during the sterilization process, which affected the subsequent normal germination.

Various studies have underscored the dramatic effect of exogenous PGRs in stimulating and inducing morphogenesis [35–37]. The combined action of cytokinin and auxin enhanced in vitro plant regeneration [38,39]. In *Dianthus chinensis*, the application of $1.0 \text{ mg} \cdot \text{L}^{-1}$ 6-BA along with $0.06 \text{ mg} \cdot \text{L}^{-1}$ NAA led to a higher leaf regeneration rate and the highest observed explant induction rate [9]. The optimal shoot induction in Indian red banana was obtained with $3.0 \text{ mg} \cdot \text{L}^{-1}$ 6-BA and $0.2 \text{ mg} \cdot \text{L}^{-1}$ NAA [37]. In addition, the greatest number of branches was stimulated in *Lallemantia iberica* by application of $1.0 \text{ mg} \cdot \text{L}^{-1}$ 6-BA and $0.05 \text{ mg} \cdot \text{L}^{-1}$ NAA [31]. In the present study, MS medium was enhanced with $3.5 \text{ mg} \cdot \text{L}^{-1}$ 6-BA and $0.1 \text{ mg} \cdot \text{L}^{-1}$ NAA, leading to a notable increase in the induction rate. These results implied that effective explant induction may require a higher level of 6-BA and a lower level of NAA. Additionally, it was indicated that optimal explant differentiation might necessitate a balanced combination of lower concentrations of 6-BA and NAA.

Agrobacterium-mediated transformation is a complex process influenced by multiple factors, including the optical density of the *A. tumefaciens* culture solution and the concentrations of AS, TMT, and Hyg, among other factors [40–44]. In the present research, Hyg was used for the selection of transgenic plants, as the pCambia1300-35S expression vector contains a gene that confers resistance to Hyg, making it an effective selection marker for identification of transgenic plants. Research has demonstrated that even among different varieties of the same species, the optimal concentrations of Hyg as a selection agent can vary. For example, 7 mg·L⁻¹ Hyg was effective for further selection and regeneration of positive transformants in *Rosa chinensis* Jacq. [45]. However, research on *Rosa hybrida* demonstrated that 50 mg·L⁻¹ Hyg inhibited excessive growth of calli, and 20 mg·L⁻¹ Hyg had no dramatic impact on the later growth stages of the plants [46]. In the present study, 9 mg·L⁻¹ Hyg efficiently eliminated most of the non-transformants and was identified as the threshold for screening transgenic *H. mitterdorffii* plants.

TMT effectively inhibits *A. tumefaciens* and is widely used in plant in vitro culture [47,48], with optimally effective concentrations of 300–500 mg·L⁻¹. In the present protocol, 300 mg·L⁻¹ TMT was added to both the sterilization medium and subsequent screening medium. It effectively inhibited *A. tumefaciens* growth, eliminated other bacterial contaminants, and prevented the contamination of *H. mitterdorffii* calli and regenerative shoots.

Previous research has indicated that AS can significantly increase the transformation rate of various plant species across a broad concentration range of 20–100 µmol·L⁻¹ [49–52]. For instance, 50 µmol·L⁻¹ AS was effective for the transformation of *Cyclamen persicum* [53], and 100 µmol·L⁻¹ AS was identified as the optimal co-cultivation agent for cucumber transformation [54]. These results are consistent with previous findings in *Tagetes erecta* and *Typha latifolia* [55,56]. Similarly, this research revealed that the highest transformation rate for *H. mitterdorffii* was achieved when the AS concentration in the *A. tumefaciens* culture solution and co-cultivation medium was 100 µmol·L⁻¹.

Furthermore, the optical density of the *A. tumefaciens* solution exerts a crucial impact on the transformation rate [54]. When the concentration is too low, *A. tumefaciens* cannot sufficiently adhere to recipients, leading to ineffective transformation. Conversely, when the concentration is too high, it can cause severe damage to the recipients [57]. In the present study, it was found that the transformation rate was promoted and the transformed recipients were relatively healthier and more robust as the OD₆₀₀ value was increased from 0.3 to 0.6. However, when OD₆₀₀ value exceeded 0.6, most of the transformed recipients exhibited browning and death, which could be attributed to the excessively high concentration of the infection solution [42].

In this study, PCR amplification using 35S promoter-specific primers successfully detected the 801 bp target fragment in the transgenic lines (Figure 5a). The absence of amplification in WT plants confirmed primer specificity. Further validation by qRT-PCR revealed that *HmFT* expression in transgenic lines was significantly upregulated compared to WT plants (Figure 6). Line 13-2 exhibited the highest expression level (100-fold increase), consistent with its enhanced root growth (Supplementary Table S2). These results preliminarily validate the possible utility of the established transformation system for functional genomics and trait improvement in *H. mitterdorffii*. To further confirm transgene functionality at the protein level, future studies will include Western blot analysis using *HmFT*-specific antibodies to detect HmFT protein accumulation in T0 plants. Additionally, Southern blot analysis of T0 plants will be performed to verify the stable integration of the transgene into the plant genome, providing critical evidence of transformation stability prior to T1 generation analysis.

5. Conclusions

In this study, an efficient and reliable protocol was developed for the regeneration and callus genetic transformation of *H. middendorffii* using the aerial parts of seed-derived plantlets as explants. By optimizing the callus induction, proliferation, subculture, differentiation, and rooting conditions, a callus induction rate of 95.6% and a regeneration rate of 84.4% were achieved. Based on this procedure, a refined *Agrobacterium*-mediated transformation system for *H. middendorffii* realized the generation of transgenic plants within a 5-month period, with the transformation rate reaching up to 11.9% and the positive rate of 32.8%. The established *Agrobacterium*-mediated transformation system for *H. middendorffii* lays a solid foundation for the genetic modification and breeding of this valuable ornamental species. Furthermore, this genetic transformation system is a robust and versatile tool for gene function and editing research in *H. middendorffii*, thereby facilitating research on trait enhancement, functional genomics, and metabolite engineering in this species.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae11040417/s1>, Figure S1: Sequence of *HmFT* gene from *H. middendorffii*; Figure S2: Sanger sequencing result using the 35S sequence-specific primers; Table S1: Sequences of primers used in the present study; Table S2: Statistics on the root growth of WT and transgenic *H. middendorffii*.

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Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. We sincerely hope that readers can further develop a rapid and efficient system for adventitious shoot regeneration based on our research.

Conflicts of Interest: The authors declare that there are no conflicts of interest.

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