# Establishment of efficient callus genetic transformation system for Hemerocallis fulva ‘Kanai’

**Jinxue Du** ( 1191579921@qq.com )
Northeast Agricultural University
**Jingbo Shi**
Northeast Agricultural University
**Yiliang Pan**
Northeast Agricultural University
**Nan Zhang**
Northeast Agricultural University
**Fazhan Hou**
Northeast Agricultural University
**Yingzhu Liu**
Northeast Agricultural University
**Wei Liu**
Northeast Agricultural University

**Research Article**

**Keywords:** Hemerocallis fulva ‘Kanai’, Regenerative,Transformation, Seed, Agrobacterium tumefaciens

**Posted Date:** December 13th, 2022

**DOI:** https://doi.org/10.21203/rs.3.rs-2349792/v1

# Abstract

*Hemerocallis* is regarded as a model plant for future breeding because of its high ornamental value and strong resistance. However, there are few reports on regeneration and genetic transformation of this plant. In this study, mature seeds of *Hemerocallis fulva* 'Kanai' were used as explants for regeneration and *Agrobacterium* infection. The results showed that the germination rate of explants with inner and outer seed coats peeled was 98% and the contamination rate was only 11%. The callus induction efficiency of MS medium supplemented with 3.5 mg•$L^{-1}$ 6-Benzylaminopurine (6-BA) and 0.1 mg•$L^{-1}$ 1-naphthylcetic acid (NAA) was 95.2%. Based on this, transformation was successfully achieved using the following protocol: callus were soaked in *Agrobacterium tumefaciens* EHA105 ($OD_{600}$ = 0.6) containing pCambia1300-35S-FT and pCambia1300-35S-GUS plasmid for 15 min. After 3 days co-culture with 100 uM Acetosyringone (AS) in MS medium, it was transferred to MS medium containing 300 mg•$mL^{-1}$ Timentin for 5 days. The Transgenic plants were obtained by hygromycin (9 mg•$mL^{-1}$) screening. The presence of transgenic plants was confirmed by histochemical GUS detection and PCR (Polymerase chain reaction). Overall, the establishment of this efficient regeneration and genetic transformation will contribute to the functional gene research and genetic improvement of *Hemerocallis fulva* 'Kanai'.


# Introduction

Plants in *Hemerocallis* set flowering, leaf viewing in one, which are resistant to cold, drought, barren, widely used in landscaping (Kong and Zhang 2021; Liu et al. 2021; Ren et al. 2017; Rodriguez-Enriquez and Grant-Downton 2013). Moreover, the demand for it is also increasing due to its medicinal and edible value development (Lin et al. 2011; Matraszek-Gawron et al. 2019; Matsumoto et al. 2016). Therefore, *Hemerocallis* breeding has broad application prospects and high economic value and is known as a model plant for future breeding (Rodriguez-Enriquez and Grant-Downton 2013; Tomkins et al. 2001). However, there are some defects of *Hemerocallis* in China such as low reproduction coefficient, long cycle, short single flowering period (Roger Joseph Sauve 2003) and lack of fragrance, which seriously limit the promotion of *Hemerocallis*. In order to solve and get rid of this situation, breeders strive to change these traits through regulatory mechanisms including genetic improvement and gene modification (Gong 2020; Liu et al. 2019; Liu et al. 2021; Ren et al. 2019; Wang 2013). In this context, tissue culture and genetic transformation constitute important tools for gene functional studies and for specific improvement of individual traits without loss of the existing characteristics (Zhang et al. 2021). In 1959, Gautheret first used tuberized roots of Daylily as explants for value-added culture, but the value-added efficiency was low and growth was slow. In recent decades, studies of regeneration systems began to be gradually reported, such as callus induction and in vitro plant using Daylily stem tissues and leaves bud (Matand et al. 2020), *Hemerocallis fulva* petal explants (Heuser 2011), and *Hemerocallis middendorfii Trautv* scapes, steams and ovaries. However, the above explants are unable to form a widely used *Hemerocallis* tissue culture system due to the seasonal restrictions and aseptic culture difficulties (Liu 2016). In this study, an efficient and stable regeneration system was established by removing *Hemerocallis fulva*'Kanai' seeds from internal and external seed coats as explants.

As a perennial monocotyledon, *Hemerocallis* is rarely reported on the genetic transformation system due to the complex genetic background and heterozygote of interspecific hybridization (Jin et al. 2012). So far, the methods of genetic transformation of *Hemerocallis* included gene gun method, pollen tube channel method and *Agrobacterium*-mediated method (Roger Joseph Sauve 2003; Zheng et al. 2015), among which the gene gun bombardmentis expensive and unstable. The transformation rate of transgenic *Hemerocallis* (Zhong et al. 2020) obtained by pollen tube channel method is relatively low, which is not conducive to wide application. *Agrobacterium*-mediated method has the advantages of simple operation, high transformation rate, good repeatability and low experimental cost. Therefore, *Agrobacterium*-mediated method is the most widely used plant transgenic method now. In previous studies on genetic transformation of *Hemerocallis*, callus induced by stem tips of *Hemerocallis* were usually used as explants for genetic transformation. To date, the regulating color gene *CHS*, salt tolerance gene *LcChi2* and green fluorescent protein (GFP) (Gao 2012) reporter gene have been successfully integrated in *Hemerocallis* (Liu 2016; Wang 2013). However, due to the low differentiation efficiency, low transformation rate and seasonal restriction, the application of genetic engineering in the directional breeding of new species of *Hemerocallis* was limited. Therefore, it is significant to improve the transformation rate of *Hemerocallis* mediated by *Agrobacterium tumefaciens*.

In this study, we first optimized several factors affecting in vitro regeneration of *Hemerocallis fulva* 'Kanai' including explant type, explant treatment and hormone concentration. The induction rate was 95.2%, which provides a sufficient source of receptors for *Agrobacterium*-mediated gene transformation. In addition, we used *GUS* as the reporter gene and *FT* as the target gene to establish an efficient *Agrobacterium*-mediated genetic transformation system for callus. The establishment of this transformation system will promote genetic improvement and functional gene studies of *Hemerocallis fulva* 'Kanai'.

# Materials And Methods

### Overexpression vector construction and *Agrobacterium* strain

Refer to previous transcriptome data of the research group, full-length cDNA of the *FT* gene with the open reading frame (ORF) of *FT* was cloned from total RNA of *Hemerocallis fulva* 'Kanai' leaves using RT-PCR. We utilized the binary pCambia1300-35S plasmid vector containing a hygromycin B phosphate transferase gene as a selection marker. The *FT* gene sequence was connected overnight to the plasmid carrier 35S by T4 ligase at the KpnI and PstI enzyme cutting sites. In addition, the *GUS* gene sequence enzymes digested on the pBI121 vetor were attached to the pCambia1300-35S vetor. The reconstructed pCambia1300-35S-FT and pCambia1300-35S-GUS overexpression vector was introduced into *Agrobacterium tumefaciens* strain EHA105 (Tsingke Biotechnology, Beijing).

### Media preparation and surface seed sterilization

Cleaned *Hemerocallis fulva* 'Kanai' seeds were soaked in distilled water for 36-48h at room temperature and were set in three modes: reataining the seed coats, removing the outer seed coats and removing the

inner and outer seed coats. First seeds was sterilized by 75% ethanol for 30 S, and cleaned with sterile water 2-3 times for 2 min each time. Subsequently, they were disinfected with 3% sodium hypochlorite shock for 20 min. Eventually seeds were rinsed with sterile water 5-6 times for 2 min each time to completely remove sodium hypochlorite. Sterilized seeds germinated and grew in MS medium (Toshio Murashige 1962) including 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 mg•$L^{-1}$ agar at 22 ± 2 ℃ in the dark. Each experiment was repeated at least three times, with each medium containing 6 inoculated seed explants. All the culture media used in this study were adjusted to PH 5.9 before autoclaving at 1.0 kg•$cm^{-2}$ at 121 ℃ for 20 min.

### Calluses induction and subculture

To investigate the efficiency of callus induction, the above explants was transferred to MS medium containing 6-BA (1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg•$L^{-1}$) and NAA (0.1, 0.2 and 0.3 mg•$L^{-1}$). After the appearance of callus around the explants, small pieces of callus with good growth status were isolated and transferred to MS proliferation medium, including plant growth regulators required for optimal induction medium and 0.1 mg•$L^{-1}$ 2,4-D. Induction and proliferation of the callus were performed at 22 ± 2 ℃ and in the dark for 15 days each subculture. Each experiment was repeated at least three times, and each culture flask contained 2-3 inoculated explants.

### Differentiation and rooting

Callus from proliferative cultures were separated into 0.5 $cm^3$ pieces and transferred into MS differentiation medium containing different concentration of 6-BA (0, 0.5, 1.0, 1.5 and 2.0 mg•$L^{-1}$) and 0.2 mg•$L^{-1}$ NAA. After subculture for 2 times, the cluster buds grew to 3-5 cm, and the single plant was separated and transferred into rooting medium. When the roots of the seedlings were 5-10 cm in length, opened the tissue culture bottle to exercise the seedlings for 3 days, washed the root agar with warm water and moved into vermiculite: carat soil: garden soil: 1:1:1 substrate, and watered once after transplanting.

### Infection and co-culture

The callus after proliferation culture was removed from yellowing and senescent parts, separated into 0.5 $cm^2$ small pieces and evenly tiled in MS induction medium for pre-culture. 3 days later, the pre-culture callus were immersed in different density of *Agrobacterium* infection solution. In this experiment, the bacterial pellet was collected after centrifuging the overnight culture solution and then resuspended with different volumes of resuspension to five density of $OD_{600}$ (0.3, 0.4, 0.5, 0.6 and 0.7), with an infection time of 15 min. After the callus was infected, the *Agrobacterium* suspension was discarded, and the callus was evenly spread on a culture dish with three pieces of filter paper at the bottom. Then, a piece of filter paper was placed on the top, and the callus was transferred to MS co-medium half an hour later. The MS medium contained various concentrations of AS, with AS concentrations set to 0, 50, 100, 150 and

200 μM. 22 ± 2℃ dark for 2 days. Each experiment was repeated at least three times with 30 samples in each dish.

### Selection culture and plant regeneration

After co-culture, they were transferred to MS differentiation medium supplemented with different concentrations of Timentine (0, 100, 200, 300 and 400 mg•$L^{-1}$) for bactericidal culture. After 5 days of growth, they were transferred to MS differentiation medium supplemented with hygromycin of different concentrations (0, 3, 6, 9, 12 mg•$L^{-1}$) to determine the threshold concentration for elimination of non-transformed plants. Clump buds grown to 3-4 cm were cut into single buds and inoculated into rooting media: 1/2MS + 0.2 mg•$L^{-1}$ NAA + 30 g•$L^{-1}$ sucrose + 6 g•$L^{-1}$ agar. Next culutred in 22 ± 2 ℃, 16/8 (light/dark) environment for 14 days. Each experiment was repeated at least three times with 10 samples per dish and 1-2 samples per culture bottle.

### Histochemical GUS assay

Histochemical GUS assay was used to initially identified putative transgenic plants using GUS staining Kit (GT0391, Cygnus, Beijing). Wild-type plants were used as control. All callus were immersed in 5 ml GUS staining solution (0.1 ml X-gluc staining solution + 5 ml GUS staining buffer) at 25-37 °C for 24h. After staining, decolorization was washed with 70% alcohol 2-3 times, until the control group was colorless. Transient GUS expression was recorded by recording the number of explants with at least one blue focus.

### Polymerase chain reaction (PCR) analysis

The presence of these transgenic plants was further identified by using a sequence on the pCambia1300-35S vector as an upstream primer and a primer downstream of the *FT* gene (F: 5'-CAACAGCTCGGAAAGCACAC-3', FT-R: 5'-TCATGTGCACATTCFCCTTC-3') and two primers for the *GUS* gene (GUS-F: 5'-TACGGCGTGGATACGTTAGC-3', GUS-R: 5'-TCATTGTTTGCCTCCCTGCT-3'). Total genomic DNA was isolated from young leaves sterile seedlings by CTAB, with non-transgenic wild-type plants as negative controls, pCambia1300-35S-FT and pCambia1300-35S-GUS plasmid vector as positive controls. PCR was performed using the E × Taq enzyme (Takara, Beijing) and 0.2 ng of DNA as a template. The PCR was set as 98 ℃ 10 s, 57 ℃ 30 s, and 72 ℃ 1 min for 35 cycles, followed by 72 ℃ for 2 min. PCR products were separated by 1% agarose gel electrophoresis and visualized in a gel graph.

## Results

### Effects of the different treatments of the explants on primary culture

After 3-5 days, the seeds started their germination. The results showed that by soaking only with 75% ethanol and 3% sodium hypochlorite, the contamination rate was as high as 90% and the germination rate was only 22%. The removal of the black leathery exotesta reduced the contamination rate to 18%,

and the germination rate was significantly increased to 70%. While removing the black leathery exotesta and brown villous endotesta, the contamination rate was significantly reduced to 11%, and the germination rate was significantly higher than that of other groups 98% (Fig. 1a-b). Therefore, removing the inner and outer seed coats during aseptic germination can significantly reduce the pollution rate and significantly increase the germination rate.

## Induction and subculture

After one month of culture, the callus started formation (Fig. 1c), which was significantly associated with 6-BA concentration, and apparently low concentrations of 6-BA slowed callus formation while NAA had less significant effect on callus formation Table 1. For the callus proliferation, 2,4-D was added to the subculture medium, which showed that the addition of 2,4-D simultaneously optimized and promoted the proliferation of the callus. The initially induced callus was mostly yellow and calloused, but became soft, pale yellow as the callus proliferation grew (Fig. 1d). The optimum MS medium for callus induction was determined to be 3.5 mg•$L^{-1}$ 6-BA and 0.1 mg•$L^{-1}$ NAA according to the induction of yellowish pure, loose granular and large callus and the proliferation medium was determined to be 4.0 mg•$L^{-1}$ 6-BA, 0.1 mg•$L^{-1}$ NAA and 0.1 mg•$L^{-1}$ 2,4-D.

**Table1** Number of callus tissues induced by seed explants.

| 6-BA mg•$L^{-1}$ | NAA mg•$L^{-1}$ | | |
|---|---|---|---|
| | 0.1 | 0.2 | 0.3 |
| 1.5 | 11.33 ± 1.53 $^{ef}$ | 13.67 ± 2.52 $^{de}$ | 8.67 ± 2.08 $^{f}$ |
| 2.0 | 15.67 ± 3.06 $^{de}$ | 15.33 ± 1.53 $^{de}$ | 12.67 ± 1.53 $^{ef}$ |
| 2.5 | 15.67 ± 3.21 $^{de}$ | 18.33 ± 1.53 $^{cd}$ | 14.67 ± 3.21 $^{de}$ |
| 3.0 | 26.33 ± 1.53 $^{ab}$ | 23.67 ± 2.31 $^{ab}$ | 21.00 ± 3.00 $^{bc}$ |
| 3.5 | 27.00 ± 1.00 $^{a}$ | 25.67 ± 1.53 $^{ab}$ | 22.67 ± 2.08 $^{abc}$ |
| 4.0 | 25.33 ± 1.53 $^{ab}$ | 24.00 ± 2.00 $^{ab}$ | 21.00 ± 2.65 $^{bc}$ |

Means with only the same letters are not signifcantly diferent at the 5% level based on Duncan's multiple range test ($p \leq 0.05$).

## Differentiation and rooting

Callus isolated into small pieces, who was cultured on MS medium with different concentrations of 6-BA (Fig. 1e), developed budlets after 14 days. When the concentration of NAA was 0.2 mg•$L^{-1}$, the shoots would grow faster as 6-BA concentration decreased from 3.0 to 1.0 mg•$L^{-1}$, but the speed of plant growth would slow down when 6-BA concentration belove 0.5 mg•$L^{-1}$ (Fig. 1j). The MS medium with 0.2 mg•$L^{-1}$

NAA and 1.0 mg•$L^{-1}$ 6-BA could not only stimulate shoot growth, but also promote formation and proliferation of small shoots. Therefore, the MS medium with 0.2 mg•$L^{-1}$ NAA and 1.0 mg•$L^{-1}$ 6-BA were demonstrated to be the best medium formulation for shoot growth (Fig. 1f-g). Roots began to generate on the 15th day. After 30 days, roots developed to 3-5 cm in length and 4 or 5 in number (Fig. 1h). The seedings were transferred to the folwerpot after proper training (Fig. 1i), and the survival rate of transplanting reached 100%.

## Genetic transformation

Five levels of the *Agrobacterium tumefaciens* concentrations $OD_{600}$ (0.3, 0.4, 0.5, 0.6 and 0.7) were measured. Calluses with good growth status were selected as the *Agrobacterium*-mediated transformation receptor (Fig. 3a-c). During the period of continuous slight shock, it is necessary to ensure that the bacterial solution fully into the transformation receptor but also to avoid damage to it due to excessive shock. The infected callus is sucked dry of the surface *Agrobacterium* solution and transferred to the co-media (Fig. 3d-f). The results show that when the density of the bacteria ($OD_{600}$) increased from 0.3 to 0.6, the transformation rate increased and the callus was in good condition. However, when the density of the bacteria ($OD_{600}$) > 0.6, most of the infection callus receptsor showed browning and death, which may be caused by the excessive concentration of the infection solution (Fig. 4a).

## The effect of AS concentration on the transformation efficiency

Many studies demonstrated that small phenolic inducer molecules AS can induce the expression of the *vir* genes, in the cause of promoting the successful transformation of the *Agrobacterium* Ti plasmid into the host cells (Krishnamohan et al. 2001; Veluthambi et al. 1989). Therefore, the effect of AS concentration on the transformation efficiency was investigated. Among the tested concentrations of AS, the transformation rate enhanced with the increasing of AS concentration up to 100 uM, while the higher concentrations of AS (>100 uM) decreases the transformation rate. The size of the AS concentration did not significantly affect the transformation rate, but the transformation rate was better when the concentration was 100 uM (Fig. 4b). Higher concentrations of AS can affect the normal growth of receptor tissues, such as causing the overgrowth of *Agrobacterium* and the browning of explants.

## Optimization of Timentin concentration

In this study, Timentin was selected as the antibiotic for inhibiting the growth of *Agrobacterium*. The co-cultured calluses were desterilized on media supplemented with different concentrations of Timentin. In this process, all the infected explants in the control group (0 concentration of Timentin) showed a large area of *Agrobacterium* growth circle on one side of the culture medium, resulting in the browning and death of the explants. When the concentration of Timentin increased by 250 mg•$L^{-1}$ and 300 mg•$L^{-1}$, the growth of *Agrobacterium* was significantly inhibited and the contamination rate was significantly reduced (Fig. 4c). However, When the concentration of Timentin was > 300 mg•$L^{-1}$, the explants showed serious

yellowing and were on the verge of death. That is, the critical concentration of Timentin bacteriostatic is selected to be 300 mg•$L^{-1}$.

### Hygromycin tolerance assay

A sensitivity assessment was performed in order to find the optimal concentration of hygromycin, since it varies from species to species. Appropriate hygromycin concentrations can promote the growth of positively transformed plants and inhibit non-transformed plants. Transformation receptors after five days of sterilizing culture were evenly tiled on differentiation media containing Timentin 300 mg•$L^{-1}$ and different concentrations of hygromycin. In the control group (0 concentration of hygromycin), all explants grown normally and were light yellow. When the concentration of hygromycin increased to 9 mg•$mL^{-1}$, some explants stopped growing and their tissues turned brown within 30 days, speculated to be receptors not successfully transferred into genes, but most survived and in good condition may be positive lines. However, when the hygromycin concentration > 9.0 mg•$L^{-1}$, the condition of the explants was poor, and they gradually turned white and stopped growing after ten days, indicating that excessive concentrations of hygromycin had a lethal effect on explants (Fig. 4d). Therefore, a concentration of 9 mg•$L^{-1}$ is the effective level of hygromycin to select a recognized transgenic plant.

### Confirmation of transgenic plants

For preliminary identification of transgenic plants, GUS staining of co-cultured transformation receptors showed that GUS staining (Fig. 5b-f) was detected at the protein expression level of the successful transformation receptor, while no obvious GUS staining was observed in non-overexpressed gene *GUS* receptors. However, some of the non-transformed receptors still show slight blue spots (Fig. 5a), presumably due to too long staining time and insufficient decolorization treatment.

Numerous regenerative buds were formed during the regeneration of the same transformation receptor in *Hemerocallis fulva*'Kanai'and separated into single plants for subculture. The leaf tissue DNA from the fused T0 transgenic plants was extracted by CTAB, and the transgenic status of the regenerated buds was further examined by PCR amplification using primers specific for the *FT* and *GUS* gene. In three stability tests, PCR product of *FT* gene (801bp) and *GUS* gene (300bp) were amplified from strains 1-1, 1-2, 1-3, 7-1, 7-2, 8-1, 8-2, 11-2, 13-2 and 13-3 in 1, 7, 8, 11, 13, and were named #1-10, respectively (Fig. 5g-h), had a transformatin rate of up to 12%. The results showed that transformation did not occur throughout the receptor and that protein expression was detected only in a fraction of it.

## Discussion

Plant tissue culture is used for large-scale plant reproduction, virus elimination, secondary metabolite synthesis, and in vitro cloning (Ebrahimzadegan and Maroufi 2022; Haida et al. 2022). Many studies have shown that the healing rate of *Hemerocallis* flower organs in tissue culture is higher than that of other parts, and the flower stem, scape, pedicel and petals are commonly used as explants (Wang 2013).

However, the above explants are restricted by the growing season, during which the plants contain more phenolic compounds and have high activity, which are easy to be released from the section of the explants (Das and Rahman 2016; Lux-endrich et al. 2000). This may be a natural defense mechanism for plants against pathogen invasion through wounds. The release of phenols from the sections into the medium has phytotoxic effects and the oxidative products may not only lead to explant development failure, but also to explant death (Uppeandra Dhar 1999). In this study, we used *Hemerocallis fulva* 'Kanai' seeds from different treatments as explants, which formed the same genetic basis for callus, serving as a good receptor for *Agrobacterium*-mediated genetic transformation. In addition, stripping the seeds inner and outer seed coats are prone to low contamination rate, while excluding the influence of small amounts of phenolic compounds that may be contained in the seed coat, which can be used as the best explant for tissue culture of *Hemerocallis fulva* 'Kanai'.

The results of many studies have proved that exogenous plant hormones play an important role in stimulating and inducing morphogenesis (Idayat Gbadamosi 2010; Preeti Rajoriya 2018; Siddique et al. 2013). The combination of cytokinin and auxin promotes plant regeneration in vitro (Geert-Jan De Klerk 2001; Huang et al. 2022). Here, we added 3.5 mg•$L^{-1}$ 6-BA and 0.1 mg•$L^{-1}$ NAA to the MS medium, which increased the explants induction rate to 95.2%, indicating that the induction of the explants may require high concentrations of 6-BA and low concentrations of NAA. Furthermore, when 6-BA concentration was 1.0 mg•$L^{-1}$, increasing of NAA concentration promoted the differentiation of explants, and when NAA concentration was 0.2 mg•$L^{-1}$, increasing 6-BA concentration inhibited exant differentiation, suggesting that differentiation of explants may require a combination of lower 6-BA and relatively higher concentrations of NAA (Yan et al. 2019).

*Agrobacterium* are major tools in plant research through overexpression and downregulation of specific genes (Wen et al. 2022). However, *Agrobacterium*-mediated transformation is also a complex process, where many factors potentially influence its efficiency, including the optical density of *Agrobacterium*, AS, Timentin and hygromycin concentration (Chen et al. 2020; Chhabra et al. 2011; CONFALONIERI et al. 1994; Yuan et al. 2009; Wang et al. 2021). In this study, the explants were infected with *Agrobacterium* at $OD_{600}$ = 0.6 for 15 min and co-cultured for 2 days, with the highest infection frequency. However, *Agrobacterium* concentrations higher than 0.6 ($OD_{600}$) significantly decreased the number of callus overexpressing *FT* and *GUS* activity due to the hypersensitive response of callus and also difficulties in the elimination of bacteria after co-culture (Chhabra et al. 2011). Secondly, the calluses used in pre-culture require complete resection of the differentiation site including the growth point, and the residue of the differentiated tissue leads to the emergence of a large number of false positive plants, causing difficulties in screening the transgenic plants. The pre-culture callus was immersed in different concentrations of infestation solution for 15 min, and then dried the *Agrobacterium* solution on the surface with filter paper to slow down the inhibition of *Agrobacterium* on the growth of recipient plants in the subsequent culture process.

Several studies have shown that the use of appropriate concentrations of AS during co-culture improves transformation efficiency (Ammara Ahad 2014; Li et al. 2017; Movahedi et al. 2014). In this experiment, adding different concentrations of AS to the pre-culture and co-culture medium had no significant effect on the transformation rate, but low concentrations of AS (100uM) induced a higher transformation rate (Li et al. 2021).

Timentin has been reported to be effective in eliminating *Agrobacterium tumefaciens* from plant somatic embryos and has long been used in plant in vitro culture (Bannikova et al. 2016; Zhang et al. 2017). Timentin with concentrations ranging from 300-500 mg•$L^{-1}$ is considered to be an ideal antibiotic. We added a concentration of 300 mg•$L^{-1}$ to the sterilization media and the following screening media, which could effectively inhibit the growth of *Agrobacterium*, kill other bacteria, and prevent the contamination of callus and regenerative buds. The results of this study are consistent with those of these studies. Furthermore, hygromycin is the expression vector pCambia1300-35S resistance gene, and hygromycin served as a selection pressure to select the transformed cells. Because the degree of hygromycin sensitivity is different from the plant and the growth stage, hygromycin (9.0 mg•$L^{-1}$) was selected for the preliminary screening of transgenic plants. The differentiation and regeneration phases gradually reduce the concentration of hygromycin to 4 mg•$L^{-1}$, reducing the inhibition of bud and root growth.

# Conclusion

In conclusion, we propose an optimized protocol for *Hemerocallis fulva* 'Kanai' regeneration and *Agrobacterium tumefaciens* mediated callus transformation. Using this system, transgenic plants can be obtained within five months, with a transformation rate of up to 12%. To our knowledge, this was the first report of *Agrobacterium*-mediated transformation of *Hemerocallis* plants, providing a practical basis for the genetic transformation and breeding of this valuable garden plant. Furthermore, gene transformation provides a powerful tool for studying gene function and gene editing, which will contribute to trait improvement, functional genomics, and metabolite engineering.

# Declarations

**Author contributions** Jinxue Du and Yingzhu Liu planned and designed the research. Jinxue Du, Jinbo Shi and Nan Zhang conducted the experiments and collected the materials. Jinxue Du, Yingzhu Liu, Yiliang Pan and Jinbo Shi analysed the data. Jinxue Du,Yingzhu Liu wrote the manuscript. Yingzhu Liu and Wei Liu revised the manuscript, provided advice on the experiments and fifinalized the manuscript. All the authors have read and approved the fifinal manuscript.

**Funding** This work was supported by National Natural Science Foundation of China (32102420), Project funded by China Postdoctoral Science Foundation (2021M700739) and Natural Science Foundation of Heilongjiang Province of China (YQ2021C014).

# References

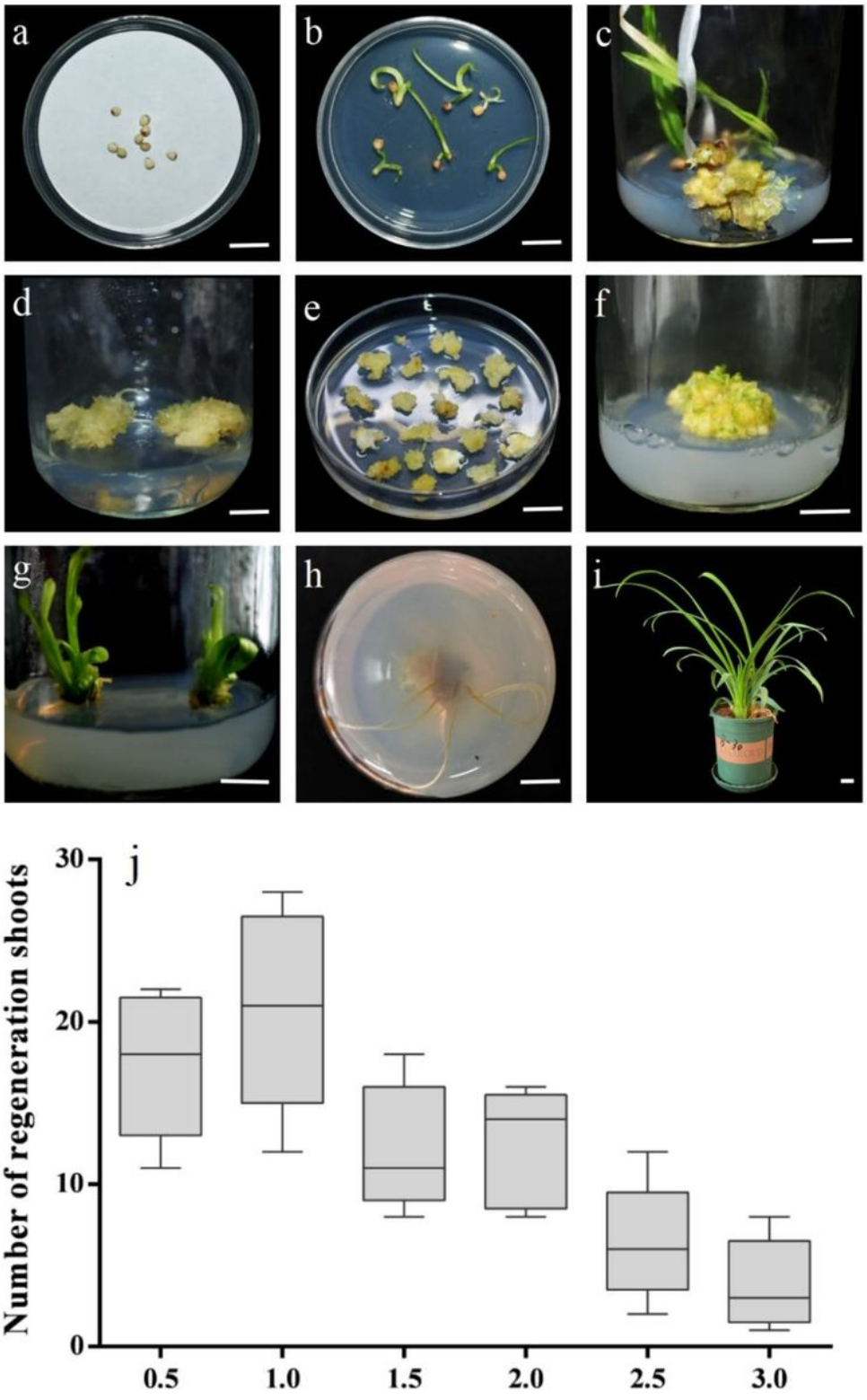
1. Ahad A, Maqbool A, Malik K (2014) Optimization of agrobacterium tumefaciens mediated transformation in eucalyptus camaldulensis. Pak J Bot 46:1079–1086
2. Aziz AN, Sauve RJ, Zhou S (2003) Genetic transformation of Stella De Oro daylily by particle bombardment. Can J Plant Sci 83:873–876. http://dio.org/10.4141/P03-025
3. Bannikova MA, Gorbatyuk IR, Hnatiuk IS, Malysheva-Otto LV, Duplij VP (2016) Effect of Ceftriaxone and Timentin antibiotics on morphogensis processes in the *in vitro* culture of bread wheat *Triticum aestivum* L. Biopolymers and Cell 32:367–376. http://doi.org/10.7124/bc.000933
4. Chen XA, He X, Zhang M, Mao XM, Li YQ (2020) An efficient genetic transformation system for Chinese medicine fungus *Tolypocladium ophioglossoides*. J Microbiol Methods 176:106032. http://dio.org/10.1016/j.mimet.2020.106032
5. Chhabra G, Chaudhary D, Sainger M, Jaiwal PK (2011) Genetic transformation of Indian isolate of *Lemna minor* mediated by *Agrobacterium tumefaciens* and recovery of transgenic plants. Physiol Mol Biol Plants 17:129–136. http://dio.org/10.1007/s12298-011-0059-5
6. Confalonieri M, Balestrazzi A, Bisoffi S (1994) Genetic transformation of *Populus nigra* by *Agrobacterium tumefaciens*. Plant Cell Rep 13:256–261. http://dio.org/10.1007/BF00233315
7. Das DK, Rahman A (2016) Phenolic Compositions of Litchi Shoot Tips and Zygotic Embryos Collected in Different Months and Their Effects on the Explant Browning and Its Control. Adv Anthropol 6:73–92. http://dio.org/10.4236/aa.2016.64008
8. Deklerk GJ, Hanecakova J, Jasik J (2001) The role of cytokinins in rooting of stem slices cut from apple microcuttings. Plant Biosystems 135:79–84. http://dio.org/10.1007/978-1-4020-5005-3_8
9. Ebrahimzadegan R, Maroufi A (2022) In vitro regeneration and *Agrobacterium-mediated* genetic transformation of Dragon's head plant (*Lallemantia iberica*). Sci Rep 12:1784. http://dio.org/10.1038/s41598-022-05776-w
10. Gao J (2012) Optimization of regeneration system and establishment of agrobacterium-mediated transformation system of daylily. Master's thesis Sichuan Agri Univ
11. Gbadamosi IT, Egunyomi A (2010) Micropropagation of Plumbago zeylanica L. (Plumbaginaceae) in Ibadan, Southwestern, Nigeria. J Med Plants Res 4:293–297
12. Gong FF (2020) Cloning and Functional Identification of Circadian Clock Gene *LHY* in *Hemerocallis* spp. Master's thesis Shanxi Agri Univ
13. Haida Z, Sinniah UR, Nakasha JJ, Hakiman M (2022) Shoot induction, Multiplication, Rooting and Acclimatization of Black Turmeric (C*urcuma caesia* Roxb.): An Important and Endangered *Curcuma* Species. Hortic 8:740. http://dio.org/10.3390/horticulturae8080740
14. Heuser CW, Apps DA (2011) In vitro plantlet formation from flower petal explants of *Hemerocallis* cv. Chipper Cherry[1]. Can J Bot 7:616–618. http://dio.org/10.1139/b76-064
15. Huang T, Zhang HH, Zhao RN, Zhu ZL (2022) Establishing an Efficient Regeneration System for Tissue Culture in *Bougainvillea buttiana* 'Miss Manila'. Plants 11:2372. http://dio.org/10.3390/plants11182372

16. Kong JY, Zhang ZG (2021) Study on the Development of Hemerocallis Culture and Its Landscape Application. IOP Conference Series: Earth and Environmental Science 768: 012082. http://dio.org/10.1088/1755-1315/768/1/012082
17. Jin LM, Zhou YZ, Guo ZH, Hang BJ (2012) Effect of inbred, hybrid and different cultivation enviroments on seed setting characteristics of Hemerocallis hybrid. Hubei Agric Sci 51:2760–2763. http://dio.org/10.14088/j.cnki.issn0439-8114.2012.13.060
18. Krishnamohan A, Balaji V, Veluthambi K (2001) Efficient *vir* Gene Induction in *Agrobacterium tumefaciens* Requires *virA*, *virG*, and *vir* Box from the Same Ti Plasmid. J Bacteriol 183: 4079–4089. http://dio.org/10.1128/JB.183.13.4079-4089.2001
19. Li SJ, Zhen C, Xu WJ, Wang C, Cheng YX (2017) Simple, rapid and efficient transformation of genotype Nisqually-1: a basic tool for the first sequenced model tree. Sci Rep 7:2638. http://dio.org/10.1038/s41598-017-02651-x
20. Li XN, Li HY, Zhao YZ, Zong PX, Zhan ZX, Piao ZY (2021) Establishment of A Simple and Efficient Agrobacterium-mediated Genetic Transformation System to Chinese Cabbage (*Brassica rapa* L. Ssp. *pekinensis*). Hortic Plant J 7:117–128. http://dio.org/10.1016/j.hpj.2021.01.006
21. Lin YL, Lu CK, Huang YJ, Chen HJ (2011) Antioxidative Caffeoylquinic Acids and Flavonoids from *Hemerocallis fulva* Flowers. J Agric Food Chem 59:8789–8795. http://dio.org/10.1021/jf201166b
22. Liu YF (2016) The Establishment of *Hemerocallis Middendorfii* Callus Induction and *Agrobacterium* Mediated Genetic Transofrmation System. Master's thesis Jilin Agri Univ
23. Liu YZ, Gao YK, Yuan L, Zhang QX (2019) Functional characterization and spatial interaction of *TERMINAL FLOWER 1* in *Hemerocallis*. Sci Hortic 253:154–162. http://dio.org/10.1016/j.scienta.2019.03.036
24. Liu YZ, Gao YK, Yuan L, Zhang QX (2021) Molecular Characterization and Expression Patterns of the *HKSVP* Gene Reveal Distinct Roles in Inflorescence Structure and Floral Organ Development in *Hemerocallis fulva*. Int J Mol Sci 22:12010. http://dio.org/10.3390/ijms222112010
25. Lux-Endrich A, Treutter D, Feucht W (2000) Influence of nutrients and carbohydrate supply on the phenol composition of apple shoot cultures. Plant cell tissue organ cult 60:15–21. http://dio.org/10.1023/A:1006406527242
26. Matand K, Shoemake M, Li CX (2020) High frequency in vitro regeneration of adventitious shoots in daylilies (*Hemerocallis sp*) stem tissue using thidiazuron. Bmc Plant Biol 20:1–10. http://dio.org/10.1186/s12870-020-2243-7
27. Matraszek-Gawron R, Chwil M, Terlecka P, Skoczylas MM (2019) Recent Studies on Anti-Depressant Bioactive Substances in Selected Species from the Genera *Hemerocallis* and *Gladiolus*: A Systematic Review. Pharmaceuticals 12:172. http://dio.org/10.3390/ph12040172
28. Matsumoto T, Nakamura S, Nakashima S, Ohta T, Yano M, Tsujihata J, Tsukioka J, Ogawa K, Fukaya M, Yoshikawa M, Matsuda H (2016) γ-Lactam alkaloids from the flower buds of daylily. J Nat Med 70:376–383. http://dio.org/10.1007/s11418-015-0963-z

29. Movahedi A, Zhang JX, Amirian R, Zhuge Q (2014) An efficient *Agrobacterium*-Mediated Transformation System for Poplar. Int J Mol Sci 15:10780–10793. http://dio.org/10.3390/ijms150610780
30. Rajoriya P, Singh VK, Jaiswal N, Lall R (2018) Optimizing the effect of plant growth regulators on *in vitro* micro propagation of Indian red banana (*Musa acuminata*).J Pharmacogn Phytochem628–634
31. Ren Y, Gao YK, Gao SY, Yuan L, Wang XQ, Zhang QX (2019) Genetic characteristics of circadian flowering rhythm in *Hemerocallis*. Sci Hortic 250:19–26. http://dio.org/10.1016/j.scienta.2019.01.052
32. Ren Y, Gao YK, Liu JJ, Fu M, Zhang QX (2017) Investigation on resources of *Hemerocallis* in north china. Acta Horti 65–72. http://dio.org/10.17660/ActaHortic.2017.1185.10
33. Rodriguez-Enriquez MJ, Grant-Downton RT (2013) A new day dawning: *Hemerocallis* (daylily) as a future model organism. Aob Plants 5:s55. http://dio.org/10.1093/aobpla/pls055
34. Siddique I, Javed SB, Al-Othman MR, Anis M (2013) Stimulation of in vitro organogenesis from epicotyl explants and successive micropropagation round in *Cassia angustifolia* Vahl.: an important source of sennosides. Agrofor Syst 87:583–590. http://dio.org/10.1007/s10457-012-9578-5
35. Tomkins JP, Wood TC, Barnes LS, Westman A, Wing RA (2001) Evaluation of genetic variation in the daylily (*Hemerocallis* spp.) Using AFLP markers. Theor Appl Genet 102:489–496. http://dio.org/10.1007/s001220051672
36. Murashige T, Skoog F (2006) A revised Medium for Rapid Growth and Bio Agsays with Tohaoco Tissue Cultures. Physoiol Plant 15:473–497. http://dio.org/10.1111/j.1399-3054.1962.tb08052.x
37. Dhar U, Upreti J (1999) In vitro regeneration of a mature leguminous liana (*Bauhinia vahlii* Wight & Arnott). Plant Cell Rep 18:664–669. http://dio.org/10.1007/s002990050639
38. Veluthambi K, Krishnan M, Gould JH, Smith RH, Gelvin SB (1989) Opines stimulate induction of the vir genes of the agrobacterium tumefaciens Ti plasmid. J Bacteriol 171:3696–3703. http://dio.org/10.1128/jb.171.7.3696-3703.1989
39. Wang XX (2013) The plant regeneration and transformation of CHS gene by grobacteria-mediated in *Hemerocallis hybrida*. Master's thesis, Gansu Agri Univ
40. Wang XH, Zhou FL, Liu JL, Liu WQ, Zhang SL, Li DL, Song JK, Wang R, Yang YJ (2021) Establishment of efficient callus genetic transformation system for *Pyrus armeniacaefolia*. Sci Hortic 289:110429. http://dio.org/10.1016/j.scienta.2021.110429
41. Wen SS, Ge XL, Wang R, Yang HF, Bai YE, Guo YH, Zhang J, Lu MZ, Zhao ST, Wang LQ (2022) An Efficient Agrobacterium-Mediated Transformation Method for Hybrid Poplar 84k (*Populus alba* × *P. Glandulosa*) Using Calli as Explants. Int J Mol Sci 23:2216. http://dio.org/10.3390/ijms23042216
42. Xu Q, Yang XM, Wang GB (2021) Transcriptome Analysis of Browning and Non-browning Callus of *Ginkgo biloba*.Mol Breed1–14
43. Yan MM, Xu C, Kim CH, Um Y, Bah AA, Guo DP (2009) Effects of explant type, culture media and growth regulators on callus induction and plant regeneration of Chinese jiaotou (*Allium chinense*). Sci Hortic 123:124–128. http://dio.org/10.1016/j.scienta.2009.07.021

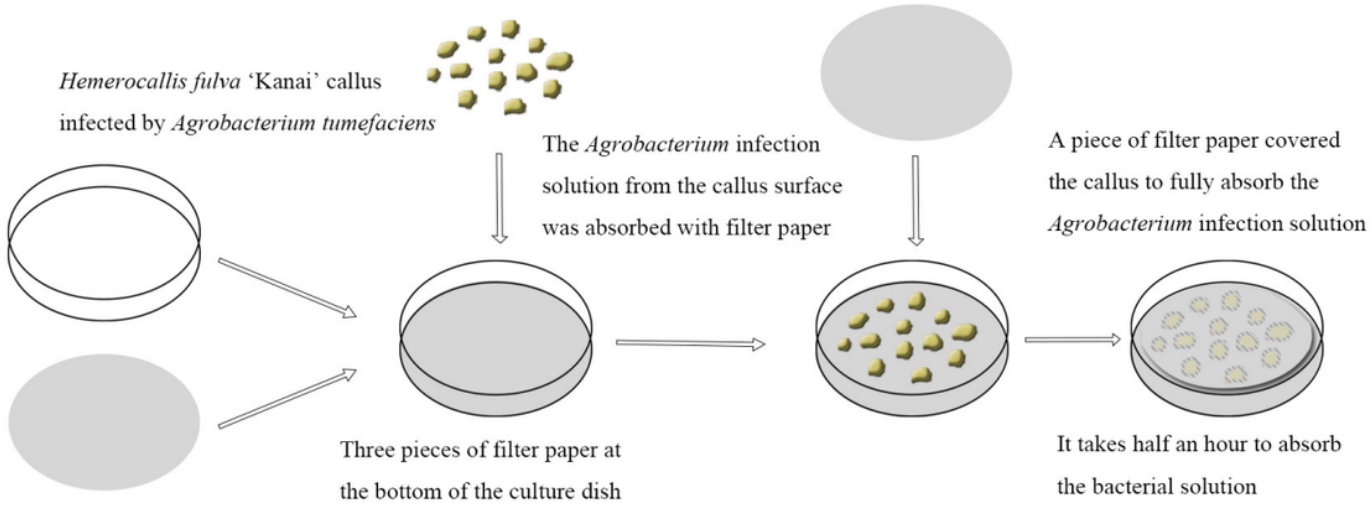
44. Yuan XJ, Wang ZY, Liu JX, She JM (2009) Development of a plant regeneration system from seed-derived calluses of centipedegrass [*Eremochloa ophiuroides* (Munro.) Hack]. Sci Hortic 120:96–100. http://dio.org/10.1016/j.scienta.2008.09.005
45. Zhang HX, Wang GL, Qiao YX, Chen C (2017) Effects of timentin and other β-lactam antibiotics on callus induction, shoot regeneration, and rooting in *Anthurium ndraeanum* Linden ex Andre. In Vitro Cell Dev-Plant 53:219–225. http://dio.org/10.1007/s11627-017-9823-8
46. Zhang XN, Wu QS, Lin SN, Zhang Z, Wang ZH, Wang QJ, Yan XL, Bendahmane M, Bao MZ, Fu XP (2021) Regeneration and *Agrobacterium*-mediated genetic transformation in *Dianthus chinensis*. Sci Hortic 287:110279. http://dio.org/10.1016/j.scienta.2021.110279
47. Zheng N, Wang XX, Cao DM (2015) Transformation of agrobacteria-mediated *CHS* gene in *Hemerocallis hybrida*. Chin Hortic Abs 31:5–11
48. Zhong R, Tan YX, Zhang Z, Yin DM, Liu X, Qin QP, Zhang ZG, Ni DA (2021) Selection of pollen electroporation transformation parameters of daylily(*Hemerocallis sp*.). Plant Physiol Commun 57:723–726. http://dio.org/10.13592/j.cnki.ppj.2020.0564

# Figures



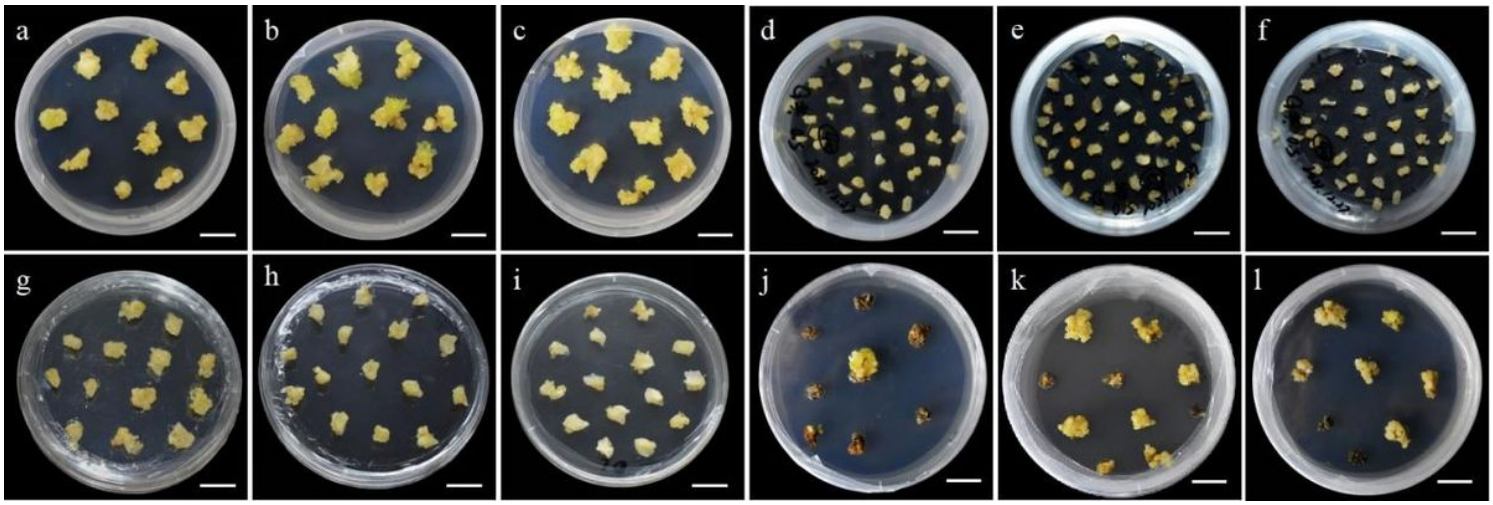

## Figure 1

The formation process of regeneration plant from seed-derived calluses. **a-b** The seeds are removed from the inner and outer seed coats and germinated; **c** callues induced from seed; **d** callus proliferation; **e** differention culture; **f-g** formation and growth of small shoot near the callus; **h** plantlets rooting; **i** transplanting survival plants; **j** number of regenerated buds in medium with different concentrations of 6-BA ($mg \bullet L^{-1}$). Bars = 1 cm.



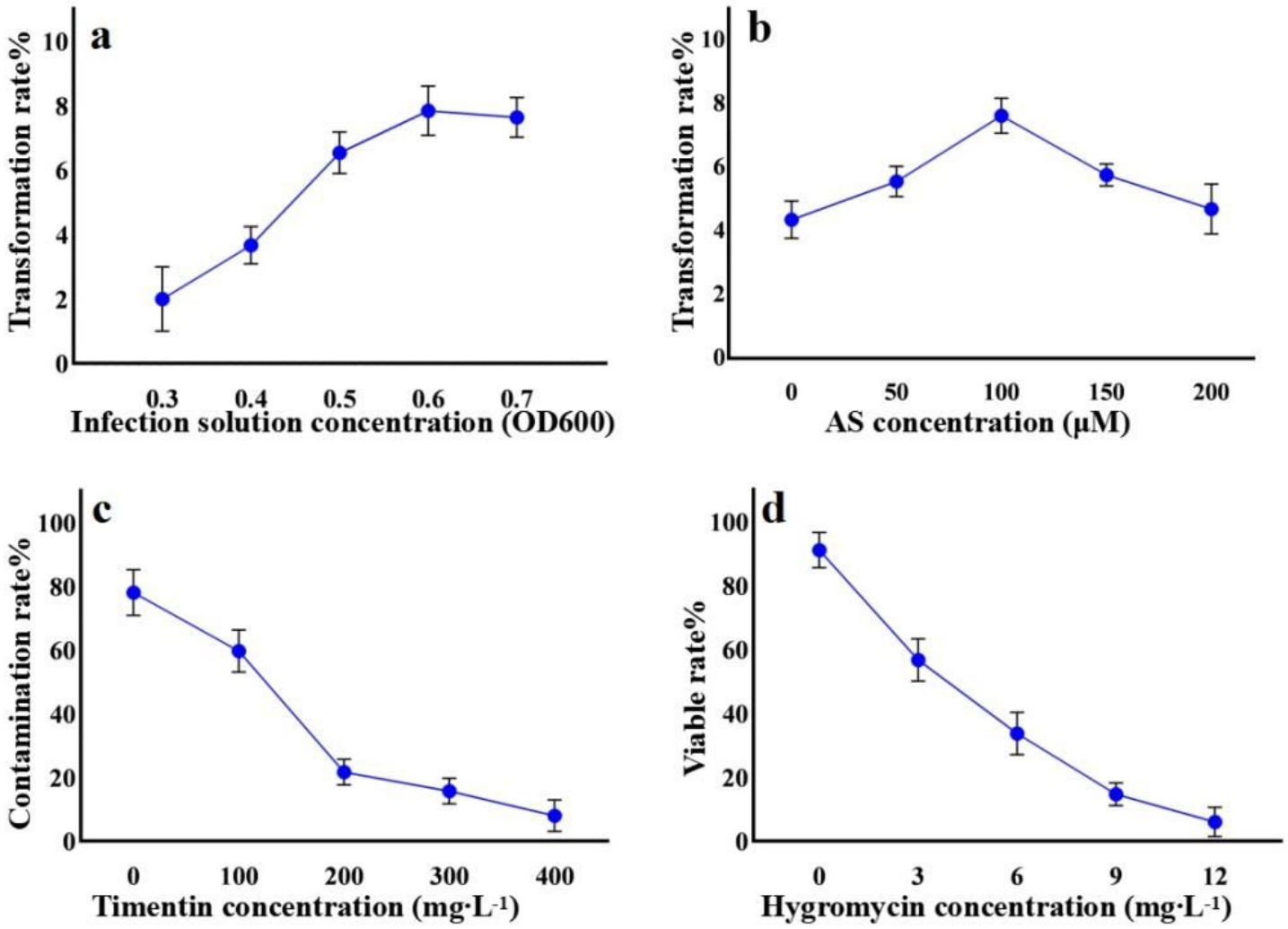

## Figure 2

Infection of callus with *Agrobacterium tumefaciens*.



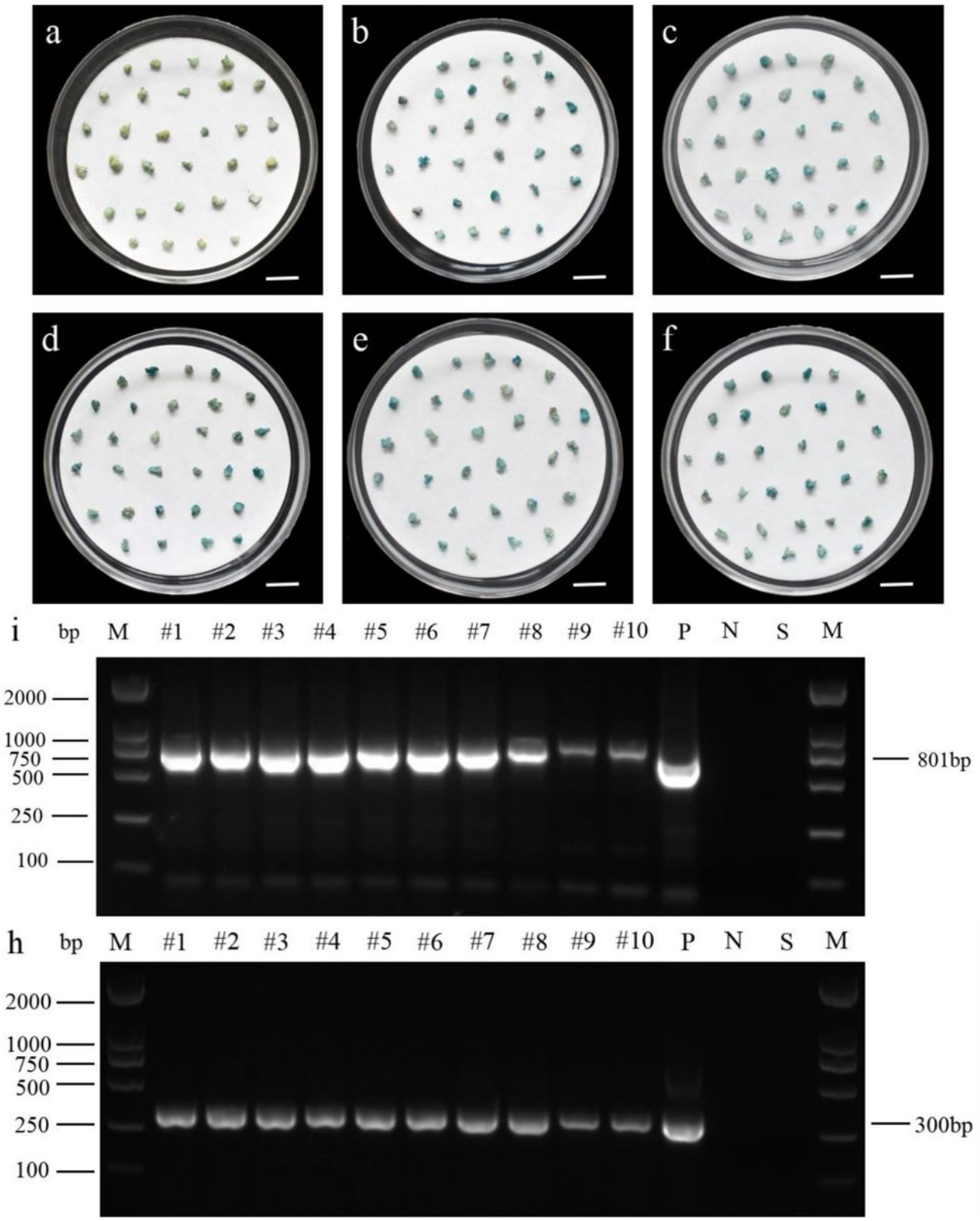

## Figure 3

Genetic transformation process of *Hemerocallis fulva* 'Kanai'. **a-c** Transformer receptors with a good growth state; **d-f** co-culture in medium for 2 days; **g-i** debacteria were cultured for 5 days; **j-l** screens were cultured for 60 days. Bars = 1 cm.




## Figure 4

**a** Effects of different density of infection solution ($OD_{600}$); **b** effect of different concentrations of AS on the transformation rate; **c** anti *Agrobacterium* ability of different concentrations of Timentin; **d** screening effect of different concentrations of hygromycin.




## Figure 5

**a**Non-transformed GUS receptors; **b-f** *Agrobacterium tumefaciens*concentrations OD600 (0.3, 0.4, 0.5, 0.6 and 0.7); **g-h** PCR detection of the *FT* and *GUS* genes putatively transformed *Hemerocallis fulva* 'Kanai' plants. Maker: DL2000 DNA marker; #1-10: putatively transformed *Hemerocallis fulva* 'Kanai' plants genomic DNA. Bars = 1 cm.