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## Research Article

**Keywords:** *Salix brachista*, Genetic transformation, FT, Flowering

**Posted Date:** April 30th, 2025

**DOI:** <https://doi.org/10.21203/rs.3.rs-6446817/v1>

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# Establishment of *Agrobacterium*-mediated genetic transformation in *Salix brachista*.

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## Abstract

*Salix brachista*, commonly known as the Cushion willow, is a plant species that is indigenous to the alpine ice edge zones. This remarkable plant is predominantly found in the Hengduan Mountains. As a quintessential example of an alpine plant, the development of a genetic transformation system for *Salix brachista* is of paramount importance for unraveling the origins and the intricate mechanisms that underpin the diversity of alpine plant species. In the course of our research, we initially focused on creating a robust regeneration system for this species, utilizing stem segments of *Salix brachista*. Through meticulous optimization of various factors, we achieved a commendable average regeneration rate of 52%. Building upon this foundational work, we then established a straightforward and highly effective genetic transformation protocol, employing the *Agrobacterium*-mediated method. The successful establishment of this genetic transformation method not only represents a significant advancement in itself but also opens up new avenues for further gene editing and functional research endeavors in *Salix brachista*.

**Keywords:** *Salix brachista*, Genetic transformation, *FT*, Flowering

**Key Message:** First *Agrobacterium*-mediated transformation for *Salix brachista* achieves 52% regeneration, enabling genetic manipulation in *Salix brachista*.

## Introduction

*Salix brachista* is a plant species native to the alpine ice edge zone, primarily distributed in the Hengduan Mountain region, which is also one of the global hotspots of biodiversity. The rapid uplift of the Hengduan Mountains, along with its complex topography and landforms, diverse habitats, and drastic climate fluctuations have been formed. These factors likely contribute to the isolation and differentiation of species populations, thereby promoting speciation. As a representative alpine plant of this region, the establishment of a genetic transformation system for *Salix brachista* is of great significance for elucidating the origins and mechanisms underlying alpine plant diversity.

Genetic transformation is a technique used to introduce exogenous genes into target organisms, thereby altering their genetic characteristics. This can be achieved through various methods, including *Agrobacterium*-mediated transformation, biolistic gene gun, electroporation, and pollen tube pathway. *Agrobacterium*-mediated transformation is often preferred by researchers due to its cost-effectiveness, ability to introduce a low copy number of genes, ability to transform large gene fragments, and the stability of exogenous genes. This method has been successfully established in the transformation of Poplar and Willow family plants (Movahedi A 2014; Yang J L 2013). Some researchers have also delivered gene editing reagents to model tree species like *Populus tremula* × *P. alba* using DNA-coated particle bombardment (Hoengenaert L 2023). Additionally, there are methods such as hairy root transformation (Gomes C 2019) and plastid transformation which have been also explored (Hyden B 2022). Studies have reported that *Salix brachista* can survive in high-altitude areas, which may be attributed to specific gene families in its genome, such as those involved in DNA repair and anthocyanin synthesis. Moreover, the sex determination region of *Salix brachista* shows significant recombination events compared to other *Salix* species, indicating that this region is evolving dynamically and is species-specific (Chen J H 2019). Therefore, the establishment of the genetic transformation system for *Salix brachista* not only provides an important tool for understanding the origins and evolution of alpine plant diversity but also lays the foundation for genetic improvement and biotechnological applications of this species.

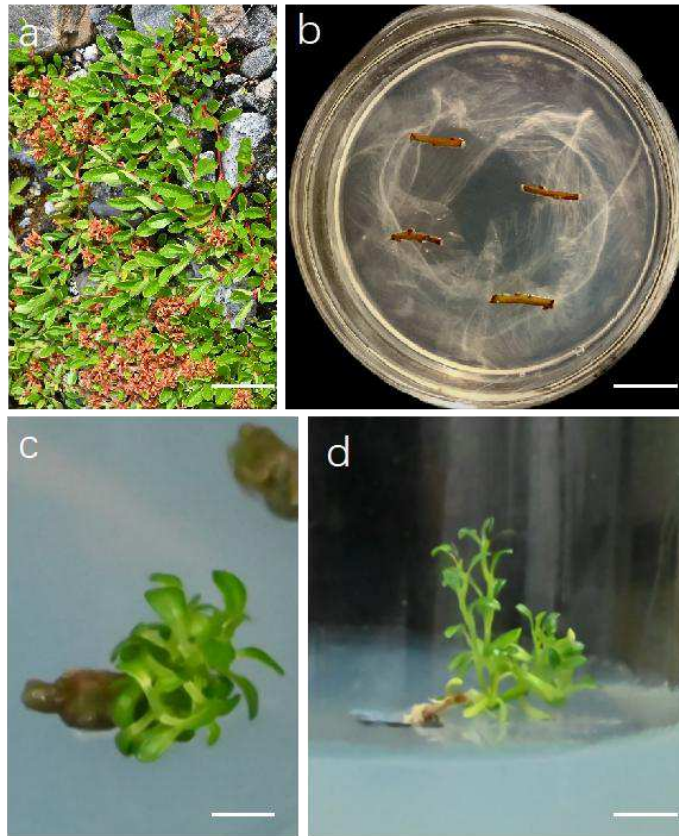
The *FT* gene (FLOWERING LOCUS T) in *Arabidopsis thaliana* is a core gene in the regulation of plant flowering (Kardailsky I 1999). As a member of the PEBP (phosphatidylethanolamine-binding protein) family, *FT* controls the photoperiod and vernalization pathways, facilitating the transition of plants from vegetative to reproductive growth. Under long-day conditions, the *FT* gene is activated, inducing flowering initiation and inflorescence formation through interactions with downstream genes (Wang Q Q 2024). Currently, homologous genes of *FT* have been cloned in various plants species (Lin M K 2007; Tamaki S 2007; Tränkner C 2010), and in some species within *Willowaceae* family, early flowering has been achieved through genetic transformation (Shen L L 2012). Overexpression of the *FT* gene in *Salix brachista* promoting its early flowering is of great significance for studying the sex

differentiation of *Salix brachista*.

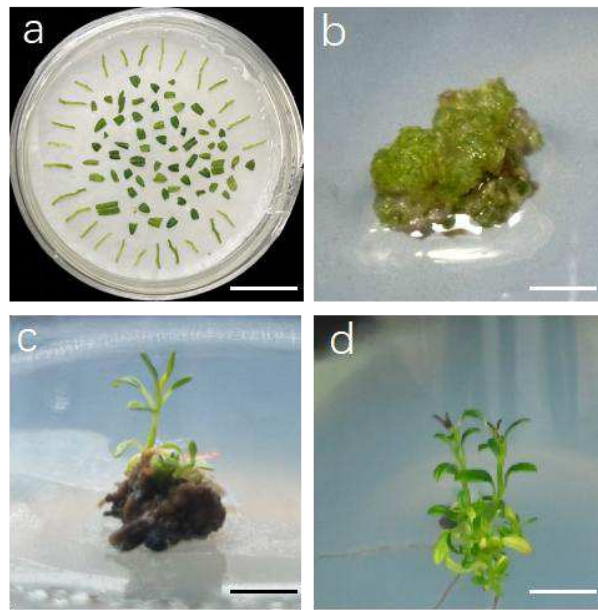
## Results and Discussion

### Establishment of Regeneration System

As a native plant of the alpine ice edge zone, *Salix brachista* is primarily found in the Hengduan Mountain region (Fig. 1a). To obtain the sterile seedlings, we selected branchlets with axillary buds and removed the leaves as explants, cutting them into 1-2 cm stem segments. After cleaning and disinfecting, the stem segments were placed on MS medium (Murashige and Skoog medium) supplemented with 100 mg/mL TMT (timentin) (Fig. 1b). After 1-2 weeks of culture, new seedlings emerged from the axillary buds (Fig. 1c). The seedlings were then transferred to bottle-contained MS medium for subsequent experiments (Fig. 1d). The results indicate that the system can efficiently support the regeneration of aseptic seedlings for *Salix brachista*. To establish a regeneration system in *Salix brachista*, the young stems of the regenerated *Salix brachista* seedlings were cut into segments, approximately 1 cm in length, and the leaves were excised into smaller sections measuring  $0.5 \times 0.5$  mm (Fig. 2a). After 3 weeks incubation, we found that stem achieves a callus formation rate of 52%, and 21% for the leaf sections (Fig. 2b, tab. 1, tab. 2 ), indicating stem is an excellent explant for *Salix brachista*. The optimal medium is 1/2 MS with 0.1 mg/L 6-BA, 0.1 mg/L NAA and 50 mg/L VC. After six weeks cultivation, shoots were formed in callus (Fig. 2c). Then, the regenerants were transferred to half-strength MS medium to generate roots (Fig. 2d). These results indicated that regeneration system of *Salix brachista* is successfully established.



**Fig. 1** Establishment of aseptic seedling regeneration system for *Salix brachista*. a Natural habitat and sampling site of *Salix brachista* in the Hengduan Mountains. Scale bar = 3 cm b Surface sterilization of stem segments (1-2 cm) on MS medium supplemented with 100 mg/mL TMT. Scale bar = 1.6 cm c Axillary bud sprouting and seedling emergence after 1-2 weeks of culture. Scale bar = 4 mm d Aseptic seedlings transferred to MS medium in culture bottles for further growth. Scale bar = 1.1 cm



**Fig. 2** Optimization of in vitro regeneration system for *Salix brachista*. a Young stems from regenerated seedlings were sectioned into segments (~1 cm in length), and leaves were dissected into explants (0.5 × 0.5 mm) and placed on callus-inducing medium for further cultivation. Scale bar = 1.7 mm b Callus formation on explants after 3 weeks of incubation. Scale bar = 4 mm c Shoot regeneration from callus after 6 weeks of culture on optimized medium. Scale bar = 3.5 mm d Rooting of regenerated shoots on half-strength MS medium. Scale bar = 8 mm

**Tab. 1** The young stem callus induction rate of *Salix brachista* cultured on callus induction medium reached approximately 52% under standard conditions (28°C, 16/8 h photoperiod).

| Number of stems | Number of healing tissues | recovery rate (%) |
|-----------------|---------------------------|-------------------|
| 112             | 67                        | 59.82             |
| 125             | 71                        | 56.80             |
| 103             | 46                        | 44.66             |
| 86              | 44                        | 51.16             |
| 125             | 60                        | 48.00             |
| 101             | 51                        | 50.50             |

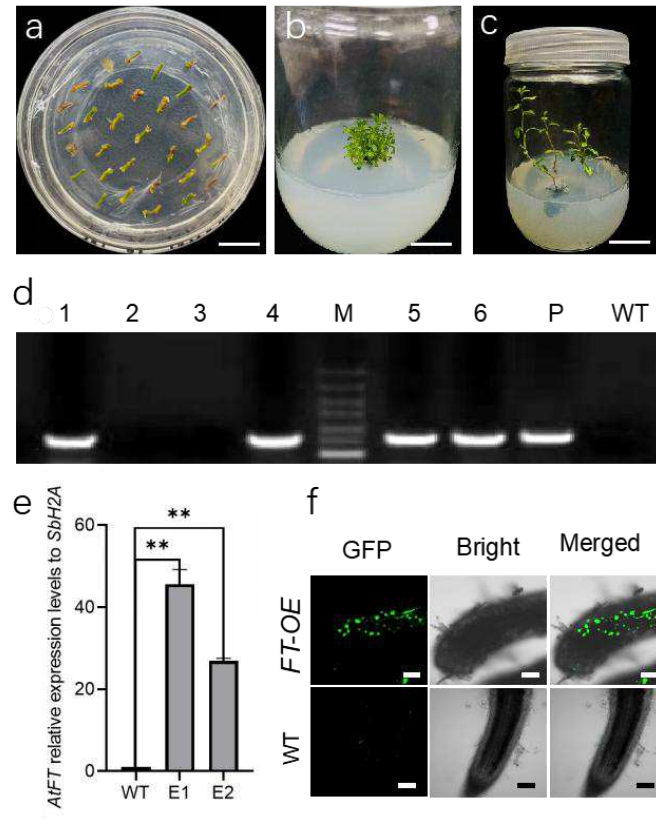
**Tab. 2** The leaf callus induction rate of *Salix brachista* cultured on callus induction medium reached approximately 21% under standard conditions (28°C, 16/8 h photoperiod).

| Number of healing tissues | number of shoots | recovery rate (%) |
|---------------------------|------------------|-------------------|
| 150                       | 32               | 21.33             |
| 144                       | 41               | 19.44             |
| 168                       | 30               | 24.40             |
| 139                       | 30               | 21.58             |
| 158                       | 36               | 22.78             |
| 133                       | 24               | 18.05             |

## Establishment of Genetic Transformation System

Tissue culture has been widely used to advance plant research by transgenesis and gene-editing technologies. Although successful regeneration of transformed seedlings has been reported in some species, it is still the bottleneck for the *Salix brachista* to produce transgenic or gene-edited plants. While *GUS* or *PDS* has been typically utilized to detect transgenic plants, these plants cannot be employed for further experimentation. Given the dioecious genus in *Salix brachista*, the *Arabidopsis* *FT* gene was selected to ascertain the efficiency of transgenesis in *Salix brachista*. The *FT* gene is recognized to be closely associated with the timing of flowering (Nakamura Y 2014). Notably, the overexpression of the *FT* gene can result in early flowering (Nobusawa T 2022). The transformants obtained thus hold significant potential for accelerating advancements in research related to flower sex and gene function. Then, the generating transgenic plants can be used to determine the role of genes in sex determination of male and female. The explants of young stems were inoculated with *Agrobacterium* containing *pRII01GFP-FT* for 17 minutes. Following inoculation, the explants were transferred to a co-cultivation medium (Fig. 3a). Four to six weeks later, the callus began to sprout (Fig. 3b), with a sprouting rate of approximately 33% (Tab. 3). Once the callus had sprouted and developed into seedlings, the regenerants were transferred to jars containing half-strength MS medium, where complete plants were formed within 30 days (Fig. 3c). Gene-specific primers targeting the upstream sequence of *pRII01GFP* vector (F) and the downstream sequence of the *FT* gene (R) successfully amplified the expected band in the regenerated lines, while no amplification was detected in the wild type plants (Fig. 3d). To assess the relative expression levels of the *FT* gene, the *Salix brachista* *H2A* gene was used as a reference gene for normalization. Data obtained from RT-qPCR analysis showed

that the expression level of the *FT* gene in the regenerated lines was significantly higher than that of the wild-type controls (Fig. 3e). Confocal laser scanning microscopy showed that GFP fluorescence was predominantly localized in the nucleus of root cells in all confirmed transformants (Fig. 3f). This data suggests that we have successfully generated *35S::GFP-FT* transgenic plants, indicating that the genetic transformation system has been effectively established.



**Fig. 3** Establishment of a genetic transformation system for *Salix brachista*. a *Agrobacterium* inoculation and co-cultivation, Stem explants inoculated with *Agrobacterium* carrying pRI101GFP-FT for 17 min, followed by transfer to co-cultivation medium. Scale bar = 1.6 cm b Callus sprouting after 4-6 weeks of culture. Scale bar = 1.6 cm c Complete plant formation on half-strength MS medium within 30 days. Scale bar = 1.8 cm d PCR validation of *FT* gene integration, Expected band amplified in transgenic lines using primers flanking pRI101GFP vector and *FT* gene, while absent in wild-type (WT). e RT-qPCR analysis of *FT* expression showed that the *FT* expression in transgenic lines was significantly higher than that in wild type (\*\* $P < 0.01$ ), normalized with *H2A* as the reference. Among them, E1 and E2 are *FT* overexpression lines, with three replicates for each line. f Subcellular localization of GFP, GFP fluorescence (green) predominantly localized in root cell nuclei by confocal microscopy. Scale bar = 50  $\mu$ m

**Tab. 3** The bud induction rate of *Salix brachista* on the bud induction medium was approximately 33%.

| Number of healing tissues | Number of buds | Bud induction rate (%) |
|---------------------------|----------------|------------------------|
| 56                        | 15             | 26.79                  |
| 33                        | 9              | 27.27                  |
| 69                        | 23             | 33.33                  |
| 77                        | 36             | 46.75                  |
| 53                        | 20             | 37.74                  |
| 46                        | 13             | 28.26                  |

In conclusion, we have successfully developed a stable regeneration system and genetic transformation protocol for *Salix brachista*, generating a novel germplasm with *35S:GFP-FT*. These systems will serve as valuable technical tools for examining the temporal dynamics of sex differentiation, exploring the expression patterns of associated genes, and assessing the impacts of environmental factors on sex differentiation.

## Materials and methods

In this experiment, we selected the monoecious *Salix brachista* specimens collected from Tianbao Mountain (Diqing, Yunnan) as the experimental material. The branches with axillary buds were cut into segments measuring 1-2 cm in length using a sterile blade. These segments were disinfected in 75% alcohol for 30 seconds to 1 minute, and then rinsed three times with dd H<sub>2</sub>O (double-distilled water). Subsequently, the segments were further disinfected with 20% sodium hypochlorite for 17-18 minutes, followed by five additional rinses with dd H<sub>2</sub>O. After drying the surface moisture, the segments were placed on MS medium supplemented with TMT for growth over 10-15 days. Young stems, approximately 5 mm in length, were then excised as explants and infected with *Agrobacterium tumefaciens* for further experimentation.

### Plasmid construction

Following the method of Li et al. (Li C 2019), the full-length coding DNA sequence of *Arabidopsis FT* was cloned and inserted into the *pRI01GFP* vector using the ClonEx-press II One Step Cloning Kit

(Vazyme Biotech).

#### Transformation

Transform the ligation product using heat shock at 42°C into competent cells of DH5 $\alpha$ . Subsequently, the recombinant plasmid *pRI01GFP -FT* was introduced into competent cells of LBA4404 using electroporation. After shaking culture at 28°C for 3 hours, 50  $\mu$ L of the culture was plated onto LB (Luria-Bertani) solid medium. Identification of successful transformants was performed using the PCR method.

#### *Agrobacterium* culture

Use a pipette to transfer the bacterial solution containing the PCR-verified band size into LB solid medium. Incubate the plate, inverted, in a 28°C incubator for 3 days.

#### Bacterial solution activation

Remove the culture plate from the 28°C incubator and scrape the bacterial solution into a 50 mL centrifuge tube with the infection solution (MS, pH 5.8, supplemented with 200 mM acetosyringone). Adjust the optical density at 600 nm (OD600) to 0.3-0.5 using a spectrophotometer. Prepare an infection solution with a final volume of approximately 40 mL. Both the culture plates and liquid cultures must be supplemented with spectinomycin and rifampicin reagents.

#### *Agrobacterium* infection

Carefully immerse the stem segments and leaves into the infection solution, ensuring they are fully submerged, and incubate for approximately 15 minutes, and gently shake the mixture every 5 minutes. After the infection period, remove the hypocotyls, blot them with filter paper to remove excess liquid, and place them on co-culture medium. Incubate the samples in the dark for 2 days, placing a layer of filter paper on the medium.

#### Bacterial washing

Remove the stem segments and leaves from the co-culture medium and transfer them to a culture dish containing sterile double distilled water. Wash the samples for 7 minutes, gently shaking the dish every 2 minutes. Repeat this washing process 4 times. After washing, blot the stem segments and leaves with

204 filter paper to remove excess water.

205

206 Callus induction and bud induction

207 Transfer the cleaned stem segments and leaves to a callus-inducing medium supplemented with  
208 hygromycin for callus screening and induction. Subculture the explants every 7 days. After 4 weeks,  
209 once callus buds have been formed, transfer them to a hormone-free medium in a culture bottle for  
210 further growth.

211 Once the plants in the culture bottle have developed numerous and sturdy roots, open the lid and  
212 continue to culture them for an additional 2 days. Subsequently, transfer the plants to a greenhouse for  
213 a further 2 days of acclimatization. After this period, carefully remove the plants from the culture bottle,  
214 discard the surrounding medium, rinse the plants with clean water, and transplant them into plastic pots  
215 filled with a mixture of limestone and sterilized humus soil. Cover the pots with a thin layer of plastic  
216 film to shield the plants from direct sunlight. Remove the plastic film after one week and water the  
217 plants regularly.

218

219 Preparation of Culture Medium System

220 Preparation of 1L half-strength MS medium: MS 2 g, sucrose 10 g, agar 3.6 g, adjust the pH to  
221 5.75-5.85.

222 Co-culture medium: 1/2 MS + 200 mg/L Cef + 0.1 mg/L 6-BA (6-Benzylaminopurine) + 0.1 mg/L  
223 NAA (Naphthaleneacetic Acid) + 50mg/L VC (Ascorbic acid) + 100 mM Acetosyringone

224 Callus induction medium: 1/2 MS + 10 mg/L Hyg (Hygromycin) + 200 mg/L Cef + 0.1 mg/L 6-BA +  
225 0.1 mg/L NAA + 50 mg/L VC (Ascorbic Acid)

226 Bud induction medium: 1/2 MS + 10 mg/L Hyg

227 Rooting medium: 1/2 MS + 0.1 mg/L NAA

228

229 PCR Identification

230 After transferring *Salix brachista* to a hormone-free medium and allowing it to grow for one week, cut  
231 off a leaf segment with a sterile blade, grind it in liquid nitrogen, and extract DNA. Design primers for  
232 PCR: select a 25 bp sequence approximately 100 bp upstream of the restriction site on the vector as the  
233 upstream primer, and a 25 bp sequence within the middle of the *FT* gene as the downstream primer.  
234 Prepare the PCR reaction mixture as follows: add 10 µL of 2×PCR Mix, 1 µL of upstream primer, 1 µL

of downstream primer, and 1  $\mu$ L of DNA to a 200  $\mu$ L PCR tube, then add 8  $\mu$ L of sterilized ultrapure water to achieve a final volume of 20  $\mu$ L. Set the PCR conditions to: pre-denaturation at 94°C for 5 minutes, 32 cycles of amplification with denaturation at 94°C for 30 seconds; annealing at 56°C for 30 seconds, and extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes. After completing the PCR, analyze the results using 1% agarose gel electrophoresis analysis and fluorescent imaging.

## Total RNA Extraction

First, perform sample grinding: Sterilize the mortar and pestle with an alcohol lamp to eliminate potential contaminants, and after natural cooling, add liquid nitrogen into the mortar for pre-cooling treatment. Then quickly take out the *Salix brachista* samples stored at -80°C from the freezer and place them into the mortar, continuously adding liquid nitrogen thorough the grinding process. Extract RNA according to the instructions of the Eastep® SupER Total RNA Extraction Kit.

Take 2  $\mu$ L of the extracted RNA for electrophoresis detection. Typically, the observation of three bright bands indicates high RNA quality. Then, use the NanoDrop1000 spectrophotometer to measure the RNA concentration for subsequent reverse transcription experiments. For short-term storage, RNA can be kept at -20 °C. For long-term use, it should be preserved in a -80 °C freezer.

Synthesize the first strand of cDNA according to the instructions of the purchased 5×All-In-One RT MasterMix reverse transcription kit.

## Real-time fluorescent quantitative PCR

The *Salix brachista* *H2A* gene was used as the reference gene in this study. The qPCR primer sequences for *FT* gene were designed using an online website (Primer designing tool (nih.gov)). Operate according to the EvaGreen 2×qPCR MasterMix-ROX kit, Each reaction was performed in triplicate to ensure reproducibility and accuracy. The qPCR reaction analysis was carried out on an Applied Biosystems StepOnePlus PCR instrument, following the specified experimental program. The relative expression levels were calculated based on the classic  $2^{-\Delta\Delta C_t}$  method using the obtained  $C_t$  values. Subsequently, statistical significance analysis was assessed through the T-test method, applying independent sample ANOVA. For data visualization, GraphPad Prism 8 software was used for charts and graphical representations.

## Data availability

The authors confirm that all data generated or analyzed during this study are included in this published

article.

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## Statements and Declarations

### Funding

This work was supported by the National Natural Science Foundation of China (32070226, 32370405), and the Chinese Academy of Sciences “Light of West China” Program (xbzg-zdsys-202110).

### Competing Interests

The authors declare no competing interests.

### Author Contributions

X.S and J.C. conceived the project and designed the experiments. D.T. and Y.L. collected and prepared the samples. D.T. and Y.L. performed the bioinformatic analyses. D.T. drafted the manuscript. X.S., J.C. and G.P. revised the manuscript. All authors contributed to the article and approved the submitted version.

