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A Method for Genetic Transformation Using Embryonic Callus of *Pinus koraiensis*

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Abstract: *Pinus koraiensis* is classified as a second-class protected wild plant in China, recognized for its considerable economic and ecological importance. However, progress in functional research and breeding applications for this species has been hindered by the lack of an effective genetic transformation system. The purpose of this study was to develop a reliable and efficient genetic transformation system for a *Pinus koraiensis* embryonic callus using somatic embryogenesis technology. The *Pinus koraiensis* embryonic callus and β-glucuronidase (GUS) were employed as the reporter gene in an Agrobacterium-mediated transformation to investigate critical transformation factors, including antibiotic type and concentration, *Agrobacterium* bacterial solution concentration, infiltration, and co-cultivation times. The findings indicated that the proliferation of the *Pinus koraiensis* embryonic callus was substantially inhibited by 10 mg·L^{-1} of Hygromycin (Hyg), and a remarkable 93.42 ± 2.13% efficiency was achieved with an OD600 absorbance value of 0.6 during transformation. Two days of optimal co-cultivation yielded a transformation rate of 82.61%, with the resistant embryonic callus exhibiting a high GUS staining rate of 88.89%. Resistant somatic embryos were effectively obtained following the optimized protocol. This research contributes to the advancement of seed resource breeding and genetic enhancement for *Pinus koraiensis*, establishing a solid foundation for the investigation of gene functions specific to this species.

Keywords: *Pinus koraiensis*; embryogenic callus; genetic transformation; somatic embryogenesis

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1. Introduction

Pinus koraiensis, also known as the sea pine, is a scientifically classified evergreen tree in the *Pinus* genus of the Pinaceae family [1]. It is primarily found in the Great and Small Xing'an Mountains and the Changbai Mountains. Beyond its timber value, *Pinus koraiensis* provides important industrial raw materials, including sapwood, bark, pine needles, and cones [2]. In addition to its industrial applications, the *Pinus koraiensis* fruit is renowned for its extremely high nutritional value, rendering it an essential part of the timber forest and nut economy in China. *Pinus koraiensis*, recognized for its ecological, economic, medicinal, and nutritional significance [3], has been extensively studied for its genetic diversity, seedling reforestation, and resource utilization. Recent years have seen growing interest in propagating and genetically improving *Pinus koraiensis*, driven by its significant ecological and economic contributions. To date, there have been no successful reports of genetic transformation in *Pinus koraiensis*. Therefore, this study uses *Pinus koraiensis* embryonic tissues to experimentally investigate and optimize key factors influencing genetic transformation efficiency, aiming to establish a foundation for future molecular breeding of the species.

Although *Pinus koraiensis* faces challenges like a lengthy growth cycle and limited asexual propagation techniques that restrict its use in large-scale silvicultural seedling

production, it shows considerable promise in somatic embryogenesis. Renowned for its stable heritability, rapid reproduction, and low mutation rate, this technique presents significant potential for advancing the regeneration and genetic improvement of *Pinus koraiensis*. Researchers such as Gao and Chen et al. [4,5] have successfully generated a *Pinus koraiensis* embryonic callus from immature seed embryos, achieving plant regeneration through somatic embryogenesis. Additionally, Peng et al. and Wang et al. [6–8] established an ultra-low-temperature preservation system using the *Pinus koraiensis* embryonic callus and investigated proliferation differences through transcriptome and metabolomics sequencing technologies. These advancements enhance key technological aspects of *Pinus koraiensis* somatic embryogenesis [9,10].

The *Agrobacterium*-mediated genetic transformation method has emerged as the most extensively studied, technically advanced, efficient, and widely adopted approach for genetic transformation in conifers [11]. Notably, Huang [12] pioneered the use of hairy *Agrobacterium* to inoculate sterile European larch seedlings, resulting in the successful production of transformed plants. Currently, *Agrobacterium*-mediated techniques have successfully produced transgenic embryogenic cultures in several tree species, including Japanese larch (*Larix kaempferi*), torch pine (*Pinus taeda*), and Norway spruce (*Picea abies*) [13–17].

Integrating the somatic embryogenesis pathway with genetic transformation technology [18] allows for the development of somatic embryos from resistant callus tissues identified during the genetic transformation process. These embryos can germinate into fully resistant plants, potentially offering greater efficiency and producing purer transformed plants compared to traditional organ explant transformation methods.

In this study, *Agrobacterium* strain GV3101, combined with the binary vector VB191103-1905rcy, was employed to transform a *Pinus koraiensis* embryonic callus. Optimizing bacterial solution concentrations and infiltration times during the transformation process led to the successful development of a resistant embryonic callus. This efficient *Agrobacterium*-mediated genetic transformation system for *Pinus koraiensis* embryonic tissue paves the way for accelerated transgenesis and advances research into the genetic improvement and molecular functions of the species.

2. Materials and Methods

2.1. Plant Material

Gao et al. [19] provided *Pinus koraiensis* embryonic material, which was cultivated on a DCR medium (Supplementary Table S1). This medium comprised 0.5 mg·L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg·L^{-1} 6-benzyladenine (6-BA), 30 g·L^{-1} sucrose, 4 g·L^{-1} gellan gum, 0.1 mg·L^{-1} inositol, 0.5 mg·L^{-1} glutamine (Gln), and 0.5 mg·L^{-1} casein hydrolyzed (CH), adjusted to a pH of 5.9. The callus was maintained in darkness at 25 °C, with media refreshed every two days to support optimal growth and development.

2.2. Agrobacterium Strains

The Agrobacterium strain (GV3101) and the plasmid (pBI121) used in this study were maintained in the laboratory. The vector strain employed was VB191103-1905rcy, which carried the β-glucosidase gene (GUS). This plasmid was sourced from the State Key Laboratory of Forest Genetic Breeding, Northeast Forestry University, Harbin, China. A detailed plasmid map information is shown below (Figure 1):

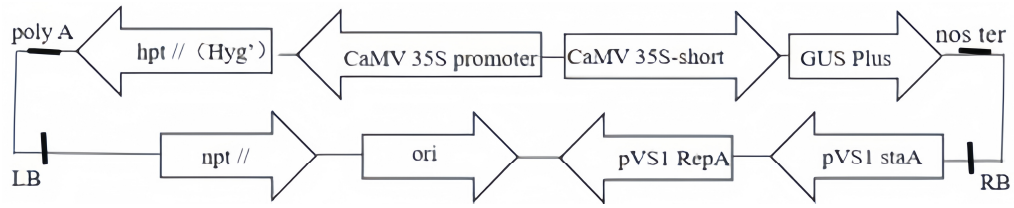

Figure 1. Plasmid map.

2.3. Antibiotic Sensitivity Test of Embryonic Callus

After a ten-day incubation in the DCR proliferation medium, approximately 0.2 g of embryonic callus was transferred to a new DCR medium supplemented with 0.5 mg·L^{-1} 2,4-D, 0.1 mg·L^{-1} 6-BA, and 30 g·L^{-1} sucrose. The medium also included 4 g·L^{-1} gellan gum, 0.1 mg·L^{-1} inositol, 0.5 mg·L^{-1} Gln, and 0.5 mg·L^{-1} casein hydrolysate, with the pH adjusted to 5.9. Cef, Kan, or Hyg were added as antibiotics. The callus was screened at 25 ± 1 °C for 15 days in darkness. In the experiment, a petri dish was inoculated with three explants, each with an initial weight of 0.2 g. Three Petri dishes were repeated. The fresh mass of the embryonic callus was measured, and the procedure was repeated three times. Gradient concentrations were employed for Kan (0, 10, 20, 40, 80, and 100 mg·L^{-1}), Hyg (0, 4, 8, 10, 20, 40, and 60 mg·L^{-1}), and Cef (0, 100, 200, 300, and 400 mg·L^{-1}).

2.4. Agrobacterium Strain Culture

A small volume of bacterial solution was drawn up using an inoculation loop and streaked onto solid yeast extract mannitol broth (YEB) medium supplemented with 50 mg·L^{-1} Kan, Hyg, and rifampicin. The culture was incubated for two days at 28 °C. Following this, single colonies were selected and transferred into 20 mL of liquid YEB medium containing 20 mg·L^{-1} Kan and Rif. The culture was shaken at 200 rpm and incubated at 28 °C in darkness for 16–18 h, or until the optical density (OD_{600}) reached from 0.8 to 1.0. Subsequently, 1 mL of the bacterial culture was inoculated into a fresh liquid YEB medium containing 50 mg·L^{-1} Kan and Rif. The culture was incubated for from five to eight hours, allowing the Agrobacterium to attain the logarithmic growth phase with an OD_{600} of from 0.6 to 0.8

2.5. Genetic Transformation

The fresh bacterial solution was transferred into a sterile centrifuge tube and centrifuged (Therom, Shanghai, China) at 4 °C and 8000 rpm for 10 min. The resulting bacterial pellet was then used to prepare the infiltration solution. The fresh embryonic callus was carefully selected and submerged in the infiltration solution containing 100 μM·L^{-1} acetosyringone (AS). The concentration of the infiltration solution was adjusted to absorbance values of 0.4, 0.6, or 1.0 at OD600. After a 20 min infiltration period, the solution was discarded, and the tissue surfaces were gently blotted with sterile filter paper. The tissues were then transferred to a co-culture medium (DCR + 250 mg·L^{-1} Cef) and incubated at 25 ± 1 °C in the dark for two, three, or four days.

After co-culture, the tissues were rinsed twice with sterile water for two minutes each, followed by two washes with a 500 mg·L^{-1} Cef suspension for 3 min each. Excess water on the cleaned callus surface was carefully blotted dry with sterile filter paper. The embryonic callus was then spread into sheets on a recovery medium and cultured in the dark at 25 ± 1 °C for seven, 14, or 21 days. The resistant tissues were then subjected to screening on a specialized medium. The number of resistant tissues obtained was recorded across three consecutive screenings, each lasting 21 days. The resistant tissues were subjected to GUS histochemical staining and PCR analysis for molecular confirmation. Once verified, the embryonic callus proceeded to successive culture and somatic embryo maturation (Figure 2).

2.6. GUS Histochemical Assay

The resistant tissues were carefully selected and immersed in a GUS staining solution composed of 100 mM sodium phosphate buffer (pH 7.0), 0.5 mg·mL^{-1} X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide), 1% Triton X-100, 1% DMSO, and 10 mM EDTA. The staining solution was infiltrated using a vacuum pump until no bubbles were visible. The embryogenic callus was stained in a water bath at 37 °C for one day. The development of the color status of the embryonic callus was observed and recorded, and non-transformed tissues were employed as the negative control. Transgenic tissues were

identified by their blue–green color, while colorless, transparent, or slightly yellow tissues were deemed false positives.

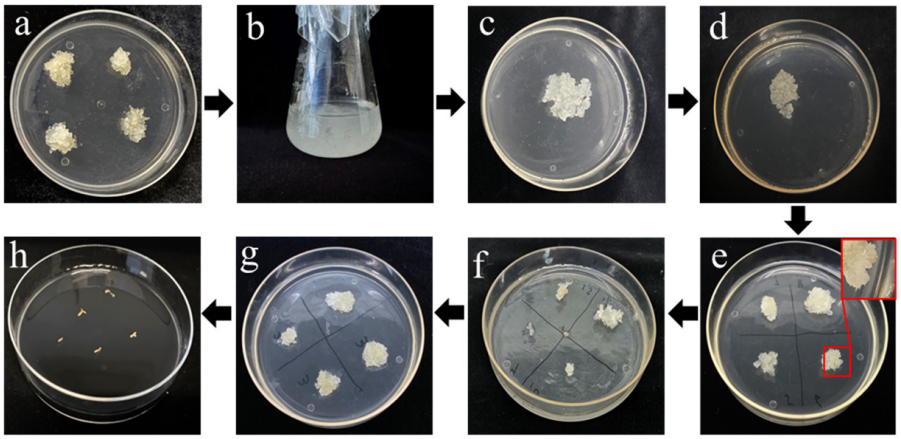

Figure 2. Genetic transformation process. (**a**) Successional culture. (**b**) Agrobacterium infestation. (**c**) Co-culture. (**d**) Recovery Culture. (**e**) First screening culture. (**f**) Second screening culture. (**g**) Third screening culture. (**h**) Obtaining resistant somatic embryos.

2.7. PCR Analysis

Genomic DNA was extracted from 14 randomly selected resistant *Pinus koraiensis* callus samples using the CTAB method. PCR assays were then performed with upstream and vector downstream primers specific to the target genes. The upstream primer sequence was 5′-CAAAGCAAGTGGATTGATGTGAT-3′, and the downstream primer sequence was 5′-AGAGAGAAAAGGGTCCTAACCAAGA-3′. The reaction mixture consisted of 10 µL of Green Taq MIX, 1 µL each of upstream and downstream primers, 1 µL of DNA, and 7 µL of ddH_2O.

The PCR reaction conditions were set as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 15 s, and extension at 72 °C for 15 s. The process concluded with a final extension at 72 °C for 10 min.

The PCR samples were separated by electrophoresis on a 1.0% (*w*/*v*) agarose gel, with wild-type *Pinus koraiensis* embryonic callus serving as the negative control and Agrobacterium solution as the positive control. The electrophoresis results were visualized and documented using a gel imager (Tanon 2500R, Shanghai, China). The resistance of the embryogenic callus was confirmed based on the presence of the expected bands for the target genes.

2.8. Somatic Embryo Maturation

The somatic embryo maturation process, adapted from Peng [20], involved modifying the original protocol. Specifically, 3 g of different transgenic *Pinus koraiensis* embryonic callus samples were incubated in 20 mL of hormone-free liquid medium (DCR + 30 g·L^{-1} sucrose) for seven days. After the incubation period, the liquid was carefully poured off, and any excess liquid was absorbed with sterile filter paper. The embryonic callus was then dried on an ultra-clean bench for 10 min.

The transgenic *Pinus koraiensis* embryogenic callus, prepared as described, was transferred to a DCR maturation medium. This medium included varying concentrations of gellan gum (8, 10, and 12 g·L^{-1}), 20 mg·L^{-1} ABA, 0.1 g·L^{-1} inositol, 0.5 g·L^{-1} Gln, 0.5 g·L^{-1} CH, and 30 g·L^{-1} maltose, with pH adjusted to 5.9. All somatic embryogenesis events were recorded after 10 weeks.

2.9. Statistical Analysis

Statistical analysis was performed using SPSS software (IBM SPSS Statistics 27.0.1), with a significance level set at $p < 0.05$. The tables and figures were generated using Microsoft Excel 2010.

The calculation methods of the various parameters in this study were as follows.

Transformation Efficiency of Embryogenic Callus (%) = Number of the Resistant Tissues after Screening/ Total Number of Infected Embryogenic Callus × 100%

Proliferation multiple = callus proliferation/callus initial weight × 100%

GUS staining rate (%) = Number of blue calli block after GUS staining/Number of resistant calli stained × 100%

PCR Positive Rate (%) = Number of Calli Amplified by PCR/Number of Resistant Tissues Detected by PCR × 100%

3. Results

3.1. Cefotaxime Effects on Embryonic Callus

Cefotaxime (Cef) had little effect on inhibiting the *Pinus koraiensis* embryonic callus (Table 1). However, as the Cef concentration increased, the proliferation rate of the callus gradually decreased after 15 days. At a concentration of 500 mg·L^{-1}, the fresh mass of the proliferating embryonic callus decreased by 10.01% compared to the control. Overall, the inhibitory effects of Cef on *Pinus koraiensis* embryonic callus were not significant across all tested concentrations ($p > 0.05$).

Table 1. Different concentrations of Cef on the proliferation rate of embryonic callus.

Cef Concentration (mg·L^{-1})	Rate of Callus Proliferation (%)
0	3.36 ± 0.32 a
100	3.28 ± 0.49 a
200	3.18 ± 0.43 a
300	3.00 ± 0.39 a
400	3.07 ± 0.75 a
500	2.87 ± 0.42 a

Note: The data have been expressed as mean ± standard deviation. The different lowercase letters in the same column numbers indicate significant differences.

3.2. Kanamycin Effects on Embryonic Callus

No significant differences were observed in the proliferation rate of *Pinus koraiensis* embryonic callus with increasing concentrations of kanamycin (Kan) (Figure 3). The proliferation rate of embryonic callus cultured for 15 days without Kan was found to be 335%. With the addition of Kan at concentrations of 10, 20, 40, 80, and 100 mg·L^{-1}, the proliferation rates of the embryonic callus were determined to be 2.89, 2.57, 2.59, 2.52, and 2.07, respectively. A significant difference ($p < 0.05$) in embryonic callus proliferation was observed only at a Kan concentration of 100 mg·L^{-1}. No significant differences in the proliferation of the *Pinus koraiensis* embryonic callus were observed at Kan concentrations below 100 mg·L^{-1}; inhibitory effects were evident only at concentrations above this level.

3.3. Hygromycin Effects on Embryonic Callus

Hygromycin (Hyg) significantly inhibited the proliferation of the *Pinus koraiensis* embryonic callus. As the Hyg concentration increased, the proliferation rate of the callus decreased progressively after 15 days (Figure 4). The results revealed that 4 mg·L^{-1} of Hyg decreased the proliferation rate of the embryonic callus by approximately 51.72%. Furthermore, a significant decrease in the proliferation of the embryonic callus was observed at Hyg concentrations of 20 mg·L^{-1} or higher, which resulted in negative growth. Hyg concentrations of 20, 40, and 60 mg·L^{-1} reduced embryonic callus proliferation rates by 9.59%, 47.94%, and 56.35%, respectively. Therefore, 10 mg·L^{-1} was determined to be the optimal concentration for screening the *Pinus koraiensis* embryonic callus ($p < 0.01$).

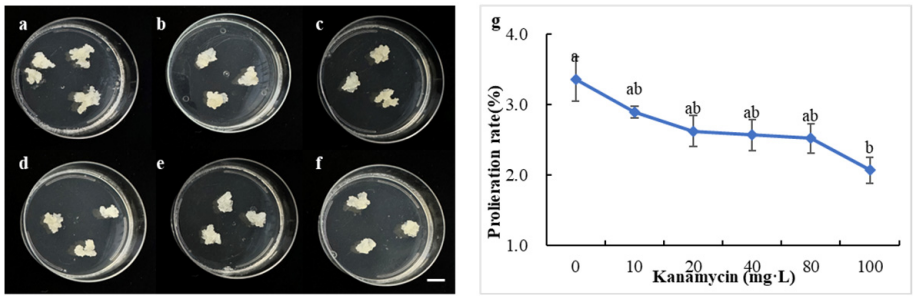

Figure 3. Effect of Kan on the embryonic callus. (**a**–**f**) Proliferation at 0, 10, 20, 40, 80, and 100 mg/L for 15 days of culture. (**g**) The proliferation rate of the callus with different Kan concentrations, bar = 1 cm. Note: The data have been expressed as mean ± standard deviation. The different lowercase letters in the same column numbers indicate significant differences.

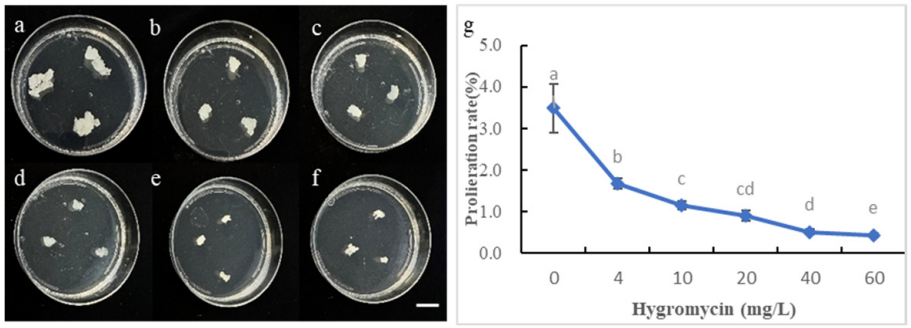

Figure 4. Effect of Hyg on the embryonic callus. (**a**–**f**) Proliferation at 0, 4, 10, 20, 40, and 60 mg/L for 15 days of culture. (**g**) The proliferation rate of callus with different Hyg concentrations, bar = 1 cm. Note: The data have been expressed as mean ± standard deviation. The different lowercase letters in the same column numbers indicate significant differences.

3.4. Effects of Infiltration Solution Concentrations

The efficiency of Agrobacterium-mediated transformation in the *Pinus koraiensis* embryonic callus initially increased with the concentration of the infiltration solution but then decreased at higher concentrations. Variation in solution concentration revealed that the highest conversion efficiency, 93.42 ± 2.13%, was achieved at an absorbance of 0.6 at OD600. The next highest conversion efficiency was determined to be 89.67 ± 5.03%, observed at an absorbance of 0.4. Conversely, transformation efficiencies were similar at an absorbance of 0.2 and 0.8, measuring 39.64 ± 4.42% and 42.63 ± 5.33%, respectively. These results highlight the significant effect of infiltration solution concentration on the transformation efficiency of the *Pinus koraiensis* embryonic callus ($p < 0.001$).

3.5. Effects of Co-Culture Cycle

Within a specified range, conversion efficiency increased with longer co-culture durations. Five different co-culture periods, ranging from zero to four days, were tested to identify the optimal co-culture duration. The results showed no resistant tissues on tissue blocks screened immediately after decolonization without co-culture following infiltration. The highest transformation efficiency, 82.61%, was achieved with a two-day co-culture period. Extending the co-culture period beyond three days resulted in higher Agrobacterium levels, which adversely affected the proliferation and growth of the embryonic callus. Furthermore, completely suppressing Agrobacterium growth during later decolonization stages proved to be challenging, even with 500 $mg \cdot L^{-1}$ of Cef. This led to decreased transformation efficiency due to repeated sterilization attempts.

3.6. Effects of Antibiotics on Callus Proliferation and Somatic Embryogenesis

The choice of antibiotics for screening significantly affects genetic transformation efficiency. According to the antibiotic susceptibility test results, Hyg exhibited a stronger inhibitory effect on the *Pinus koraiensis* embryonic callus compared to Kan. This study aimed to evaluate the screening efficacy of these antibiotics during genetic transformation. The findings revealed that the false positive rate for resistant tissues on medium containing 100 mg·L^{-1} Kan was approximately 71.05% while screening with 10 mg·L^{-1} Hyg resulted in a much lower false positive rate of 13.89%.

Furthermore, Hyg demonstrated a more immediate inhibitory effect on the embryonic callus compared to Kan. Screening with 10 mg·L^{-1} Hyg for ten days caused significant browning of untransformed tissues, whereas there was no significant change with 100 mg·L^{-1} Kan. In summary, Hyg demonstrated greater effectiveness and efficiency compared to Kan, making it better suited for screening transformed tissues of *Pinus koraiensis*.

3.7. GUS Staining of Resistant Tissues

A resistant *Pinus koraiensis* embryonic callus, cultured for 63 days on a screening medium with 10 mg·L^{-1} Hyg, was subjected to β-glucosidase gene (GUS) histochemical staining, with uninfiltrated tissues used as negative controls. Blue staining in the GUS-positive embryonic callus confirmed the successful integration of the GUS reporter gene into the *Pinus koraiensis* embryonic callus (Figure 5). Out of 36 samples tested, 32 showed blue staining, yielding an 88.89% staining rate.

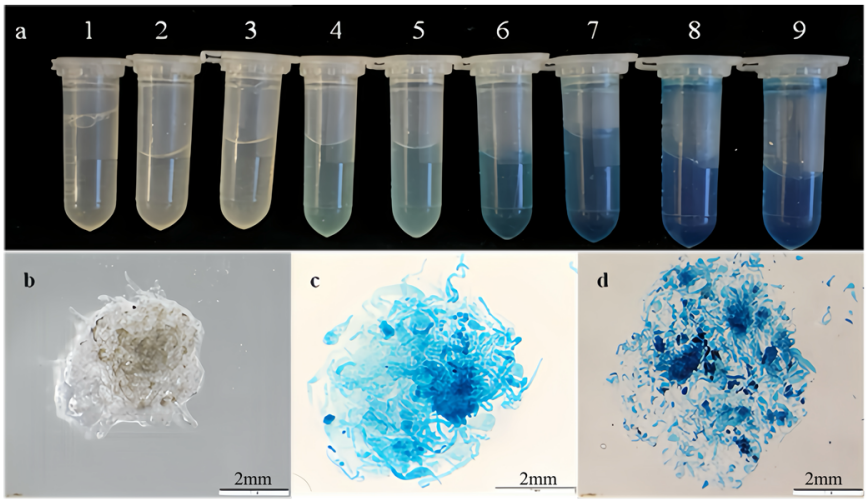

Figure 5. GUS staining of resistant embryonic callus. (**a1**–**3**) GUS staining of wild-type embryonic callus. (**a4**–**6**) GUS staining of resistant callus. (**a7**–**9**) GUS staining of LobHLH gene. (**b**) GUS staining of wild-type embryonic callus. (**c**) GUS staining of resistant callus. (**d**) GUS staining of *LobHLH* gene.

3.8. PCR of Resistant Tissues

From the 32 Hyg-resistant *Pinus koraiensis* embryonic callus samples, 14 were randomly selected for total DNA extraction. This DNA was then used as a template in PCR to verify the successful transfer of the GUS reporter gene. The primers were designed based on the 35S promoter sequence. Uninfiltrated tissues served as negative controls, while Agrobacterium tumefaciens was used as a positive control. The results revealed that all 14 resistant embryonic callus samples showed a target band with a fragment length of 256 bp (Figure 6a).

The *LobHLH34* gene, previously cloned in the authors' laboratory with a size of 696 bp, was transformed using the aforementioned procedure to assess the stability of the genetic transformation in this study. The transformed material was analyzed through GUS staining and PCR. The results confirmed the successful integration of the *LobHLH34* gene into the

Pinus koraiensis embryonic callus, validating the stability of the genetic transformation system (Figure 6b).

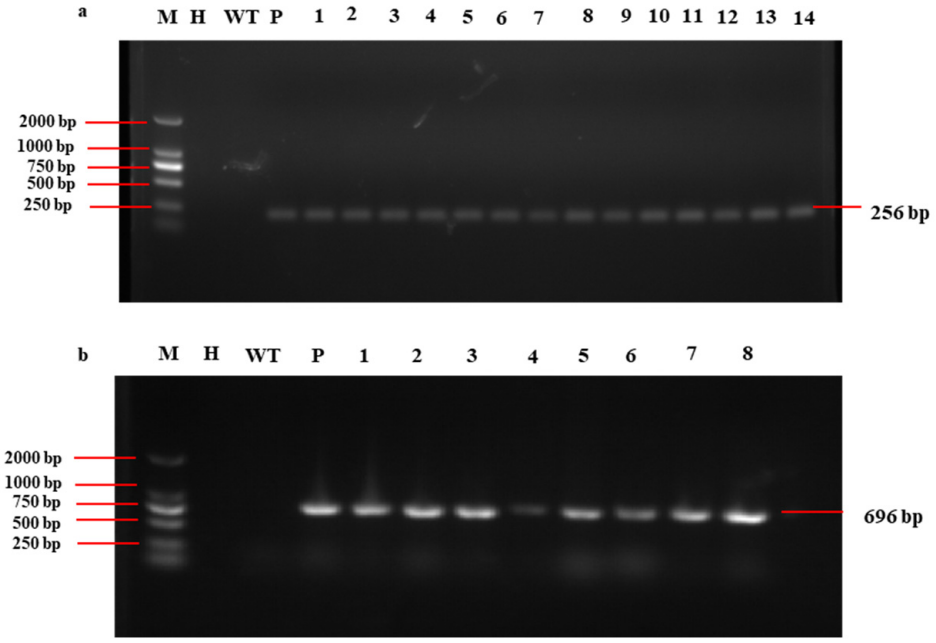

Figure 6. PCR Assay; (**a**) PCR assay of resistant callus with GV3101 vector; (**b**) PCR assay of *LobHLH* gene; M: 2000 DNA Marker; H: ddH_2O; WT: Wild Type; P: Plasmid.

3.9. Effects of Gellan Gum on Somatic Embryo Maturation

The influence of gellan gum concentration on somatic embryo maturation is presented in Figure 6a. The *Pinus koraiensis* esistant embryogenic callus was placed on a gellan gum medium containing 12 g·L^{-1} following screening and testing. The *Pinus koraiensis* embryogenic callus showed significant drying on the medium with 12 g·L^{-1} gellan gum, with most of the tissue turning dark brown and a somatic embryogenesis rate of 4.33 g·L^{-1}. On a medium containing 10 g·L^{-1} gellan gum, the callus appeared dry and granular, with about half of it turning dark brown. Somatic embryos were observed as white globular or elongated structures, often appearing interconnected, resulting in a somatic embryogenesis rate of 17.33 g^{-1}. The *Pinus koraiensis* embryogenic callus demonstrated a dry and powdery texture on gellan gum medium containing 8 g·L^{-1}. The callus was characterized by the uniform distribution of predominantly white long strips. The somatic embryogenesis rate reached 21.67 g^{-1} (Table 2).

Table 2. Somatic embryogenesis at different concentrations of gellan gum.

Gellan Gum Concentration (g/L)	Amount of Somatic Embryogenesis (g·L^{-1})	
	DCR Medium	mLV Medium
8	21.67 ± 4.41 a	11.67 ± 4.16 a
10	17.33 ± 6.03 a	11.00 ± 4.35 a
12	4.33 ± 1.53 b	1.33 ± 1.53 b

Note: The data have been expressed as mean ± standard deviation. The different lowercase letters in the same column numbers indicate significant differences.

Maintaining the gellan gum concentrations at 8, 10, and 12 g·L^{-1} (Figure 7b) and changing the basal medium from DCR to mLV (Supplementary Table S2), the somatic embryo formation was reduced. Somatic embryo formation decreased compared to DCR culture, with rates of 11.67, 11, and 1.33 g·L^{-1}, respectively. However, the number of mature malformed *Pinus koraiensis* somatic embryos was higher than that of normal somatic embryos in both DCR and mLV.

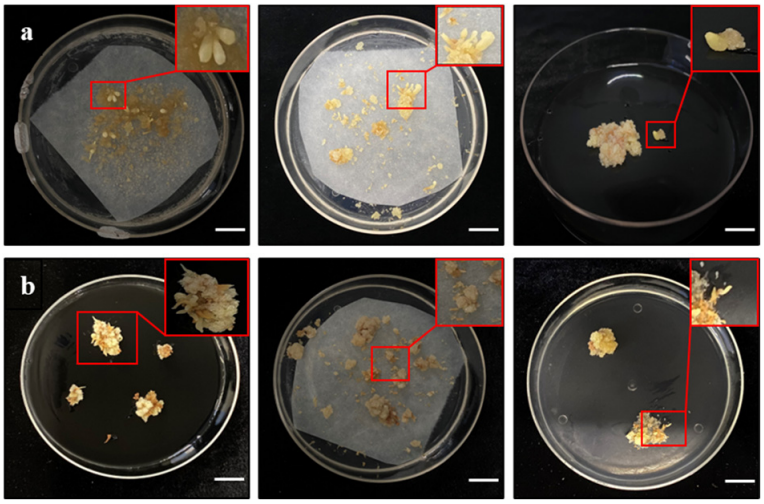

Figure 7. Occurrence of resistant somatic embryos. (**a**) Somatic embryogenesis in DCR medium with Gellan gum concentrations of 8, 10, and 12 g/L. (**b**) Somatic embryogenesis in mLV medium with Gellan gum concentrations of 8, 10, and 12 g/L, bar = 1 cm.

4. Discussion

Agrobacterium-mediated genetic transformation involves the integration of target genes into a plant's genome through the infiltration of the plant with Agrobacterium, which enables the expression of the target genes within the plant genome. This technique is widely used because of its high transformation efficiency and the well-established mechanisms and technologies associated with it [21].

Several factors affect the efficiency of Agrobacterium-mediated transformation, including the concentration of the bacterial solution, the duration of infiltration and co-culture, the type of antibiotic used for screening, and the selection of explants. Optimizing these factors and determining the ideal thresholds can enhance transformation efficiency; however, excessive adjustments may lead to reduced effectiveness.

Genetic transformation can be carried out using various plant tissues or organs, including roots, stems, leaves, somatic embryos, mature embryos, and immature embryos [22,23]. In studies on conifer genetic transformation, mature and immature zygotic embryos have been commonly used [24]. For example, genetic transformation has been achieved in *Pinus massoniana* [25] and slash pine [26] using mature zygotic embryos. Furthermore, Gao [9] employed immature *Pinus koraiensis* zygotic embryos to induce embryonic callus tissue, thereby establishing a regeneration system through somatic embryogenesis. In the current study, the *Pinus koraiensis* embryonic callus was selected for genetic transformation, resulting in a high transformation rate of 88.89%. The resistant embryonic callus was subsequently cultured to develop into resistant somatic embryos.

Both infiltration duration and bacterial solution concentration are crucial for successful transformation. Insufficient bacterial concentration can result in poor adherence of *Agrobacterium* to the plant material, leading to reduced transformation efficiency. Conversely, excessively high concentrations can lead to over-proliferation of *Agrobacterium* in the plant material during later co-culture stages, making decolonization more challenging and potentially risking plant senescence. Infiltration duration also plays a role in transformation efficiency. If the duration is too short, *Agrobacterium* does not have enough time to adequately attach to the plant material. Conversely, an excessively long infiltration period can lead to an overaccumulation of Agrobacterium, which can be detrimental to the transformation process.

In conifer genetic transformation systems, bacterial solution concentrations during infiltration usually vary from an OD_{600} absorbance of from 0.3 to 0.8, while infiltration times typically range from 10 to 40 min. For instance, in certain Larix species, the highest transformation rate was achieved using a bacterial solution concentration of approximately

OD_{600} = 0.6 and an infiltration time of 20 min [27]. Similarly, optimal results in the genetic transformation of *Pinus bungeana* were observed with an OD_{600} = 0.4 and an infiltration time of 30 min [28]. In *Pinus taeda*, transformation yielded a transient expression rate of around 70% when using a bacterial solution concentration of OD_{600} = 0.8 and infiltration times ranging from 15 to 30 min [29]. In this study, the optimal results were achieved by infiltrating a *Pinus koraiensis* embryonic callus at an OD_{600} absorbance of 0.5 for 20 min.

The co-culture phase is crucial for T-DNA integration into the plant genome. For conifers, the optimal co-culture conditions were from two to four days in dark culture at 25 °C. Deviations from this timeframe, whether shorter or longer, can significantly reduce conversion efficiency [11]. For instance, the co-culture period was established at two days for the genetic transformation of the embryonic callus of hybrid fir [30] and white spruce [31]. In comparison, a three-day co-culture period was ideal for mature zygotic embryos of *Pinus massoniana* [25]. The findings of this study demonstrated that tissue transformation was more effective in a two-day co-culture period compared with a one-, three-, or four-day co-culture. Prolonged co-culture beyond three days resulted in repetitive decolonization of tissues due to elevated bacterial concentrations, ultimately leading to tissue death.

Selecting the appropriate antibiotic type and concentration is crucial for influencing the transformation process. Sensitivity tests on other plant species have demonstrated that using multiple screenings enhances the transformation rate and reduces the false positive rate. In conifer genetic transformation, Kan and Hyg are the two primary antibiotics predominantly used for screening. Yaupon [32] achieved resistance in tissues by conducting Kan screening at concentrations ranging from 50 to 75 $mg \cdot L^{-1}$. In hybrid larch, three consecutive screenings with 20 $mg \cdot L^{-1}$ of Kan resulted in a positive rate of 66.2% for obtaining a resistant embryogenic callus [33]. *Larix olgensis* showed a false-positive rate of only 11.66% with Hygromycin screening [34].

In this study, sensitivity experiments on the *Pinus koraiensis* embryonic callus revealed that it was not effectively inhibited by Kan. Even at a concentration of 100 $mg \cdot L^{-1}$, no significant differences in the proliferation rate of the callus were observed. In contrast, using Hyg for screening led to a marked reduction in the proliferation rate of *Pinus koraiensis* embryonic callus. At 4 $mg \cdot L^{-1}$, the proliferation rate was reduced to 68.42%, and higher concentrations severely inhibited proliferation, with tissues ceasing to grow at 20 $mg \cdot L^{-1}$ and dying at 40 $mg \cdot L^{-1}$.

Furthermore, the number of screening rounds was hypothesized to influence the detection of positive conversions. Given the sensitivity of the embryonic callus, initial screenings resulted in fewer resistant tissues and a higher rate of false positives. Subsequent screenings, especially the second and third rounds, significantly reduced the false positive rate and improved the conversion rate. Therefore, this study successfully obtained 36 resistant embryonic callus using Hyg as the antibiotic, applying three consecutive screenings over 21 days each, resulting in a significantly low false positive rate of 11.11%.

5. Conclusions

In this study, a highly efficient genetic transformation system for a *Pinus koraiensis* embryonic callus was successfully established and optimized, representing a significant advancement in this field. *Agrobacterium* strain GV3101, in combination with the binary vector VB191103-1905rcy, yielded 36 resistant embryogenic calluses under optimized conditions: an OD_{600} absorbance of 0.6 for the infiltrating solution, a two-day co-culture period, and 10 $mg \cdot L^{-1}$ Hyg as the screening antibiotic. The transformation rate achieved an impressive 88.89%, confirmed through GUS and PCR analyses. This study not only introduced a novel approach for investigating gene function in *Pinus koraiensis* but also laid a crucial foundation for developing a regenerative genetic transformation system for these plants.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f15122058/s1, Table S1: Composition of DCR basic medium; Table S2: Composition of mLV basic medium.

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References

1. Zhang, Z.; Zhang, H.G.; Mo, C.; Zhang, L. Transcriptome Sequencing Analysis and Development of EST·SSR Markers for *Pinus koraiensis*. *Sci. Silvae Sin.* **2015**, *51*, 114–120.
2. Yan, P.; Xie, Z.; Feng, K.; Qiu, X.; Zhang, L.; Zhang, H. Genetic diversity analysis and fingerprint construction of *Korean pine* (*Pinus koraiensis*) clonal seed orchard. *Front. Plant Sci.* **2023**, *13*, 1079571. [CrossRef] [PubMed]
3. Wolff, R.L.; Pédrono, F.; Pasquier, E.; Marpeau, A.M. General characteristics of *Pinus* spp. seed fatty acid compositions, and importance of delta5-olefinic acids in the taxonomy and phylogeny of the genus. *Lipids* **2000**, *35*, 1–22. [CrossRef] [PubMed]
4. Gao, F.; Peng, C.X.; Wang, H.; Tretyakova, I.N.; Nosov, A.M.; Shen, H.L.; Yang, L. Key Techniques for Somatic Embryogenesis and Plant Regeneration of *Pinus koraiensis*. *Forests* **2020**, *11*, 912. [CrossRef]
5. Chen, S.G.; Qin, C.Y.; Wang, C.H.; Cai, J.F.; Tao, J. Study on Somatic Embryogenesis induction from immature seeds of five family lines of *Pinus koraiensis*. *For. Eng.* **2021**, *37*, 13–17. [CrossRef]
6. Shen, X.; Jiang, X.; Yill, S.P.; Mahedrappa, M.K. Establishment of a technical system for somatic cell embryo culture of *Pinus koraiensis*. *J. Chengdu Univ.* **2005**, *01*, 11–14.
7. Peng, C.X.; Gao, F.; Wang, H.; Shen, H.L.; Yang, L. Optimization of maturation process for somatic embryo production and cryopreservation of embryogenic tissue in *Pinus koraiensis*. *Plant Cell Tissue Organ Cult.* **2020**, *144*, 185–194. [CrossRef]
8. Wang, G.; Fan, X.L.; Shen, X.H.; Sun, Y.L. Ultra-low temperature preservation of red pine embryonic healing tissue. *J. Shanghai Jiao Tong Univ.* **2009**, *27*, 223–225+230.
9. Gao, F.; Shi, Y.J.; Wang, R.R.; Tretyakova, I.N.; Nosov, A.M.; Shen, H.L.; Yang, L. Exogenous Glutathione Promotes the Proliferation of *Pinus koraiensis* Embryonic Cells and the Synthesis of Glutathione and Ascorbic Acid. *Plants* **2022**, *11*, 2586. [CrossRef]
10. Castander Olarieta, A.; Moncalean, P.; Montalban, I.A. Somatic Embryogenesis in *Pines*. *Methods Mol. Biol.* **2022**, *25*, 41–56. [CrossRef]
11. Ramkumar, T.R.; Lenka, S.K.; Arya, S.S.; Bansal, K.C. A Short History and Perspectives on Plant Genetic Transformation. *Methods Mol. Biol.* **2020**, *2124*, 39–68. [CrossRef] [PubMed]
12. Huang, Y.; Diner, A.M.; Karnosky, D.F. *Agrobacterium* rhizogenes-mediated genetic transformation and regeneration of a conifer: *Larix decidua*. *In Vitro Cell. Dev. Biol.-Plant* **1991**, *27*, 201–207. [CrossRef]
13. Maruyama, T.E.; Hosoi, Y. Progress in Somatic Embryogenesis of *Japanese Pines*. *Front. Plant Sci.* **2019**, *10*, 31. [CrossRef]
14. Dang, S.F.; Zhang, L.F.; Han, S.Y.; Qi, L.W. *Agrobacterium*-Mediated Genetic Transformation of *Larix kaempferi* (*Lamb.*) *Carr*. Embryogenic Cell Suspension Cultures and Expression Analysis of Exogenous Genes. *Forests* **2022**, *13*, 1436. [CrossRef]
15. Tang, W. *Agrobacterium*-mediated transformation and assessment of factors influencing transgene expression in loblolly pine (*Pinus taeda* L.). *Cell Res.* **2001**, *11*, 237–243. [CrossRef]
16. Cui, Y.; Zhao, J.; Gao, Y.; Zhao, R.; Zhang, J.; Kong, L. Efficient Multi-Sites Genome Editing and Plant Regeneration via Somatic Embryogenesis in *Picea glauca*. *Front. Plant Sci.* **2021**, *12*, 751–891. [CrossRef]
17. Hazubska-Przybyl, T.; Wawrzyniak, M.K.; Kijowska-Oberc, J.; Staszak, A.M.; RRatajczak, E. Somatic Embryogenesis of *Norway Spruce* and *Scots Pine*: Possibility of Application in Modern Forestry. *Forests* **2022**, *13*, 155. [CrossRef]
18. Liang, Y.; Xu, X.; Shen, H.L.; Gao, M.L.; Zhao, Y.; Bai, X. Morphological and endogenous phytohormone changes during long-term embryogenic cultures in *Korean pine*. *Plant Cell Tissue Organ Cult.* **2022**, *151*, 253–264. [CrossRef]
19. Gao, F.; Peng, C.X.; Wang, H.; Shen, H.L.; Yang, L. Selection of culture conditions for callus induction and proliferation by somatic embryogenesis of *Pinus koraiensi*. *J. For. Res.* **2021**, *32*, 483–491. [CrossRef]
20. Peng, C.X.; Gao, F.; Wang, H.; Tretyakova, I.N.; Nosov, A.M.; Shen, H.L.; Yang, L. Suspension Culture and Somatic Embryogenesis of *Korean pine*. Phyton. *Int. J. Exp. Bot.* **2022**, *91*, 223–238. [CrossRef]
21. Peng, C.X.; Gao, F.; Wang, H.; Shen, H.L.; Yang, L. Physiological and Biochemical Traits in *Korean pine* Somatic Embryogenesis. *Forests* **2020**, *11*, 577. [CrossRef]

22. Wang, B.; Zhang, Z.; Yin, Z.; Feng, C.; Wang, Q. Novel and potential application of cryopreservation to plant genetic transformation. *Biotechnol. Adv.* **2011**, *30*, 604–612. [CrossRef] [PubMed]
23. Yao, R.; Wang, Y. An advanced protocol for the establishment of plantlets originating from somatic embryos in *Pinus massoniana*. *3 Biotech* **2020**, *10*, 394. [CrossRef] [PubMed]
24. Zhang, Y. Establishment of Genetic Transformation Receptor Systems and Agrobacterium-Mediated Genetic Transformation in Three Coniferous Species. Ph.D. Thesis, Graduate School of Chinese Academy of Sciences, Shanghai, China, 2006.
25. Maleki, S.S.; Mohammadi, K.; Ji, K.S. Study on factors influencing transformation efficiency in *Pinus massoniana* using *Agrobacterium* tumefaciens. *Plant Cell Tissue Organ Cult.* **2018**, *133*, 437–445. [CrossRef]
26. Tang, W.; Xiao, B.; Fei, Y.J. *Slash pine* genetic transformation through embryo cocultivation with a tumefaciens and transgenic plant regeneratio. *In Vitro Cell. Dev. Biol. Plant* **2014**, *50*, 199–209. [CrossRef]
27. Lin, X.; Zhang, W.; Takechi, K.; Takio, S.; Ono, K.; Takano, H. Stable genetic transformation of *Larix gmelinii* L. by particle bombardment of zygotic embryos. *Plant Cell Rep.* **2005**, *24*, 418–425. [CrossRef]
28. Zhang, L.; Qi, L.W.; Han, S.Y. Differentially expressed genes during *Larix* somatic embryo maturation and the expression profile of partial genes. *Yi Chuan Hered.* **2009**, *31*, 540–545. [CrossRef]
29. Tang, W.; Newton, R.J. Genetic transformation of conifers and its application in forest biotechnology. *Plant Cell Rep.* **2003**, *22*, 1–15. [CrossRef]
30. Salaj, T.; Moravcikova, J.; Vookova, B.; Salaj, J. Agrobacterium-mediated transformation of embryogenic tissues of *hybrid firs* (*Abies* spp.) and regeneration of transgenic emblings. *Biotechnol. Lett.* **2009**, *331*, 647–652. [CrossRef]
31. Le, V.Q.; Belles-Isles, J.; Dusabenyagasani, M.; Trembiay, F.M. An improved procedure for production of *white spruce* (*Picea glauca*) transgenic plants using *Agrobacterium* tumefaciens. *J. Exp. Bot.* **2001**, *52*, 2089–2095. [CrossRef]
32. Liu, S.; Ma, J.; Liu, H.; Guo, Y.; Li, W.; Niu, S. An efficient system for *Agrobacterium*-mediated transient transformation in *Pinus* tabuliformis. *Plant Methods* **2020**, *16*, 52. [CrossRef] [PubMed]
33. Gleeson, D.; Lelu-Walter, M.; Parkinson, M. Overproduction of proline in transgenic *hybrid larch* (*Larix* x *leptoeuropaea* (Dengler)) cultures renders them tolerant to cold, salt and frost. *Mol. Breed.* **2005**, *15*, 21–29. [CrossRef]
34. Song, Y.; Bai, X.; Dong, S.; Yang, Y.; Dong, H.; Wang, N.; Zhang, H.; Li, S. Stable and Efficient *Agrobacterium*-Mediated Genetic Transformation of *Larch* Using Embryogenic Callus. *Front. Plant Sci.* **2020**, *11*, 584492. [CrossRef] [PubMed]