A method for permanent genetic transformation using embryonic callus of Pinus koraiensis

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Abstract

Pinus koraiensis holds the designation of a second-class protected wild plant in China, valued for its significant economic and ecological importance. However, the lack of a well-established genetic transformation system has hindered progress in functional research and breeding applications for this species. This study aimed to establish a reliable and efficient genetic transformation system of Pinus koraiensis embryonic callus, building upon somatic embryogenesis technology. Utilizing Pinus koraiensis embryonic callus and GUS as the reporter gene, Agrobacterium-mediated transformation was employed to investigate key transformation factors, such as antibiotic type and concentration, Agrobacterium bacterial solution concentration, infiltration, and co-cultivation times. The results revealed that 10 mg·L-1 of Hygromycin (Hyg) significantly inhibited Pinus koraiensis embryonic callus proliferation, while an OD600 absorbance value of 0.6 during transformation led to a remarkable 93.42±2.13% efficiency. Optimal co-cultivation for two days resulted in a transformation rate of 82.61%, with a high GUS staining rate of 88.89% in the resistant embryonic callus. Following the optimized protocol, resistant somatic embryos were successfully obtained. This research contributes to the advancement of seed resource breeding and genetic enhancement for Pinus koraiensis, providing a solid foundation for investigating gene functions related to this species.

Introduction

Scientifically classified as an evergreen tree within the *Pinus* genus of the Pinaceae family, *Pinus koraiensis*, also known as the sea pine, is naturally distributed across China, particularly in the Great and Small Xing'an Mountains and the Changbai Mountains[1]. In northeastern China, *Pinus koraiensis* holds significant importance as a native timber species. Its significance extends beyond timber, as its sapwood, bark, pine needles, and cones serve as crucial industrial raw materials[2].

Aside from its industrial utility, *Pinus koraiensis* fruit is renowned for its rich nutritional content, making it a cornerstone of both the nut economy and timber forest sectors in China. Recognized for its ecological, economic, medicinal, and nutritional value [3], *Pinus koraiensis* has been the subject of research focusing on genetic diversity, seedling reforestation, and resource utilization. Recent years have seen increased attention towards propagating and genetically enhancing *Pinus koraiensis* due to its substantial ecological and economic contributions.

Despite facing challenges in widespread use for large-scale silvicultural seedling production due to its extended growth cycle and limited asexual propagation techniques, *Pinus koraiensis* shows promise in somatic embryogenesis. This technique, known for its stable heritability, rapid reproduction, and low mutation rate, offers potential for both the regeneration and genetic improvement of *Pinus koraiensis*. Researchers like Gao[4,5] have successfully generated *Pinus koraiensis* embryonic callus from immature seed embryos, leading to plant regeneration through somatic embryogenesis. Additionally, Peng et al. [6-9] utilized *Pinus koraiensis* embryonic callus to establish an ultra-low temperature preservation system and explored proliferation differences using transcriptome and metabolomics

sequencing technology. These advancements refine crucial technological aspects of *Pinus koraiensis* somatic embryogenesis[10].

The *Agrobacterium*-mediated genetic transformation method has emerged as the most extensively studied, technically advanced, efficient, and widely utilized approach for genetic transformation in conifers[11]. Notably, Huang [12] pioneered the inoculation of sterile European larch seedlings with hairy *Agrobacterium*, leading to the successful production of transformed plants. Presently, transgenic embryogenic cultures have been effectively developed using the *Agrobacterium*-mediated technique in various tree species, including Japanese larch (*Larix kaempferi*), torch pine (*Pinus taeda*), and Norway spruce (*Picea abies*) [13-17].

By integrating the somatic embryogenesis pathway with genetic transformation technology [18], the utilization of somatic embryogenesis enables the conversion of resistant callus tissues identified during genetic transformation into somatic embryos. These embryos can subsequently germinate to yield fully resistant plants, offering potentially higher efficiency compared to traditional organ explant transformation and generating purer transformed plants.

In this study, *Agrobacterium* strain GV3101 was utilized along with the binary vector VB191103-1905rcy, employing *Pinus koraiensis* embryonic callus as the primary material. Through optimization of bacterial solution concentrations and infiltration times during the transformation process, resistant embryonic calli were successfully obtained. This established a straightforward and efficient *Agrobacterium*-mediated genetic transformation system for *Pinus koraiensis* embryonic tissue is poised to accelerate transgenesis development and enhance research into genetic improvement and molecular functions of *Pinus koraiensis*.

Results

Cef Effects on Embryonic Callus

Cef did not significantly impact the inhibition of *Pinus koraiensis* embryonic callus (Table 1). With increasing Cef concentration, the proliferation rate of *Pinus koraiensis* embryonic callus gradually decreased after 15 days. At an additional concentration of 500 $mg \cdot L^{-1}$, the fresh mass of the proliferating embryonic callus decreased by 10.01% compared to the control. The inhibitory effects on the embryonic callus of *Pinus koraiensis* were not significant across all tested Cef treatment concentrations ($p > 0.05$).

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

Cef concentration ($mg \cdot L^{-1}$)	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

Kan Effects on Embryonic Callus

There were no significant differences in the proliferation rate of *Pinus koraiensis* embryonic callus with increasing Kan concentration (Figure 1). The proliferation ploidy of embryonic callus cultured for 15 days without Kan was 3.35. Upon adding Kan at concentrations of 10, 20, 40, 80, and 100 $mg \cdot L^{-1}$, the proliferation rates were 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 when the Kan concentration in the medium reached 100 $mg \cdot L^{-1}$. However, no significant difference in the proliferation of *Pinus koraiensis* embryonic callus was noted when Kan concentration was below 100 $mg \cdot L^{-1}$. Inhibitory effects were evident only at Kan concentrations exceeding 100 $mg \cdot L^{-1}$.

Hyg Effects on Embryonic Callus

Hyg exerted a significant inhibitory effect on the proliferation of *Pinus koraiensis* embryonic callus. With increasing Hyg concentration, the proliferation ploidy of *Pinus koraiensis* embryonic callus showed a declining trend after 15 days (Figure 2). The results revealed that 4 $mg \cdot L^{-1}$ of Hyg reduced the proliferation ploidy of embryonic callus by approximately 51.72%. Furthermore, Hyg concentrations equal to or exceeding 20 $mg \cdot L^{-1}$ led to a notable reduction in embryonic callus proliferation, resulting in negative growth. Specifically, at Hyg concentrations of 20, 40, and 60 $mg \cdot L^{-1}$ in the medium, the proliferation rates of embryonic callus were reduced by 9.59, 47.94, and 56.35%, respectively. Consequently, 10 $mg \cdot L^{-1}$ of Hyg was considered the optimal screening antibiotic concentration for *Pinus koraiensis* embryonic callus ($p < 0.01$).

Effects of Infiltration Solution Concentrations

The efficiency of *Agrobacterium*-mediated transformation in *Pinus koraiensis* embryonic callus demonstrated a trend of increasing and then decreasing with the concentration of the infiltration solution. Upon varying the concentration of the solution, the highest conversion efficiency, reaching 93.42 ± 2.13%, was observed at an absorbance of 0.6 at OD_{600}. Following closely, the next highest

conversion efficiency was 89.67 ± 5.03% at an absorbance of 0.4 for the infiltration solution. Conversely, the transformation efficiencies were similar at 0.2 and 0.8, measuring 39.64 ± 4.42% and 42.63 ± 5.33%, respectively. These findings underscore the significant impact of infiltration solution concentration on the transformation efficiency of *Pinus koraiensis* embryonic callus ($p < 0.001$).

Effects of Co-culture Cycle

In a specified range, the efficiency of conversion increased with a prolonged co-culture duration. Five gradients of co-culture periods ranging from zero to four days were devised to determine the optimal co-culture cycles. The results indicated that absence of resistant tissues on tissue blocks screened immediately following decolonization without co-culture post-infiltration. The highest transformation efficiency, at 82.61%, was attained with a two-day co-culture. Extending the co-culture beyond three days resulted in a larger *Agrobacterium* presence, impacting the proliferation and growth of embryonic callus. Additionally, it became challenging to completely suppress *Agrobacterium* growth during the later stages of decolonization, even with the application of 500 $mg \cdot L^{-1}$ of Cef. Subsequent repeated sterilization further decreased transformation efficiency.

Effects of Antibiotic Species Screening

The selection of antibiotics for screening significantly impacts the efficiency of genetic transformation. Based on the results of the antibiotic susceptibility test, it was noted that Hyg demonstrated a stronger inhibitory effect on the embryonic callus of *Pinus koraiensis* compared to Kan. This study aimed to evaluate the screening efficacy of these antibiotics during genetic transformation. The findings demonstrated that the false positive rate of resistant tissues screened on medium containing 100 $mg \cdot L^{-1}$ Kan was approximately 71.05%, whereas the false positive rate with 10 $mg \cdot L^{-1}$ Hyg screening was only 13.89%.

Furthermore, Hyg displayed a more rapid inhibitory effect on embryonic callus compared to Kan. Screening on medium containing 10 $mg \cdot L^{-1}$ Hyg for ten days led to significant browning of untransformed tissues, whereas the changes observed with medium containing 100 $mg \cdot L^{-1}$ Kan were not significant. In summary, Hyg proved more effective and efficient than Kan and was better suited for screening transformed tissues of *Pinus koraiensis*.

GUS Staining of Resistant Tissues

Resistant *Pinus koraiensis* embryonic callus, cultured on a screening medium containing 10 $mg \cdot L^{-1}$ Hyg for 63 days, underwent the β-glucosidase gene (GUS) histochemical staining, with uninfiltrated tissues serving as negative controls. GUS-positive embryonic callus appeared blue, indicating the successful transfer of the GUS-containing reporter gene into *Pinus koraiensis* embryonic callus (Figure 3). Along with the 36 embryonic callus samples examined, 32 displayed blue staining, resulting in a staining rate of 88.89%.

PCR of Resistant Tissues

14 out of the 32 Hyg-resistant embryonic callus samples from *Pinus koraiensis* were randomly selected, and total DNA was extracted to serve as a template for PCR, aiming to confirm the successful transfer of the GUS reporter gene. The primers were designed based on the 35S promoter sequence, while uninfiltrated tissues and *Agrobacterium tumefaciens* served were used as negative and positive controls, respectively. The results revealed that all 14 resistant embryonic callus samples displayed a target band with a fragment length of 256 bp (Figure 4a).

To evaluate the stability of the genetic transformation in this study, the *LobHLH34* gene, previously cloned in the authors' laboratory with a size of 696 bp, was transformed using the aforementioned procedure. The resulting material was subjected to GUS staining and PCR analysis. The outcomes confirmed the successful transfer of the *LobHLH34* gene into *Pinus koraiensis* embryonic callus, thereby validating the stability of the established genetic transformation system for *Pinus koraiensis* (Figure 4b).

Effects of Gellan Gum on Somatic Embryo Maturation

The influence of gellan gum concentration on somatic embryo maturation is presented in Figure 5a. Post-screening and testing, the *Pinus koraiensis* resistant embryogenic callus was placed on a gellan gum medium containing 12 $g \cdot L^{-1}$. The *Pinus koraiensis* embryogenic callus exhibited significant drying, with the majority of tissue turning dark brown, resulting in a somatic embryogenesis rate of 4.33 g^{-1}. On gellan gum medium containing 10 $g \cdot L^{-1}$, the *Pinus koraiensis* embryogenic callus appeared dry and granular, with approximately half turning dark brown. Somatic embryos were observed as white globular or elongated structures, with multiple somatic embryos appearing connected, leading to a somatic embryogenesis rate of 17.33 g^{-1}. On gellan gum medium containing 8 $g \cdot L^{-1}$, the embryogenic callus of *Pinus koraiensis* exhibited a dry and powdery texture, with predominantly white long strips distributed uniformly. The somatic embryogenesis rate reached 21.67 g^{-1} (Table 2).

Without altering the concentration of the gellan gum medium (8, 10, and 12 $g \cdot L^{-1}$) (Figure 5b), the basal medium was switched from DCR to mLV (Supplementary File 2). The occurrence of somatic embryos was lower than observed in DCR culture, with rates of 11.67, 11, and 1.33 $g \cdot L^{-1}$, respectively. However, the number of mature malformed *Pinus koraiensis* somatic embryos was higher than that of normal somatic embryos in both DCR and mLV.

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

Discussion

Agrobacterium-mediated genetic transformation entails integrating target genes into a plant's genome by infiltrating the plant with *Agrobacterium*, facilitating their expression within the plant genome. This technique has been widely utilized due to its notable transformation efficiency and the well-established mechanisms and technologies associated with it [19].

Key factors influencing efficiency in *Agrobacterium*-mediated transformation include bacterial solution concentration, infiltration duration, co-culture duration, antibiotic screening, and the choice of explants. Fine-tuning these factors and identifying optimal thresholds can amplify transformation efficiency, yet excessive adjustments may potentially diminish efficiency.

Various plant tissues or organs, such as roots, stems, leaves, somatic embryos, mature embryos, and immature embryos, can function as recipients for genetic transformation [2021]. In studies on conifer genetic transformation, mature and immature zygotic embryos are commonly utilized [22]. For example, *Pinus massoniana* [23] and slash pine [24] have been genetically transformed using mature zygotic embryos. Gao[9] employed immature *Pinus koraiensis* zygotic embryos to induce embryonic callus tissue, establishing a regeneration system via somatic embryogenesis. In· the current study, the *Pinus koraiensis* embryonic callus was selected for genetic transformation, achieving a high transformation rate of 88.89%, with the resistant embryonic callus cultured into resistant somatic embryos.

During the transformation process, both infiltration duration and concentration are critical factors for successful outcomes. Insufficient concentration of the bacterial solution leads to inadequate adherence of *Agrobacterium* to the plant material, resulting in low transformation efficiency. Conversely, excessively high concentrations result in the over-proliferation of *Agrobacterium* within the plant material during later co-culture stages, posing challenges in subsequent decolonization and potentially risking plant senescence. Infiltration duration also plays a role in transformation efficiency. Too short a duration does not permit sufficient attachment of *Agrobacterium* to the plant material, while excessively long infiltration periods lead to an unnecessary buildup of *Agrobacterium*[21].

In conifer genetic transformation systems, the bacterial solution concentrations during infiltration typically range from an OD_{600} absorbance of 0.3 to 0.8, with

infiltration times ranging from ten to 40 minutes. For example, some *Larix* species have achieved the highest transformation rate with a bacterial solution concentration of approximately OD_{600}=0.6 and an infiltration time of 20 min25. 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 26. Transformation of *Pinus taeda* exhibited a transient expression rate of around 70% with a bacteriophage concentration OD_{600}=0.8 and infiltration times ranging from 15 to 30 min [27]. In this study, the most favorable outcomes were attained by using an OD_{600} absorbance of 0.5 for infiltrating *Pinus koraiensis* embryonic callus for 20 min.

The co-culture phase signifies the stage at which T-DNA integration is conveyed into the plant genome. Optimal co-culture conditions for conifers have been determined to be two to four days in dark culture at 25°C. Straying from this timeframe, either too short or too long, can diminish conversion efficiency [28]. For example, in the genetic transformation of the embryonic callus of hybrid fir [29] and white spruce [30], the co-culture period was fixed at two days. For mature zygotic embryos of *Pinus massoniana*[23], incubation spanned a total of three days. The results of this study indicated that a two-day co-culture duration yielded superior efficiency in tissue transformation compared to one, three, or four days. Prolonged co-culture beyond three days led to repetitive decolonization of tissues due to elevated bacterial concentrations, ultimately leading to tissue death.

The selection of antibiotic type and its concentration are crucial factors that influence the transformation process. Sensitivity tests conducted on other plant species have revealed that employing multiple screenings enhances the transformation rate while reducing the false positive rate. In conifer genetic transformation, two primary antibiotics, Kan and Hyg, are predominantly utilized for screening. Yaupon [31] achieved resistance in tissues by conducting Kan screening at concentrations ranging from 50 to 75 $mg{\cdot}L^{-1}$. A positive rate of 66.2% in obtaining resistant embryogenic callus was achieved in hybrid larch under three consecutive screens with 20 $mg{\cdot}L^{-1}$ of Kan [32]. The false positive rate of resistance obtained via Hyg screening in *Larix olgensis* was only 11.66%[33].

In this study, sensitivity experiments were carried out on the *Pinus koraiensis* embryonic callus, demonstrating its insensitivity to Kan. At a Kan concentration of 100 $mg{\cdot}L^{-1}$, significant differences in the proliferation rate of *Pinus koraiensis* embryonic callus were not detected. Conversely, when screening with Hyg as the antibiotic, the proliferation rate of *Pinus koraiensis* embryonic callus significantly decreased. At a concentration of 4 $mg{\cdot}L^{-1}$, the proliferation rate was 68.42%, and the embryonic callus barely proliferated at concentrations surpassing 20 $mg{\cdot}L^{-1}$, with the group tissues dying at 40 $mg{\cdot}L^{-1}$.

Furthermore, the number of screenings was hypothesized to affect the detected positive conversion rate. Due to the sensitivity of embryonic callus, the initial screening yielded fewer resistant tissues and a higher false positive rate. Subsequent screenings, particularly the second and third rounds, effectively decreased the false positive rate and enhanced the conversion rate. Consequently, 36 resistants

embryonic calli were obtained in this study using Hyg as the antibiotic, subjected to three consecutive screenings lasting 21 days each, resulting in a significantly low false positive rate of 11.11%.

Conclusions

In this study, a highly efficient genetic transformation system for *Pinus koraiensis* embryonic callus was successfully established and optimized, marking a significant breakthrough in this field. *Agrobacterium* strain GV3101 was utilized in combination with the binary vector VB191103-1905rcy, resulting in the acquisition of 36 resistant embryogenic calli under specific conditions: an OD_{600} absorbance value of 0.6 for the infiltrating solution, a two-day co-culture duration, and 10 $mg{\cdot}L^{-1}$ Hyg as the screening antibiotic. The transformation rate reached an impressive 88.89%, as validated by GUS and PCR analyses. This innovative study not only introduced a new method for investigating the gene function of *Pinus koraiensis* but also established a crucial foundation for developing a regenerative genetic transformation system for *Pinus koraiensis* plants.

Methods

Plant material

Ling [34] provided *Pinus koraiensis* embryonic material, which underwent induction and was then inoculated in a DCR medium (Supplementary File 1). This medium consisted of 0.5 $mg{\cdot}L^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 $mg{\cdot}L^{-1}$ 6-benzyladenine (6-BA), 30 $g{\cdot}L^{-1}$ sucrose, 4 $g{\cdot}L^{-1}$ gellan gum, 0.1 $mg{\cdot}L^{-1}$ inositol, 0.5 $mg{\cdot}L^{-1}$ glutamine (Gln), 0.5 $mg{\cdot}L^{-1}$ casein hydrolyzed (CH), with a pH of 5.9. Cultivation occurred in darkness at 25°C, with reproductive successions carried out every two days.

Agrobacterium strains

The *Agrobacterium* strain (GV3101) and the plasmid (pBI121) used in this study were maintained in the laboratory. The vector strain utilized was VB191103-1905rcy, with the plasmid, it carried containing the β-glucosidase gene (GUS), sourced from the State Key Laboratory of Forest Genetic Breeding, Northeast Forestry University, China.

Antibiotic sensitivity test of embryonic callus

Following a ten-day incubation in the DCR proliferation medium, about 0.2 g of embryonic callus was transferred to the DCR medium supplemented with 0.5 $mg{\cdot}L^{-1}$ 2,4-D, 0.1 $mg{\cdot}L^{-1}$ 6-BA, and 30 $g{\cdot}L^{-1}$ sucrose. The medium also contained additional components such as 4 $g{\cdot}L^{-1}$ gellan gum, 0.1 $mg{\cdot}L^{-1}$ inositol, 0.5 $mg{\cdot}L^{-1}$ Gln, 0.5 $mg{\cdot}L^{-1}$ casein hydrolyzed, along with cefotaxime (Cef), kanamycin (Kan), or hygromycin (Hyg), at pH 5.9. Screening was conducted at 25±1°C for 15 days under dark conditions. The fresh mass of the embryonic callus was measured, and this process was repeated three times. Gradient

concentrations were employed for Kan (0, 10, 20, 30, and 40 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}).

Agrobacterium strain culture

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

Genetic transformation

The fresh bacterial solution was transferred into a sterile centrifuge tube and centrifuged (Therom, China) at 4℃ and 8,000 rpm for ten minutes to collect the bacterial pellet for the infiltration solution. Fresh embryonic callus was carefully chosen and submerged in the infiltration solution, which contained 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 OD_{600}. After a 20-min infiltration period, the solution was poured off, and the tissue surface was gently blotted with sterile filter paper before being transferred to the co-culture medium (DCR+250 mg·L^{-1} Cef) for two, three, or four days at 25±1 ℃ in the dark.

Following co-culture, the tissues were rinsed twice with sterile water for two minutes each, followed by two washes with a suspension containing 500 mg·L^{-1} Cef for 3 min each. Any excess water on the surface of the cleaned callus was carefully blotted dry with sterile filter paper. The embryonic callus was spread out into sheets on a recovery medium at 25±1°C and cultured in the dark for seven, 14, or 21 days. Following this, the resistant tissues underwent screening using a specialized screening medium. The number of resistant tissues obtained was documented over three successive screenings, each lasting 21 days. These resistant tissues were then subjected to GUS histochemical staining and PCR analysis at the molecular level. Once appropriately confirmed as resistant, the embryonic callus underwent succession culture and somatic embryo maturation (Figure 6).

GUS histochemical assay

The resistant tissues were carefully selected and immersed in an appropriate volume of GUS staining solution, formulated with 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 underwent

staining in a water bath at 37℃ for one week. Non-transformed tissues were used as the negative control, and the color development status of the embryonic callus was observed and documented. Among these, tissues displaying a blue-green color were identified as transgenic tissues, whereas colorless transparent or slightly yellow tissues were considered false-positive tissues.

PCR analysis

Using *Pinus koraiensis* resistant callus DNA as a template, genomic DNA was extracted from 14 randomly selected resistant cell lines employing the CTAB method. PCR molecular assays were carried out with upstream and downstream primers for 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 10μL of Green Taq MIX, 1μL each of upstream and downstream primers, 1μL of DNA, and 7μL of ddH_2O. The reaction conditions included denaturation at 94℃ for 3 min, followed by denaturation at 94℃ for 30 sec, annealing at 60℃ for 15 sec, extension at 72℃ for 15 sec, repeated for 35 cycles. The final extension was at 72℃ for 10 min.

The PCR samples were then separated using a 1.0% (w/v) agarose gel electrophoresis with wild-type *Pinus koraiensis* embryonic callus as the negative control and *Agrobacterium* solution as the positive control. The electrophoresis results were observed and captured using a gel imager (Tanon 2500R, China). The resistant embryogenic calli were identified based on the presence of correct destination bands.

Somatic embryo maturation

The approach for somatic embryo maturation was adapted from Peng [35] with some changes. Three grams of various transgenic *Pinus koraiensis* embryonic callus samples were placed into 20 mL of liquid medium (DCR+30 $g{\cdot}L^{-1}$ sucrose) devoid of any hormones and incubated for seven days. Subsequently, the mixture was poured off and excess liquid was absorbed using sterile filter paper. The embryonic callus was then dried on an ultra-clean bench for ten minutes.

The transgenic *Pinus koraiensis* embryogenic callus obtained through the aforementioned methods was transferred to a DCR maturation medium, comprising varying concentrations of gellan gum (8, 10, and 12 $g{\cdot}L^{-1}$), 20 $mg{\cdot}L^{-1}$ ABA, 0.1 $g{\cdot}L^{-1}$ inositol, 0.5 $g{\cdot}L^{-1}$ Gln, 0.5 $g{\cdot}L^{-1}$ CH, 30 $g{\cdot}L^{-1}$ maltose, with pH adjusted to 5.9. All somatic embryogenesis events were tallied after a 10-week period.

Statistical analysis

SPSS statistical analysis software was used for variance analysis, setting the significance level at $p < 0.05$. The tables and figures were generated using Microsoft Excel 2010.

Declarations

Data Availability

All data in this study are available in the manuscript or the Supplementary materials.

Ethical declarations

The plant collection and use was in accordance with all the relevant guidelines.

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Author contributions statement

S.L. conceived and designed the experiments; H.Z. provided the financial support; L.Y. and H.S. provided the *Pinus koraiensis* embryonic callus; H.H. and H.D. performed the experiments, analyzed the data, prepared the figures and tables, and they contributed equally to this work; Y.W and W.Z. reviewed drafts of the paper. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare no competing interests

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Figures

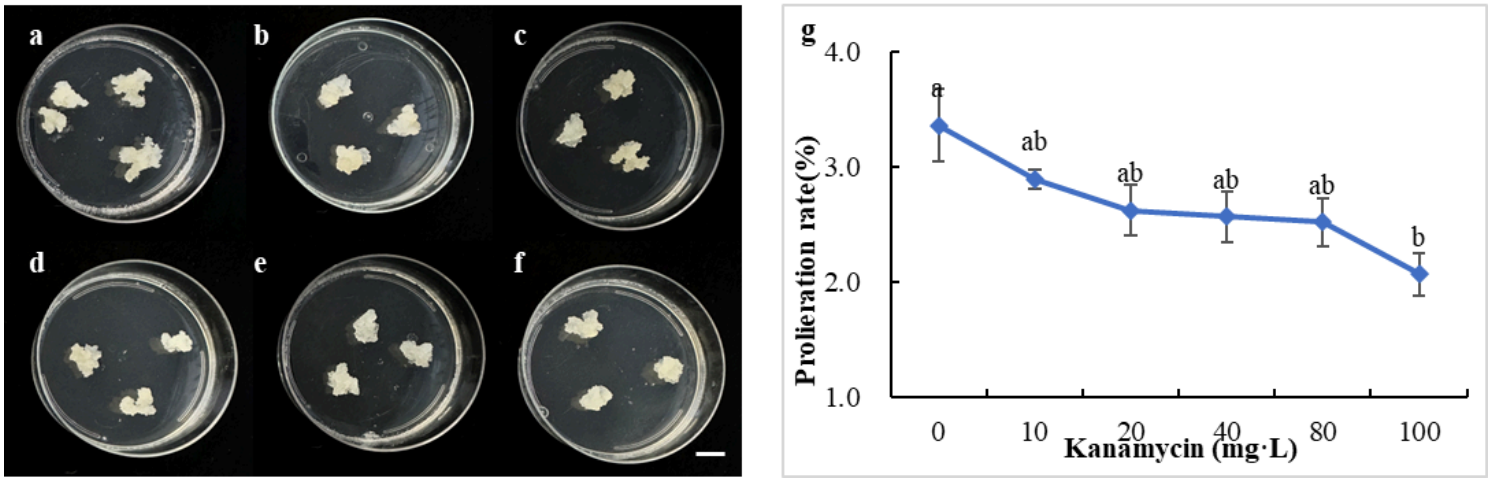

Figure 1

Effect of Kan on 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 the callus with different Kan concentrations, bar=1 cm.

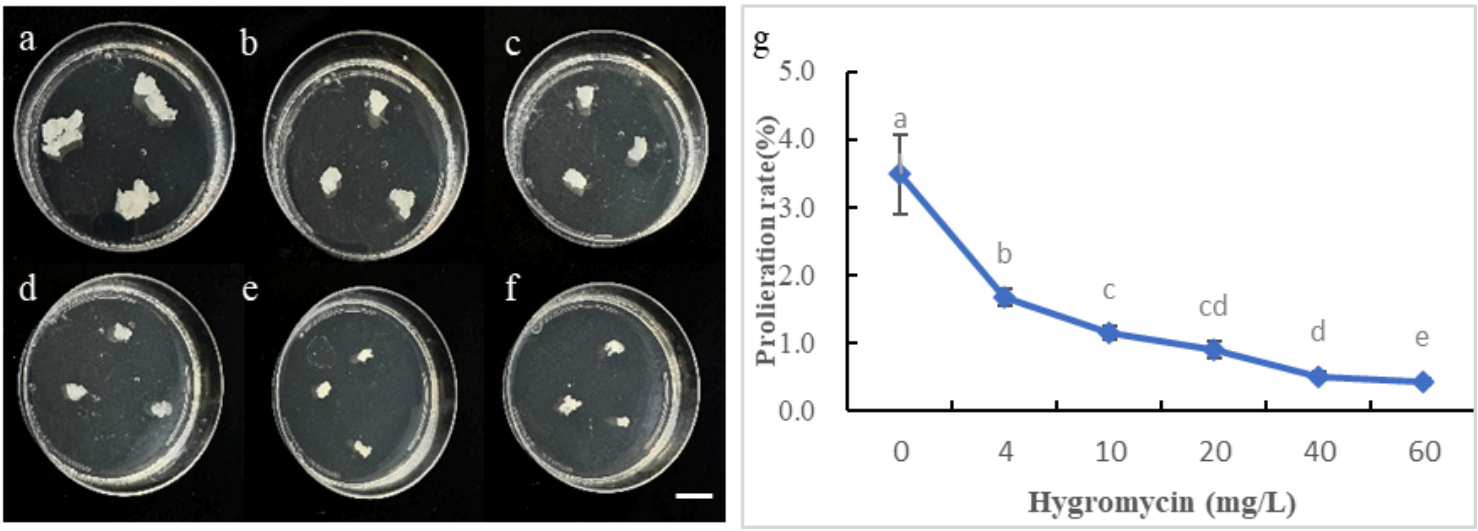

Figure 2

Effect of Hyg on 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.

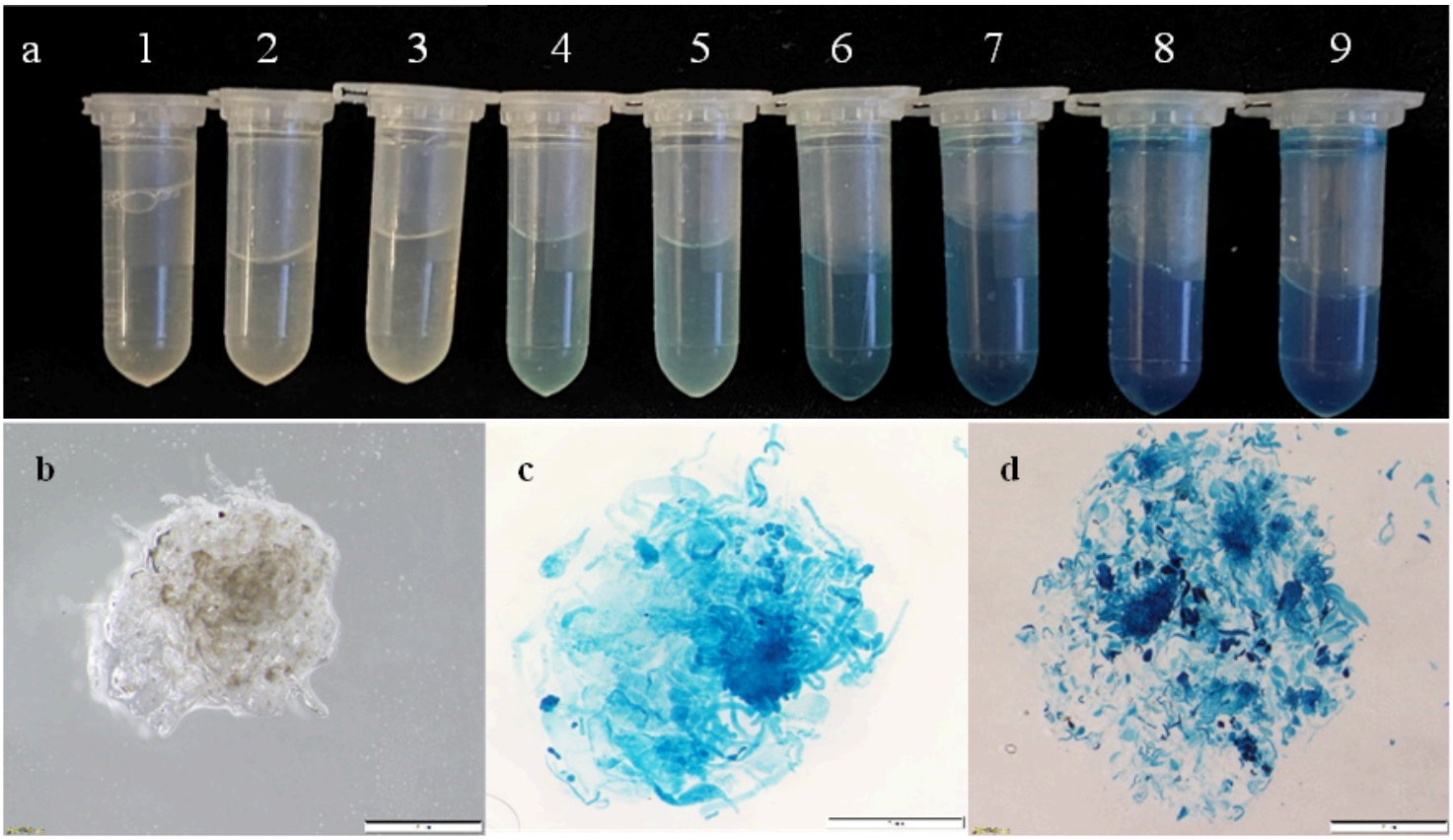

Figure 3

GUS staining of resistant embryonic callus. (a,1-3) GUS staining of wild-type embryonic callus (a,4-6) GUS staining of resistant callus(a,7-9) 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.

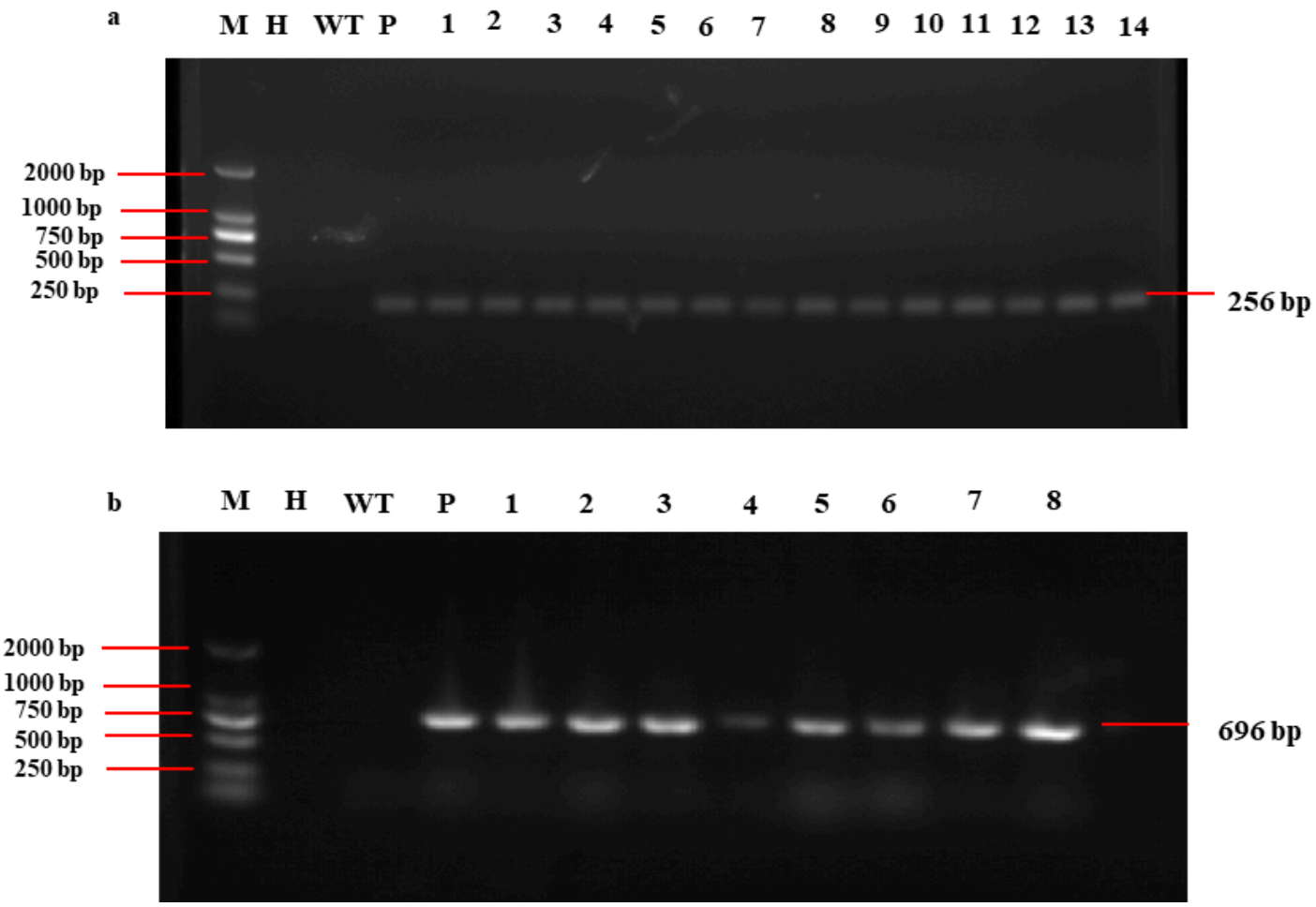

Figure 4

PCR Assay. (a) PCR assay of resistant callus with GV3101 vector (b) PCR assay of *LobHLH*gene.

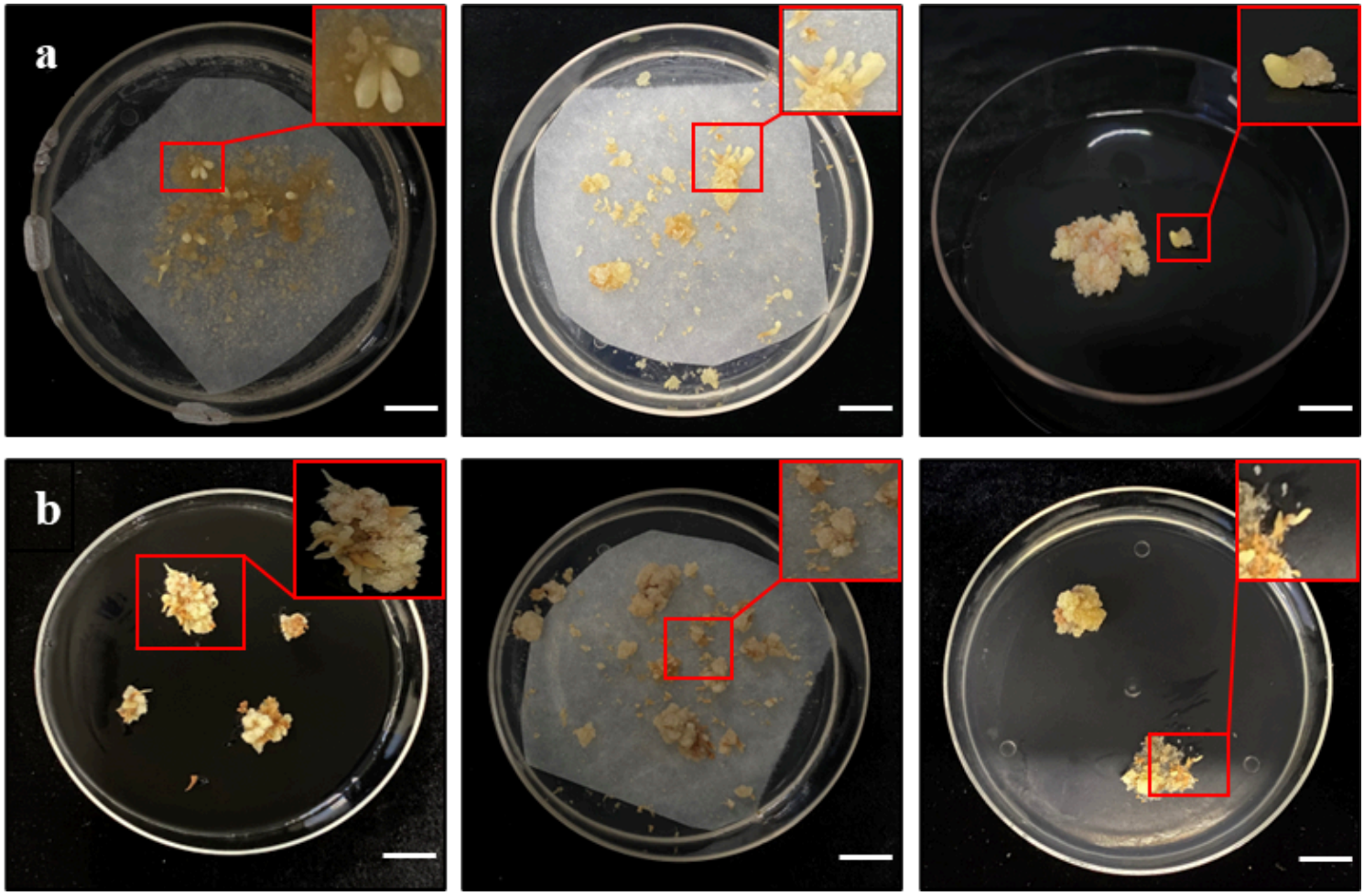

Figure 5

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.

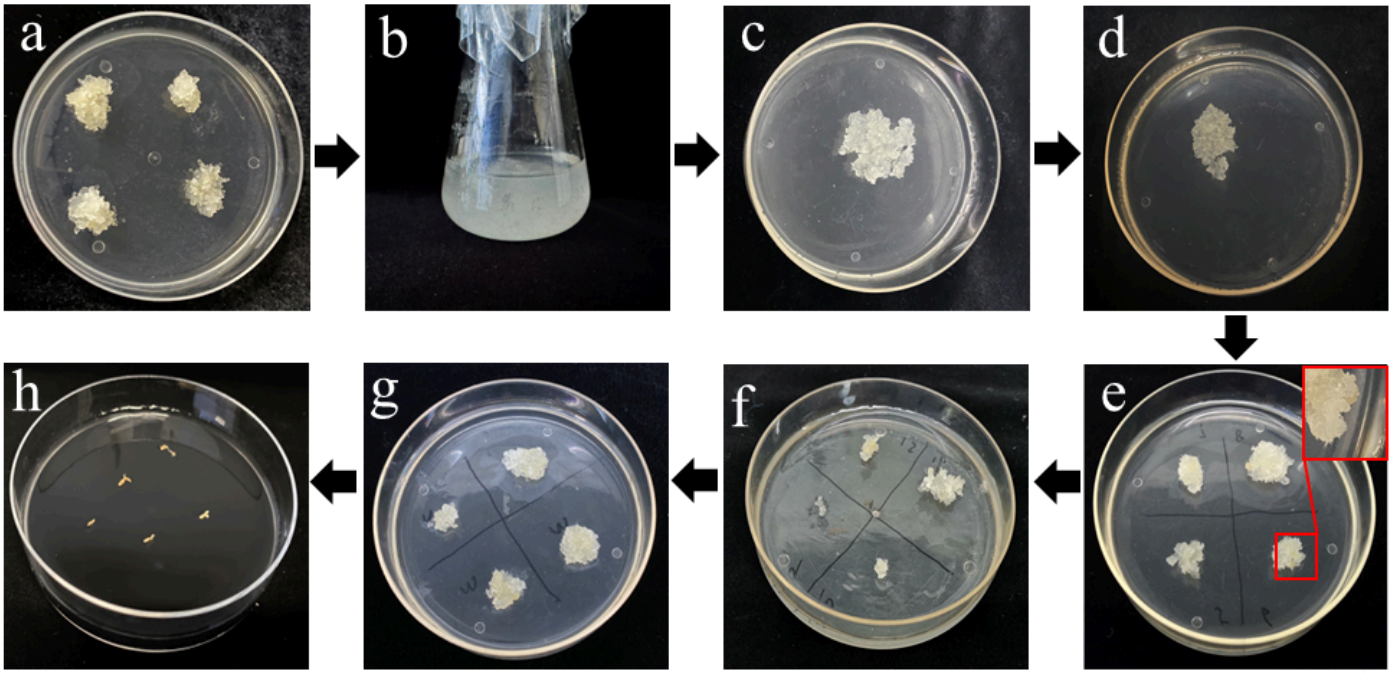

Figure 6

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.

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