

## Article

# Agrobacterium-Mediated Genetic Transformation of *Larix kaempferi* (Lamb.) Carr. Embryogenic Cell Suspension Cultures and Expression Analysis of Exogenous Genes

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**Abstract:** A simple and efficient protocol for the genetic transformation of Japanese larch (*Larix kaempferi* (Lamb.) Carr.) was developed by altering the infection method and duration and the bacterial removal process. More than 600 hygromycin-resistant embryonal masses with the vector pCAMBIA1301 were obtained, with an average of 20 transgenic lines per gram of fresh weight. Nine hygromycin-resistant transformation events (designated P1–P9) were analyzed using PCR, quantitative real-time PCR, and histochemical  $\beta$ -glucuronidase (*GUS*) assays. The *GUS* transcript abundance in each transformed cell line ranged from  $10^1$  to  $10^3$  magnitudes, with a maximum abundance of  $2.89 \times 10^3$ . In addition, the pLaTCTP::*GUS* vector, which contains *GUS* under the control of the *L. kaempferi* LaTCTP promoter, led to constitutive expression of *GUS* in embryonal-suspensor mass and somatic embryos. The transcript abundance of the exogenous genes *HPT* and *GUS*, driven by the CaMV 35S or LaTCTP promoter, ranged from  $10^1$  to  $10^4$ , which was equivalent to genes with moderate and low abundances in Japanese larch. The relatively low expression levels of exogenous genes in transformants might reflect the large genome of Japanese larch. Additional transgenic cell lines need to be screened to obtain transformants with higher expression levels of foreign genes for further functional research in Japanese larch.

**Keywords:** larch; genetic transformation; somatic embryogenesis; *Agrobacterium tumefaciens*



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## 1. Introduction

Larches (*Larix* spp.) are conifers indigenous to the Northern Hemisphere, comprising approximately 10 species distributed in North America, Europe, and Asia [1]. Among these species, Japanese larch (*Larix kaempferi* (Lamb.) Carr.) is characterized by rapid juvenile growth [2]. However, because of the long life history and complex genetic background of individual trees, the use of conventional breeding methods is difficult and propagation by seed inevitably leads to the segregation of desirable characteristics [3]. The application of modern breeding technologies to achieve rapid breeding of superior larch cultivars has become a top priority. Somatic embryogenesis may provide a novel procedure for the rapid multiplication of commercial hybrid larch genotypes.

Somatic embryogenesis is a valuable experimental system for the investigation of the regulatory mechanisms involved in plant development. However, somatic embryogenesis in conifers presents a number of challenges, such as difficulties with embryonal-suspensor mass (ESM) induction, a high frequency of abnormal embryo development during the maturation stage, and difficulties with in vitro synchronization [4–6]. Overcoming these problems requires knowledge of the molecular mechanisms of crucial genes that regulate

the different developmental stages of somatic embryogenesis. MicroRNAs (miRNAs) of *L. kaempferi* have been identified using high-throughput sequencing [7,8] and might participate in the regulation of synchronism through responses to abscisic acid (ABA) during larch somatic embryogenesis [9]. Among these miRNAs, miR171, -159, -169, and -172 play important regulatory roles in the transformation of embryogenic tissue to non-embryogenic tissue [10]. Thus, an efficient transformation procedure is a prerequisite for functional studies of the aforementioned genes.

Somatic embryogenesis is the basis of the genetic transformation system used for the majority of gymnosperms. Particle bombardment and *Agrobacterium*-mediated transformation (AMT) are the transformation methods most frequently used in conifers [11]. The advantages of the latter over the former include the production of lower copy numbers, greater stability in transgene expression levels, and fewer instances of gene silencing [11–15]. The AMT approach has been successfully applied to a variety of tissues in numerous coniferous species, including Norway spruce (*Picea abies* (L.) Karst.), white spruce (*Picea glauca* (Moench) Voss), black spruce (*Picea mariana* (Mill.) Britt., Sterns and Poggenburg), loblolly pine (*Pinus taeda* L.) [14,16], maritime pine (*Pinus pinaster* Ait.) [17], and Korean fir (*Abies koreana* Wilson) [18], through the use of embryogenic masses. The first successful procedure for the stable transformation of hybrid larch (*L. kaempferi* × *L. decidua* Mill.) involved co-cultivation with an *Agrobacterium tumefaciens* strain C58/pMP90/pMRKE70Km [19]. However, the efficiency of stable transformations was low, averaging 1–2 transgenic lines from 50 co-cultivated embryogenic masses [19]. By modifying the initial protocol, such as the procedure by which the embryogenic masses were subcultured and co-cultivated on filter paper, and by pre-culturing the embryogenic masses with the bacterial suspension for 6 h, the transformation efficiency was significantly increased to 42 kanamycin-resistant embryogenic masses per gram of fresh weight (FW) [2]. However, the protocol involves multiple handling steps, such as multiplication procedures on filter paper and pre-culture for 6 h before co-cultivation, which are time consuming and increase the risk of culture contamination. Only three to five transformed lines per gram of FW of the co-cultivated embryogenic tissue were retrieved when a similar procedure was applied to other Pinaceae species, such as white pine (*Pinus strobus*) [20]. In our laboratory, only five *LamiR166a* overexpressed embryonic cell lines were recovered from the embryogenic tissue initially co-cultured with the *A. tumefaciens* strain GV3101 [21]. The embryogenic cells did not survive after co-cultivation and an excessive growth of bacterial cells occurred. Thus, the exploration of the specific AMT parameters required for the improved transformation of different larch species is necessary.

The present study aimed to establish a simple and efficient genetic transformation system for *Larix kaempferi* using the *A. tumefaciens* strain GV3101 in conjunction with the binary vector pCAMBIA1301 (Figure S1). This novel transformation system may accelerate the development of genetically modified *L. kaempferi* and promote further molecular functional studies.

## 2. Materials and Methods

### 2.1. Plant Materials and Culture Conditions

The highly embryogenic cell line S287 of *Larix kaempferi* was induced from immature embryos on the induction medium (S basal medium [22], supplemented with 450 mg/L glutamine, 500 mg/L casein hydrolysate, 1000 mg/L inositol, 2.2 mg/L 2,4-dichlorophenoxyacetic acid, and 0.8 mg/L 6-benzylaminopurine) [6,23] and subcultured at 3-week intervals on a solid proliferation medium (S + B basal medium [24], supplemented with 450 mg/L glutamine, 500 mg/L casein hydrolysate, 1000 mg/L inositol, 0.11 mg/L 2,4-dichlorophenoxyacetic acid, and 0.04 mg/L 6-benzylaminopurine). Approximately 2 g of embryogenic culture was dissociated into a suspension by transfer to a 250 mL flask containing 100 mL liquid proliferation medium and culture in a 10-day cycle at 25 °C in the dark on a gyratory shaker at 100–110 rpm [6,23]. Once grown to 200 mg/mL FW, the embryogenic suspensions were used for AMT.

## 2.2. Hygromycin Sensitivity of Embryogenic Line S287

The hygromycin sensitivity of line S287 was assessed by transferring the embryogenic cultures to the solid proliferation medium supplemented with hygromycin (0, 3, 4, 5, 7, or 9 mg/L). The FW of the ESM was recorded at the start of the culture period (day 0) and after 20 days. The percentage ESM proliferation was calculated to determine the hygromycin concentration that effectively prohibited cell proliferation using the following formula: ESM proliferation rate (%) = [(ESM FW after 20 days–ESM FW at day 0)/ESM FW at day 0] × 100. Three petri dishes, each containing seven pieces of callus as the inoculum, were used for each experiment, and the experiment was repeated twice. The means of three replicates were analyzed with Student's *t*-test to assess the significance of the differences between hygromycin concentrations at  $\alpha = 0.05$  significance level. Data are presented as the mean  $\pm$  standard deviation.

## 2.3. Agrobacterium Strains and Binary Vectors

The binary vector Pcambia1301, 35S::GUS, and recombinant Pcambia-LaTCTP promoter-GUS binary vector (pLaTCTP::GUS) were used. The first two vectors harbor the hygromycin phosphotransferase (HPT) gene as a selection marker and the  $\beta$ -glucuronidase (GUS) reporter gene driven by the *Cauliflower mosaic virus* (CaMV) 35S promoter. The latter vector harbors the GUS gene driven by the endogenous LaTCTP promoter (pLaTCTP). The *Agrobacterium tumefaciens* strain GV3101, harboring one of the three binary vectors, was cultured at 28 °C for 36 h on a solid LB medium supplemented with 50 mg/L kanamycin, 50 mg/L rifampicin, and 50 mg/L gentamicin. A single *Agrobacterium* clone was selected and cultured in 2 mL liquid LB medium supplemented with the same antibiotics at 28 °C for 24 h on a rotary shaker (200 rpm). The culture was transferred to 50 mL LB medium and grown under the same conditions for 5–6 h to OD<sub>600</sub> = 0.6–0.8. The bacterial cells were collected by centrifugation at 4000 rpm for 10 min. The pellet was resuspended to OD<sub>600</sub> = 0.05–0.1 in 100 mL sterile water, and 200  $\mu$ M acetosyringone was added for transformation.

## 2.4. Agrobacterium-Mediated Transformation

Approximately 10 g (~50 mL) of the embryogenic suspension was added to an equal volume of the agrobacterial suspension. Based on the characteristics of the embryogenic cell suspension cultures, we improved the vacuum filter (Figure S2), which comprised a Buchner funnel with 400-mesh stainless steel screens instead of filter paper and a microvacuum pump, for the removal of excess liquid from the embryogenic cell suspensions. The filtered cultures were spread on a solid proliferation medium supplemented with 100  $\mu$ M acetosyringone and co-cultivated for ~46–120 h in the dark at 25 °C. *Agrobacterium* cells were then removed from the cultures with the vacuum filter. The co-cultivated embryogenic suspensions were washed three times with 100 mL sterile distilled water, and then three times with 100 mL cefotaxime solution (500 mg/L). The suspensions were transferred to a selective medium (a solid proliferation medium supplemented with 5 mg/L hygromycin and 500 mg/L cefotaxime) for the selection of transgenic materials. After ~4–8 weeks, hygromycin-resistant embryonal masses 3 mm in diameter were transferred to a fresh medium for further selection. The surviving putative transformants were maintained on the selective medium during all of the following steps until the completion of somatic embryogenesis. Nine independent putative hygromycin-resistant lines representing transformation events with the binary vector pCAMBIA1301, designated P1–P9, were selected for PCR detection, qRT-PCR, and histochemical GUS analysis.

## 2.5. PCR Detection

The selected hygromycin-resistant lines were subjected to molecular analysis using a PCR approach. Genomic DNA was isolated from 100 mg fresh culture of the hygromycin-resistant lines and the non-transgenic ESM using PlantZol (TransGen, Beijing, China). For the PCR analysis, three combinations of primers were used (Table 1). The primers HPT-F

and HPT-R amplified a 480 bp fragment of the *HPT* gene. The primers *virG-F* and *virG-R* amplified a 485 bp fragment of the *virG* sequence, which is a sequence of the Ti plasmid located outside of the T-DNA. The primers *GUS-F* and *GUS-R* amplified a 450 bp fragment of the *GUS* gene. The amplifications were conducted using the following PCR program: one cycle at 94 °C for 5 min, then 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, followed by one cycle at 72 °C for 5 min. The PCR products were analyzed by electrophoresis in 1% agarose gel. Control DNA from the non-transformed *ESM* and *Agrobacterium* strain GV3101 harboring pCambia1301 were included in the analysis to ensure that the reagents were not contaminated.

**Table 1.** Oligonucleotide primers used in the study.

Primer Name	Sequence (5'-3')	Application
HPT-F	5'-AGCGAGAGCCTGACCTATTG-3'	PCR analysis for <i>HPT</i>
HPT-R	5'-CTCCATACAAGCCAACCACG-3'	PCR analysis for <i>HPT</i>
<i>virG-F</i>	5'-ACGAAGTCCGATGTTCCAAT-3'	PCR analysis for <i>virG</i>
<i>virG-R</i>	5'-TCAGCGTCAAAGAAATAGCC-3'	PCR analysis for <i>virG</i>
<i>GUS-F</i>	5'-GTCAATGTAATGTTCTGCGACG-3'	PCR analysis for <i>GUS</i>
<i>GUS-R</i>	5'-GAAGTTCATGCCAGTCCAGC-3'	PCR analysis for <i>GUS</i>
qEF-F	5'-GACTGTACCTGTTGGTCGTG-3'	Real-time PCR for <i>EF</i>
qEF-R	5'-CCTCCAGCAGAGCTTCAT-3'	Real-time PCR for <i>EF</i>
qeIF-F	5'-CCTGCCCTTTGGGTTACT-3'	Real-time PCR for <i>eIF</i>
qeIF-R	5'-TTCAGTGTTACGAGGATGCTAA-3'	Real-time PCR for <i>eIF</i>
qAPm-F	5'-TGACCCTGGAAAGCCTATC-3'	Real-time PCR for <i>APm</i>
qAPm-R	5'-AGCCGAAGAGTCCCTGTTA-3'	Real-time PCR for <i>APm</i>
qHPT-F	5'-CTCCAACAATGTCCTGAC-3'	Real-time PCR for <i>HPT</i>
qHPT-R	5'-TCTGCTGCTCCATACAAGC-3'	Real-time PCR for <i>HPT</i>
qGUS-F	5'-AAGCGTGGTGATGTGGAGTA-3'	Real-time PCR for <i>GUS</i>
qGUS-R	5'-CGTAATAACGGTTCAGGCAC-3'	Real-time PCR for <i>GUS</i>
qTCTP-F	5'-ATTGTCAGAGGAACGCCA-3'	Real-time PCR for <i>TCTP</i>
qTCTP-R	5'-CATAGACCCATCATCGTGC-3'	Real-time PCR for <i>TCTP</i>

## 2.6. Histochemical *GUS* Assay

Histochemical assays for *GUS* staining were performed using the  $\beta$ -Galactosidase Reporter Gene Staining Kit (HUAYUEYANG, Beijing, China). The putatively transformed cell lines and somatic embryos at different developmental stages were stained by incubation for 12 h at 37 °C. Chlorophyll was removed by soaking in 70% ethanol for 2–3 times until the negative control material was white.

Based on the results of qRT-PCR analyses and *GUS* staining of the transformed cell lines, the lines P2, P5, and pLaTCTP::*GUS*-4 were selected for somatic embryogenesis. The *ESM* of the lines P2 and P5 were cultured on the proliferation medium for 16 days and were then transferred to a differentiation medium (S + B basal medium, supplemented with 450 mg/L glutamine, 500 mg/L casein hydrolysate, 100 mg/L inositol, 7.5% (*w/v*) PEG4000, and 20 mg/L ABA) and cultured in the dark at 25 ± 2 °C [23]. Based on morphological traits, somatic embryos at different developmental stages were sampled for the *GUS* assay and qRT-PCR.

## 2.7. Gene Expression Analysis by Quantitative Real-Time PCR

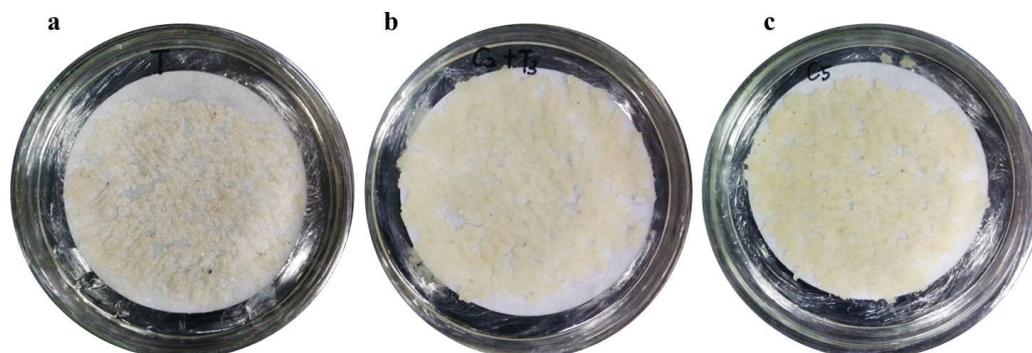
Total RNA was extracted from various tissues with the RNAiso Plus and RNAiso-mate for Plant Tissue kits (TaKaRa, Dalian, China). Then, 1  $\mu$ g RNA was reverse transcribed using the iScript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad, Cat. No. 172-5035) following the manufacturer's protocol. The *GUS* expression level was determined by conducting quantitative real-time PCR (qRT-PCR) analyses with the SYBR Premix EX Taq Kit (TaKaRa) and the CFX96™ Real-Time System. Four fragments, 1638 bp of Elongation factor (*EF*; GenBank accession No. JX157845), 782 bp of Translation initiation factor (*eIF*; GenBank accession No. JR153705), 805 bp of Adaptor-related protein complex subunit mu (*APm*;

GenBank accession No. JR140253), and 504 bp of Translationally controlled tumor protein (*TCTP*; GenBank accession No. JR140253), were amplified and independently cloned into the pEASY-Blunt Zero vector (TransGen, Beijing, China), which were used as plasmid standards for the above endogenous genes in absolute quantitative analysis, respectively. The *EF*, *eIF*, *APm*, *HPT*, *GUS*, and *TCTP* gene-specific primers used are listed in Table 1. The specificity of the primers designed for qRT-PCR was confirmed by separating the PCR products on agarose gels and sequencing clones. Each reaction was performed with 2  $\mu$ L diluted cDNA in a total reaction volume of 25  $\mu$ L. Using the aforementioned four plasmid DNAs and pCAMBIA1301 as standard materials, standard curves for *EF*, *APm*, *eIF*, *TCTP*, *HPT*, and *GUS* were generated using a gradient serial dilution of the plasmid as the template. Relative quantifications were normalized against the expression of *EF* [7]. Expression levels were estimated using absolute and relative quantification methods. Data are presented as the mean  $\pm$  standard deviation of three replicates.

### 3. Results

#### 3.1. Optimization of the Transformation Procedure

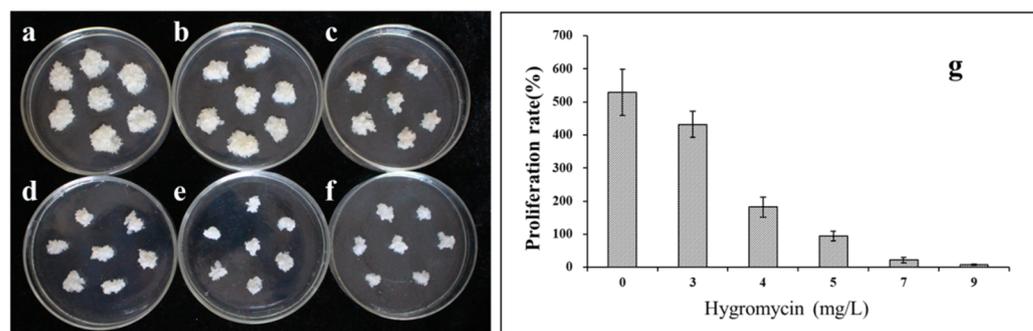
Liquid medium-based suspension cultures have been established and used for the AMT of many gymnosperm species, such as Norway spruce, loblolly pine, and white pine [16,20]. The approach has the advantage of providing a large amount of high-quality materials with vigorous growth within a short period. In the present study, a suspension culture for larch embryogenic cells in a liquid medium was established. The embryogenic suspensions cultured to 200 mg/mL FW were mixed with a lower concentration of bacteria ( $OD_{600} = 0.05\text{--}0.1$ ) to achieve infection without the pre-culturing of mixtures [2,25,26]. The co-cultivation time was determined according to whether the turbidity of the embryogenic masses was slightly turbid. When the turbidity of the culture became turbid or too turbid, the *Agrobacterium* cells had grown excessively and the embryonic cells were damaged, which affected the screening of the transformed cells in the next step. In most experiments performed, the co-cultivation period of 46–120 h was conducive to cell recovery and acquiring hygromycin-resistant cell lines. To eliminate the *Agrobacterium* cells, the vacuum filter was replaced to remove excess liquid containing the bacteria (Figure S2). The improved filter enabled the large-scale removal of bacteria from the suspension cultures in less than 15 min. It was found that 500 mg/L cefotaxime was conducive to the recovery of embryogenic cells co-cultured with *Agrobacterium*, whereas 300 mg/L timentin was detrimental to the recovery (Figure 1). Therefore, 500 mg/L cefotaxime was used to inhibit bacterial growth. The adjustment of these factors did not increase the transformation efficiency, but enabled the development of a simplified system that achieved a reasonable transformation efficiency (Figure S3).



**Figure 1.** The recovery of embryogenic cell co-cultured with *Agrobacterium* with different combinations of Timentin and cefotaxime. (a) 300 mg/L Timentin, (b) 300 mg/L Timentin and 200 mg/L cefotaxime, (c) 500 mg/L cefotaxime. It was conducive to the recovery of embryonic cells on medium supplemented with 500 mg/L cefotaxime.

### 3.2. Selection of Transformed Embryogenic Lines

To determine the optimal concentration of the antibiotic for the selection of transformed lines, the sensitivity of the S287 line was evaluated by culturing callus pieces on a proliferation medium supplemented with hygromycin at concentrations ranging from 0 to 10 mg/L. The proliferation of the untransformed embryogenic masses was slightly inhibited by a hygromycin concentration as low as 3 mg/L (Figure 2a–g). An increase in the antibiotic concentration to 5 mg/L was sufficient to prevent cell proliferation. Thus, the latter concentration was chosen for the selection of putative transformed embryogenic cell lines in the transformation procedure.



**Figure 2.** Proliferation frequency of non-transformed embryogenic tissue from hybrid larch line S287 on medium supplemented with various hygromycin concentrations. (a) 0, (b) 3, (c) 4, (d) 5, (e) 7, and (f) 9 mg/L of hygromycin. When the antibiotic concentration was raised to 5 mg/L, it was sufficient to prevent cell proliferation. (g) The proliferation rate of embryogenic cultures after 20 days of culturing on medium containing various hygromycin concentrations.

We performed three independent transformation experiments using the pCAMBIA1301 vector. After the co-cultivation of the S287 line with the *A. tumefaciens* strain GV3101 harboring the binary vector pCAMBIA1301, the embryogenic cells were washed with sterile distilled water and cefotaxime solution. The embryogenic cells were then cultured on the selective medium. The number of transformation events per gram FW was recorded after 60 days (Figure S4). In three separate experiments, ~600 hygromycin-resistant embryonal masses were obtained, with an average of 20 transgenic lines per gram FW (Table 2).

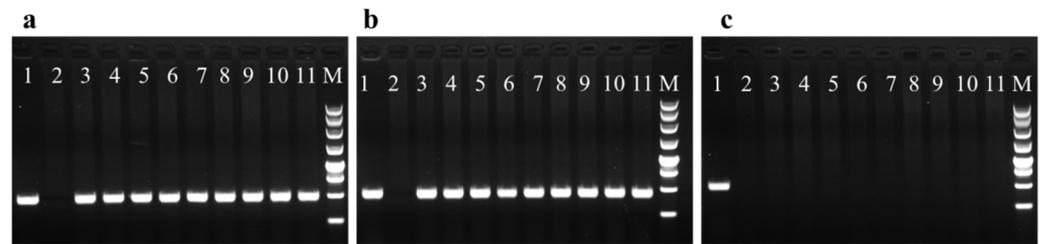
**Table 2.** Transformation efficiency in three separate experiments with pCAMBIA1301. Experiments with the pCAMBIA1301 vectors were carried out with 9.2, 10.3, and 11.7 g fresh weight of embryogenic tissue, respectively. The transformation efficiencies are expressed as the numbers of hygromycin-resistant cell lines per gram fresh weight of co-cultivated embryogenic tissue. Oligonucleotide primers used in the study.

pCAMBIA1301	Number of Hygromycin-Resistant Cell Lines		
	1	2	3
Total	183	213	216
per gram fresh weight	19.9	20.7	18.5

### 3.3. Molecular Analysis of the Transformed Lines

After more than three rounds of selection, more than 80% of the hygromycin-resistant embryonal masses were lost. In addition, only 5% of the putative transformants were stably grown on the selective medium, which may reflect the *HPT* expression level. Nine hygromycin-resistant cell lines harboring the pCAMBIA1301 vector were chosen for molecular characterization (Figure 3). The *HPT*- and *GUS*-specific primers amplified a 480 bp fragment for *HPT* and a 450 bp fragment for *GUS* (Figure 3a,b). No fragment was amplified in the non-transformed controls. In addition, the amplification of *virG* was not detected

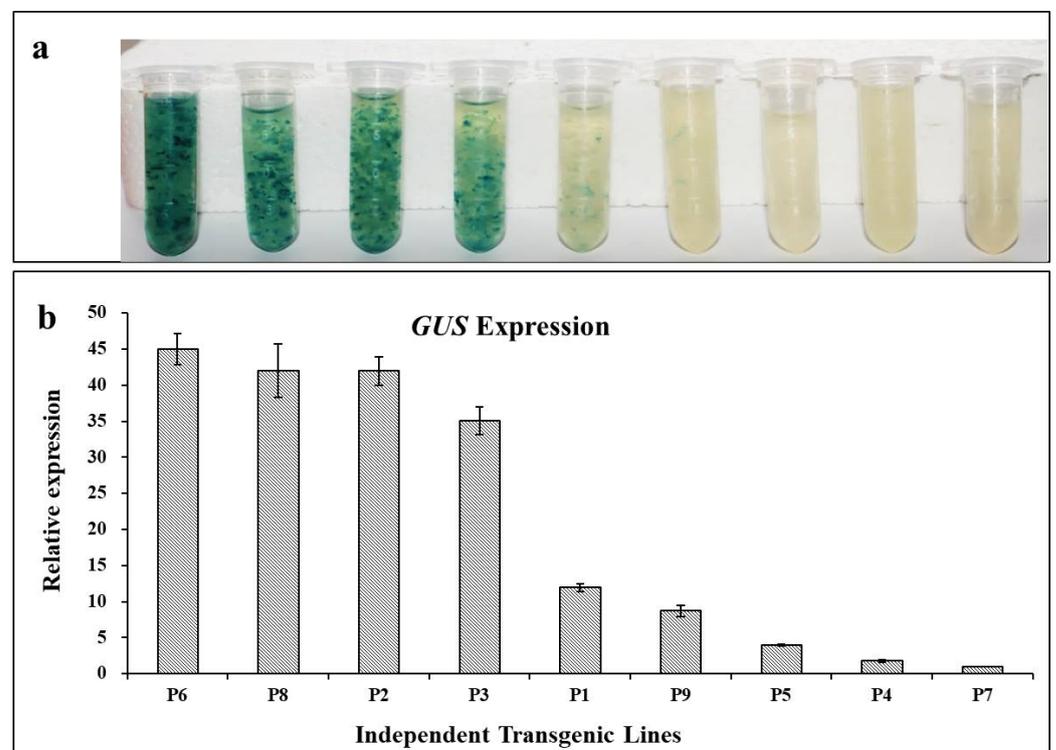
in any of the hygromycin-resistant lines (Figure 3c). Thus, the hygromycin-resistant lines were not contaminated with *Agrobacterium*.



**Figure 3.** PCR analysis of hygromycin-resistant lines P1 to P9 transformed by *Agrobacterium tumefaciens* GV3101. (a) PCR amplification with primers for *HPT* (480 bp). (b) PCR amplification with primers for *GUS* (450 bp). (c) PCR amplification with primers for *virG* (485 bp). Lane 1 positive control (GV3101 containing the plasmid pCAMBIA1301), Lane 2 negative control (Total DNA from non-transformed embryogenic tissue), Lanes 3–11 Total DNA from the nine different hygromycin-resistant lines P1 to P9, M 250-bp marker.

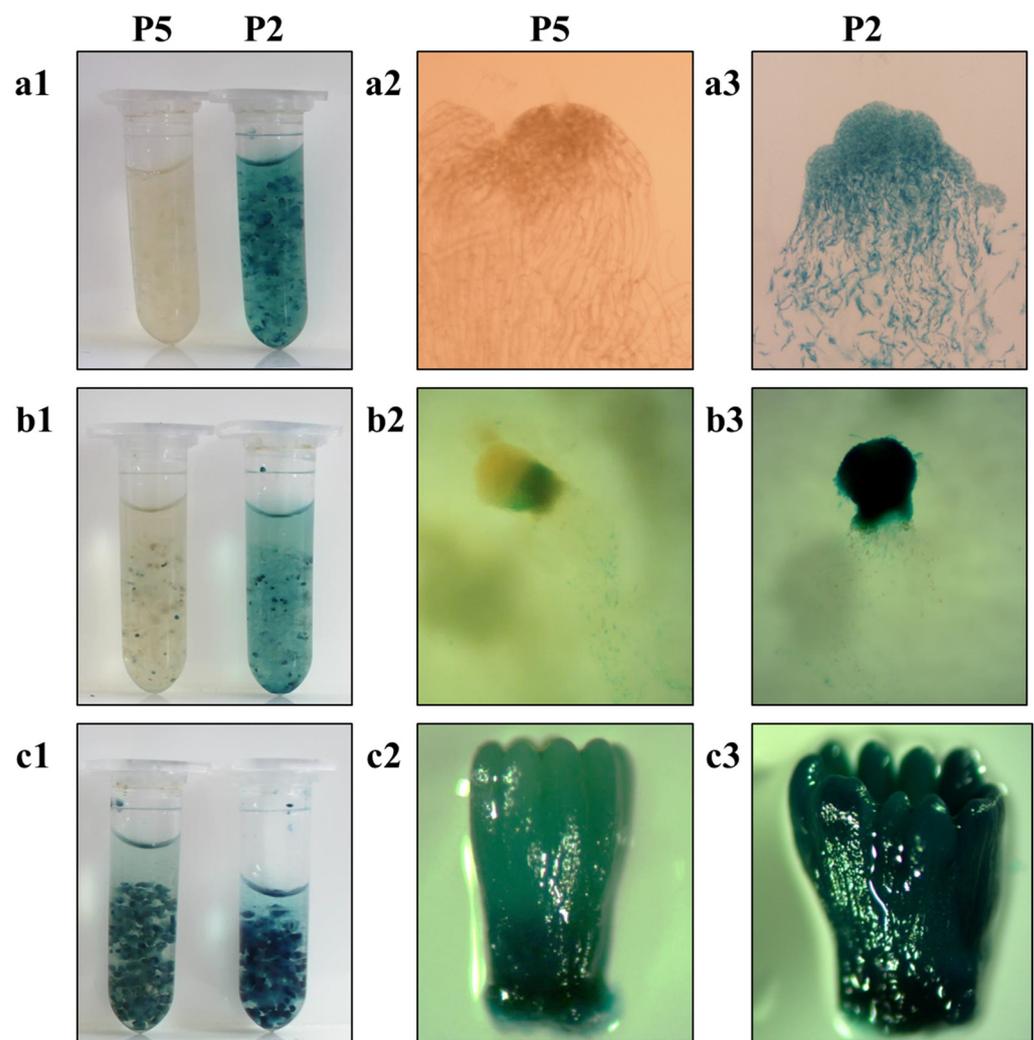
### 3.4. *GUS* Assay and *Gus* Transcript Abundance in Stably Transformed Lines

The nine hygromycin-resistant cell lines were used to visualize *GUS* staining. Four cell lines (P6, P8, P2, and P3) showed intense *GUS* staining and two cell lines (P1 and P9) exhibited lighter staining. No *GUS* staining was detected in the remaining three cell lines (P5, P4, and P7) (Figure 4a). Based on the staining intensity (Figure 4), the *GUS* activity differed in each cell line. In addition, the *GUS* transcript abundance was determined in comparison with the endogenous control *EF* using qRT-PCR (Figure 4b). The *GUS* transcript level in each putative transformed cell line was consistent with the *GUS* staining intensity (Figure 4).



**Figure 4.** Histological *GUS* assay and *GUS* expression in different transformed lines. (a) Histochemical staining for *GUS* staining in transgenic lines P1 to P9. (b) Expression of *GUS* in transgenic lines P1 to P9 as assessed by qRT-PCR.

Based on the qRT-PCR results, the transformed cell lines that did not show *GUS* staining (P5, P4, and P7) still exhibited a low level of *GUS* expression (Figure 4b). Thus, the cell lines P2 (intense *GUS* staining) and P5 (no *GUS* staining) were selected for somatic embryogenesis on a differentiation medium supplemented with ABA, and *GUS* staining was performed at different developmental stages (Figure 5a–c). When the cell line P5 was cultured for 15 days, early single embryos had formed and *GUS* staining was visible at the junction of the embryo and suspensor, which might be related to its vigorous physical activity. The *GUS* staining of P5 was also detected in mature somatic embryos and was significantly weaker than that in P2. The *GUS* transcript abundance in P2 and P5 did not change significantly at different stages of somatic embryogenesis as assessed by qRT-PCR (Figure S5).



**Figure 5.** Histochemical detection of the *GUS* staining in somatic embryos at different developmental stages from transgenic cell lines P2 and P5. (a) Embryogenic tissues, (b) early embryos cultured for 15 days, (c) mature somatic embryos cultured for 42 days on differentiation medium supplemented with abscisic acid. (a1,b1,c1) Panoramic images of *GUS* staining of the above three tissues of P2 and P5, (a2,b2,c2) microscopic images of *GUS* staining of the above three tissues of P5, (a3,b3,c3) microscopic images of *GUS* staining of the above three tissues of P2.

The expression level of the CaMV 35S-driven *GUS* gene in the stable transformation lines was further estimated using an absolute quantification method. A standard curve for *GUS* expression was generated using a gradient serial dilution of the plasmid as the qRT-PCR template. Using the measured  $C_t$  values, the *GUS* transcript abundance in each

transformed cell line was calculated as shown in Table 3. The *GUS* transcript abundance in each transformed cell line ranged from  $10^1$  to  $10^3$  magnitudes, with the highest transcript abundance of  $2.89 \times 10^3$ , which was consistent with the histochemical assessment of *GUS* staining (Table 3).

**Table 3.** Transcript abundance of *GUS*, *HPT*, *EF*, *eIF*, and *APm* by qRT-PCR in stably transformed lines.

Lines	<i>GUS</i>	<i>HPT</i>	<i>EF</i>	<i>eIF</i>	<i>APm</i>
P1	$4.47 \times 10^2$	$1.11 \times 10^4$	$9.97 \times 10^4$	$3.35 \times 10^4$	$7.35 \times 10^2$
P2	$2.01 \times 10^3$	$1.35 \times 10^4$	$1.27 \times 10^5$	$4.08 \times 10^4$	$9.06 \times 10^2$
P3	$1.25 \times 10^3$	$1.27 \times 10^4$	$9.50 \times 10^4$	$2.88 \times 10^4$	$6.78 \times 10^2$
P4	$7.36 \times 10^1$	$1.78 \times 10^4$	$1.11 \times 10^5$	$3.62 \times 10^4$	$8.45 \times 10^2$
P5	$1.82 \times 10^2$	$1.80 \times 10^3$	$1.22 \times 10^5$	$3.60 \times 10^4$	$1.14 \times 10^3$
P6	$2.09 \times 10^3$	$1.45 \times 10^4$	$1.23 \times 10^5$	$3.64 \times 10^4$	$1.17 \times 10^3$
P7	$3.81 \times 10^1$	$4.27 \times 10^3$	$1.01 \times 10^5$	$3.09 \times 10^4$	$8.46 \times 10^2$
P8	$2.89 \times 10^3$	$1.98 \times 10^4$	$1.83 \times 10^5$	$5.08 \times 10^4$	$1.71 \times 10^3$
P9	$4.70 \times 10^2$	$1.59 \times 10^4$	$1.44 \times 10^5$	$4.06 \times 10^4$	$1.15 \times 10^3$
35S:: <i>GUS</i> -1	$2.05 \times 10^3$	$2.81 \times 10^4$	$1.15 \times 10^5$	$3.37 \times 10^4$	$9.64 \times 10^2$
35S:: <i>GUS</i> -2	$5.94 \times 10^2$	$2.67 \times 10^4$	$1.02 \times 10^5$	$2.39 \times 10^4$	$6.81 \times 10^2$

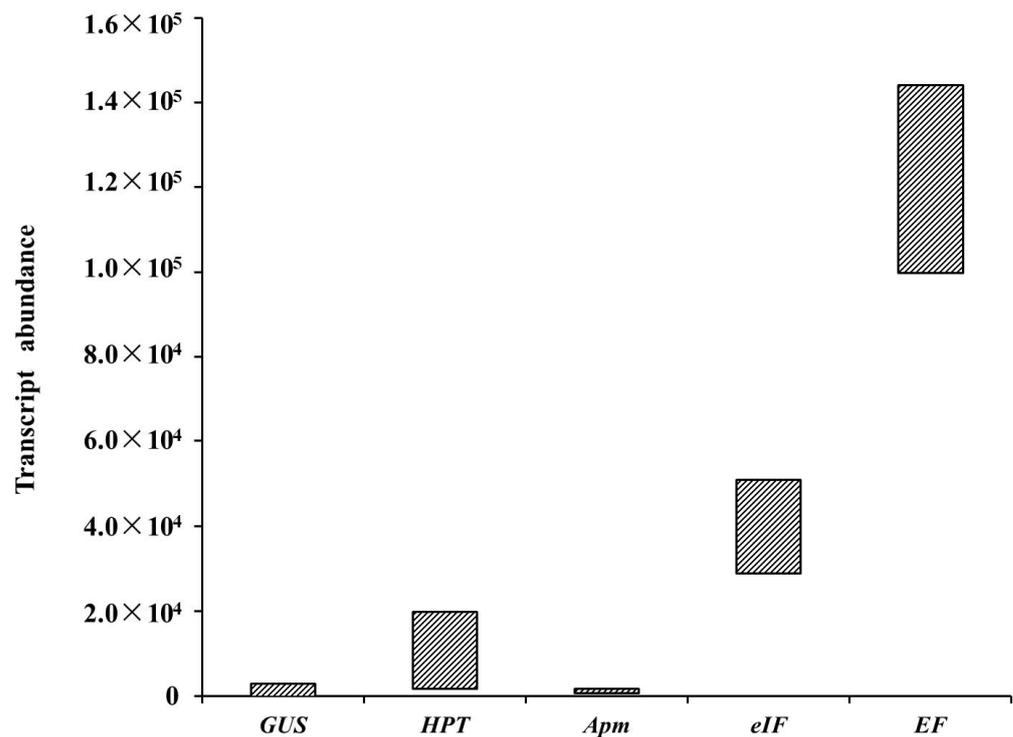
In addition, an intronless *GUS* gene from 35S::*GUS* was transferred and integrated into the genome of larch embryogenic cells (Figure S1), resulting in resistant cell lines (Figure S6). Two hygromycin-resistant cell lines harboring the vector 35S::*GUS* were selected for the detection of *GUS* staining (Figure S7). The two cell lines were stained and the *GUS* staining intensity was generally consistent with the *GUS* transcript abundance in each cell line (Table 3).

### 3.5. *HPT* Transcript Abundance in Stable Transformation Lines

The *HPT* transcript abundance in different samples was calculated using the measured  $C_t$  values (Table 3). The *HPT* transcript abundance in each transformed cell line ranged from  $10^3$  to  $10^4$  magnitudes, with the highest and lowest transcript abundances of  $1.98 \times 10^4$  and  $1.80 \times 10^3$ , respectively. Three candidate reference genes (*EF*, *eIF*, and *APm*) differed in abundance, as assessed by qRT-PCR, which was used to quantify their mRNA levels (Table 3). *EF* had the highest transcript abundance, *eIF* was moderately abundant, and the *APm* transcripts were detected at a low level (Figure 6). The transcript levels of *HPT* and *GUS* driven by the CaMV 35S promoter were equivalent to those of reference genes with moderate and low transcript abundances, respectively (Figure 6).

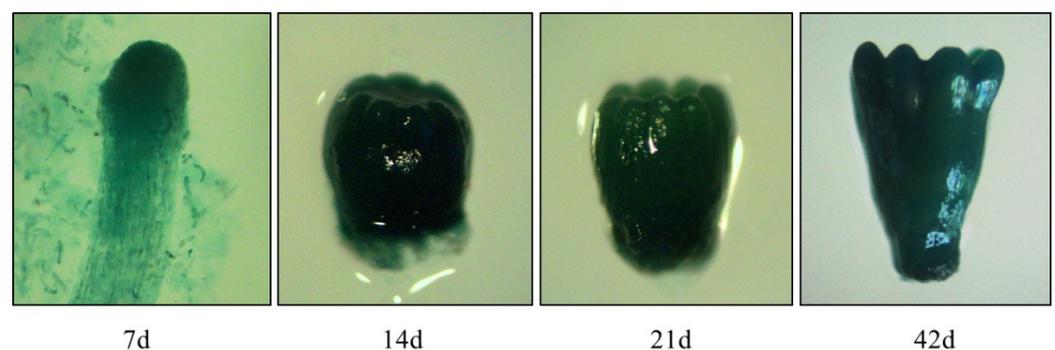
### 3.6. *GUS* Transcript Abundance under the Control of the *LaTCTP* Promoter during Somatic Embryogenesis

To further validate the accuracy of the aforementioned results, *GUS* was expressed under the control of the *L. kaempferi* *pLaTCTP* promoter in the ESM and somatic embryos at different developmental stages (Figure S1). The detailed flowchart of transformation is shown in Supplementary Figure S3. TCTPs are ubiquitously expressed in many eukaryotic organisms and are multifunctional proteins that perform vital functions, particularly in a variety of complex life processes [27]. Expression analysis has previously demonstrated that *LaTCTP* is constitutively and highly expressed during somatic embryogenesis [5]. The TCTP promoter can be used as a constitutive promoter to drive *gusA* expression in monocotyledons and dicotyledons [28], and thus is useful for the production of recombinant proteins that require constitutive expression in a plant system.



**Figure 6.** Schematic diagram of transcript abundance distributions of *GUS*, *HPT*, *EF*, *eIF*, and *Apm* as assessed by qRT-PCR in stably transformed lines.

In the present study, five hygromycin-resistant cell lines harboring the pLaTCTP::*GUS* vector were chosen for further analysis. The transgenic ESM and somatic embryos at different developmental stages were stained intensely with X-Gluc, indicating that *GUS* expression was driven by the *LaTCTP* promoter (Figure 7). Based on the staining intensity, the *GUS* activity differed in each cell line and showed lighter staining (Figure S8). Except for pLaTCTP::*GUS*-5, the early embryos of the other four cell lines cultured for 15 days showed dark *GUS* staining, which was similar to that of the P5 cell line. Dark *GUS* staining was detected at different stages of somatic embryogenesis in pLaTCTP::*GUS*-4 (Figure 7).



**Figure 7.** Histochemical detection of the *GUS* staining in somatic embryos from transgenic cell lines pLaTCTP::*GUS* at different developmental stages detected in 7 days (7 days, 14 days, 21 days, and 42 days).

The transcript abundances of *HPT*, *LaTCTP*, and *GUS* driven by the *LaTCTP* promoter in the five hygromycin-resistant cell lines (pLaTCTP::*GUS*) were estimated (Table 4). The *LaTCTP* transcript abundance in each transformed cell line was at  $10^5$  magnitudes, whereas the *GUS* transcript abundance driven by the *LaTCTP* promoter was of  $10^2$  magnitudes, which was similar to the transcript abundance of *GUS* in cell lines transformed with the pCAMBIA1301 vector.

**Table 4.** Transcript abundance of *GUS*, TCTP by qRT-PCR in stably transformed lines.

Lines	<i>GUS</i>	TCTP
7-1	$1.63 \times 10^2$	$3.72 \times 10^5$
7-2	$4.85 \times 10^2$	$1.91 \times 10^5$
7-3	$2.59 \times 10^2$	$2.61 \times 10^5$
7-4	$3.10 \times 10^2$	$2.46 \times 10^5$
7-5	$1.38 \times 10^2$	$3.55 \times 10^5$

#### 4. Discussion

In this study, several factors affecting the *Agrobacterium*-mediated genetic transformation of Japanese larch were adjusted, including the infection method and duration, and the bacterial removal process (Figure S3). The co-cultivation time is an important factor that affects transformation frequency [29,30]. A two-day co-cultivation period was previously considered to be the maximum because excessive bacterial growth causes culture death [16]. In the present study, under infection with a reduced concentration of bacteria, it can prevent excessive bacterial growth causing culture death in co-cultivation, and the co-cultivation time can be appropriately extended. Because embryonic cell suspension cultures are sensitive to bacteria, a higher concentration of bacteria or prolonged infection time will increase the likelihood of excessive bacterial growth causing culture death in co-cultivation and will increase the difficulty of maintaining uncontaminated cultures. Thus, infection with a reduced concentration of bacteria and elimination of the pre-culture step can appropriately prolong the co-cultivation time and achieve reasonable transformation efficiency in *Larix kaempferi*, which can be attained with an average of 20 transgenic lines per gram FW (Table 2).

The antibiotics kanamycin and hygromycin are efficient selective agents for the AMT of conifer embryonic cultures [2,16]. In spruce and loblolly pine, 2.5 mg/L hygromycin has been used for the selection of transformed embryonic lines [16]. However, in hybrid larch (*L. kaempferi* × *L. decidua*), 5 mg/L hygromycin is sufficient to inhibit the growth of non-transformed embryonal masses [2,19], which was subsequently chosen for further selection (Figure 2). This is consistent with the optimal hygromycin concentration for the screening of *L. kaempferi* in the present study, and slightly higher than the optimal concentration (4 mg/L hygromycin) for *L. olgensis* and hybrid larch (*L. kaempferi* 3 × *L. gmelinii* 9) [25,26]. The cell lines that were able to grow on the selective medium were transformation positive, which indicated that hygromycin is an efficient selective agent for larch transformation [2,19,25,26].

In the present experiments, the percentage detection of *GUS* staining was 66.67% among the nine hygromycin-resistant cell lines harboring the pCAMBIA1301 vector, indicating that the *GUS* expression level differed in each cell line. The *GUS* staining intensity in each transformed cell line was consistent with the *GUS* transcript abundance (Figure 4). The transcript abundance of *GUS* in the transformed lines P2 and P5 did not change significantly at different stages of somatic embryogenesis, as assessed by qRT-PCR (Figure S5), which demonstrated that *GUS* expression was not dependent on the developmental stage of the somatic embryo.

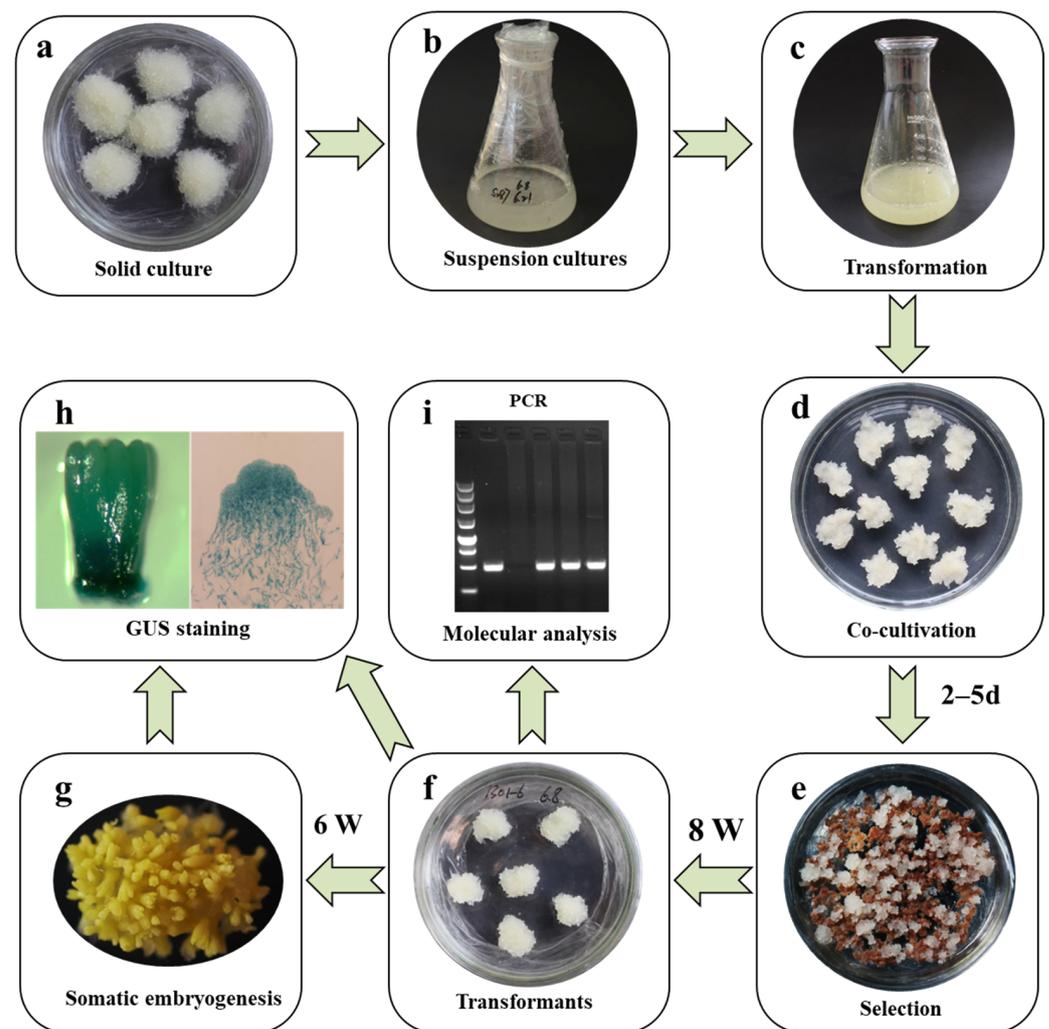
Previous studies have shown that the *GUS* intron-containing reporter gene [31] has an extremely low expression level, probably because the intron is poorly spliced in larch and thus cannot be used to monitor early transformation events following co-cultivation, such as the appearance of transformed cells and their multiplication [19]. In the current study, the *GUS* gene, with or without introns, was normally expressed in larch. However, most of the hygromycin-resistant embryonal masses were lost during further selection processes, which may be associated with the *HPT* expression level. The transformed lines were used to detect the transcript abundance of foreign genes. It was observed that the expression level of *HPT* in the surviving transformation lines must be greater than  $10^3$ , and the *GUS* transcript level in each transformed cell line ranged from  $10^1$  to  $10^3$  (Table 3). Thus, the *GUS* expression level, as represented by the *GUS* staining intensity, observed with the naked eye must be greater than  $10^3$  in the ESM (Figure 4). Based on the *HPT*

and *GUS* expression levels, it was observed that the expression levels of exogenous genes driven by the CaMV 35S promoter ranged from  $10^1$  to  $10^4$  (Figure 6). Compared with the expression of a housekeeping gene, the expression levels of *HPT* and *GUS* driven by the CaMV 35S promoter were equivalent to those of genes with moderate and low abundances, respectively, in Japanese larch (Figure 6). The expression levels of exogenous genes driven by the CaMV 35S promoter in the Japanese larch ESM were relatively low, which might be related to the insertion site and *GUS* copy number in the genome, or to the large genome of Japanese larch.

The *GUS* gene under the control of the *pLaTCTP* promoter was integrated into the genome of *L. kaempferi* embryogenic cells by *Agrobacterium*-mediated genetic transformation. The *LaTCTP* promoter could effectively drive the ectopic expression of *GUS* in larch, and constitutive expressions of *GUS* was observed in the ESM and somatic embryos at different developmental stages (Figure 7). The *GUS* transcript abundance driven by the *LaTCTP* promoter was of  $10^2$  magnitudes (Table 4), which was similar to the abundance in nine hygromycin-resistant cell lines harboring the pCAMBIA1301 vector, whereas the *LaTCTP* transcript abundance in each transformed cell line was of  $10^5$  magnitudes. The expression levels of *GUS* or *HPT* driven by the CaMV 35S or *pLaTCTP* promoters in Japanese larch were relatively low, which may be due to the relatively small number of transgenic samples. Therefore, additional transgenic cell lines need to be screened to obtain transformants with higher expression levels of foreign genes in Japanese larch for further functional research. This may be the reason why there are few reports of gene overexpression in gymnosperms.

## 5. Conclusions

In the present study, a simple and efficient protocol for the laboratory-scale genetic transformation of *Larix kaempferi* was established using the *A. tumefaciens* strain GV3101 in conjunction with the binary vector pCAMBIA1301 (Figure 8a–i). Several factors affecting the *Agrobacterium*-mediated genetic transformation of Japanese larch were adjusted, including the infection method and duration, and the bacterial elimination process. A relatively stable transformation efficiency was achieved by simplifying the infection method and improving the bacterial removal procedure. In the current study, the exogenous genes *HPT* and *GUS*, driven by the CaMV 35S, were normally expressed in Japanese larch. Additionally, its transcript abundances ranged from  $10^1$  to  $10^4$ , which was equivalent to genes with moderate and low abundances, respectively. In addition, the endogenous *pLaTCTP* drove the constitutive expression of *GUS* in the ESM and somatic embryos at different developmental stages. The transcript abundance of *GUS* driven by the *pLaTCTP* promoter in Japanese larch verified the aforementioned results, which might be related to the insertion site and *GUS* copy number in the genome, or to a large genome of Japanese larch. A higher level of *GUS* expression was not detected, which may be due to the relatively small number of transgenic samples. Therefore, it is necessary to develop a new vector construction strategy or efficiently expressed promoter that can drive the ectopic expression of crucial genes to facilitate studies of gene function and quality modifications in Japanese larch.



**Figure 8.** Overview of the simplified protocol for the genetic transformation of *Larix kaempferi*. (a) Embryogenic masses, (b) suspension cells, (c) suspension cells mixed with *Agrobacterium* ( $OD_{600} = 0.05\text{--}0.1$ ), (d) embryogenic masses co-cultivated with *Agrobacterium* for 46–120 h, (e) the selection of transformed embryogenic masses with cefotaxime (500 mg/L) and hygromycin (5 mg/L), (f) transgenic embryogenic masses, (g) somatic embryo maturation, (h) histochemical detection of *GUS* staining in somatic embryos, (i) molecular analysis of transgenic embryogenic lines.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/f13091436/s1>, Figure S1: Physical map of T-DNA region of the binary plasmids pCAMBIA1301, 35S::*GUS*, and pLaTCTP::*GUS* used for genetic transformation of *Larix kaempferi*; Figure S2: Schematic diagram of the improved vacuum filter; Figure S3: Principle of the simplified protocol for genetic transformation of *Larix kaempferi*; Figure S4: Development of hygromycin-resistant embryonal masses derived from line S287 co-cultivated with *A. tumefaciens* GV3101 harboring the binary vector pCAMBIA1301 on selective medium (supplemented with hygromycin 5 mg/L) after 60 day of culture; Figure S5: Accumulation of *GUS* transcripts at different developmental stages of somatic embryos from transgenic cell lines P2 (a) and P5 (b); Figure S6: Development of hygromycin-resistant embryonal masses derived from line S287 co-cultivated with *A. tumefaciens* GV3101 harboring the binary vector 35S::*GUS* on selective medium (supplemented with hygromycin 5 mg/L) after 60 days of culture; Figure S7: Histochemical staining for *GUS* activity in transgenic lines 35S::*GUS*-1 and 35S::*GUS*-2; Figure S8: *GUS* staining in the transgenic lines pLaTCTP::*GUS*.

**Author Contributions:** S.D. and L.Z. designed the experiments and drafted the manuscript; L.Z. carried out all the experiments and data analysis; S.H. and L.Q. provided suggestions on the experimental design and analyses. All authors have read and agreed to the published version of the manuscript.

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