An efficient system for Agrobacterium-mediated transient transformation in Pinus tabuliformis

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Abstract

Background: Functional genomic studies using genetics approaches of conifers are hampered by the complex and enormous genome, long vegetative growth period, and exertion in genetic transformation. Thus, the research carried out on gene function in Pinus tabuliformis is typically performed by heterologous expression based on the model plant Arabidopsis. However, due to the evolutionary and vast diversification from non-flowering (gymnosperms) to flowering (angiosperms) plants, several key differences may alter the underlying genetic concerns and the analysis of variants. Therefore, it is essential to develop an efficient genetic transformation and gene function identification protocol for P. tabuliformis.

Results: In the present study we established a highly efficient transgene Agrobacterium-mediated transient expression system for P. tabuliformis. Using a β-glucuronidase gene (GUS) as a reporter gene expression, the highest transformation efficiency (70.1%) was obtained by co-cultivation with Agrobacterium strain GV3101 at an optical density at 600 nm of 0.8, with 150 μM acetosyringone for 30 min followed by 3 days in the dark at 23 ± 1 °C. This protocol would be applied to other conifers; GUS staining was observed 24 h post-infection.

Conclusions: We report a simple, fast, and resilient system for transient Agrobacterium-mediated transformation high-level expression of target genes in P. tabuliformis, which will also improve transformation efficiency in other conifer species.

Keywords: Pinus tabuliformis callus, Efficient transformation system, Agrobacterium-mediated transformation, Transient gene expression, GUS staining

Background

Pinus tabuliformis belongs to conifer species, native to northern and central China that has considerable economic and ecological valuable forest plants [1–4]. Functional genomic research in P. tabuliformis is typically performed by heterologous expression based on the Arabidopsis as a model plant. However, due to the evolutionary and genetic developmental divergence leap from non-flowering (gymnosperms) to flowering (angiosperms) plants, and key differences may alter the underlying gene expression and genetic programs [5]. Therefore, it is essential to develop an efficient genetic transformation and gene-function identification system for P. tabuliformis. The Agrobacterium-mediated method, including stable transformation and transient gene expression, has been functional in number of plant species [6–10]. Although stable and resilient transformation protocols have been successfully carried out in a few conifer species, such as P. massoniana [11], Korean fir [12], Slash pine [13], Maritime pine [14], Norway spruce, and Loblolly pine [15]. However, there is still major technical obstacle...
for most conifers. Transient genetic transformation is a simple and rapid technique for investigating protein localisation and gene function [16], and enables high-throughput analysis [17, 18]. Among various methods, Transient expression methods including particle bombardment, protoplast transformation using polyethylene glycol, electroporation, and Agrobacterium-mediated transformation have been used in conifer plants [19]. Transgenic plants have been produced by particle bombardment in *Larix gmelinii* [20], Norway spruce [21], Radiata pine [22, 23] and Black spruce [24]. Transient expression was observed in electroporated protoplasts of Douglas fir and Loblolly pine [25, 26]. Compared to commonly used particle bombardment and protoplast transformation, Agrobacterium-mediated transformation is used more frequently in many plant species. This method is one such versatile simple, rapid and transient expression of target genes can be detected within a few hours [8, 16, 27]. However, the average transformation efficiency is affected by the strain [13], Agrobacterium explant type [28], Agrobacterium density [29], acetyl-syringone (AS) concentration [30], and time period [11]. In this study, we obtained a hypocotyl-derived callus from *P. tabuliformis* seedlings, which promoted transient transformation. We developed a highly efficient and buoyant Agrobacterium-mediated transient transformation system for *P. tabuliformis* and assessed the influence of Agrobacterium density, infection time, AS concentration, co-cultivation duration, and sonication. The transformation system for *P. tabuliformis* in combination with high-throughput sequencing technologies are capable to improve the transformation efficiency for other conifer species.

**Methods**

**Plant materials and treatments**

Dry and mature seeds close to natural dispersal of *P. tabuliformis* were collected from first-generation clonal seed orchard located in Pingquan City, Hebei Province, China (GPS recordings: 40° 99′ N, 118° 45′ E, 560 m above sea level) and stored in plastic bags at 4 °C with optimal storage conditions. Seeds were spread out on sphagnum moss, soaked with water and germinated in a growth chamber under a 16/8 h (light/dark) photoperiod at 23 ± 2 °C. After 20 days. Seedlings were sterilised with 75% ethanol (v/v) for 1 min and rinsed three times with sterile distilled water. Further seedlings were cleaned in 17% NaClO (v/v) for 10 min followed by rinsing three to five times in double distilled water. Subsequently, the roots were removed by using a sterilised scalpel under aseptic conditions. The callus induction medium supplemented with hormones is prepared. The pH of the medium was adjusted to 5.8 prior to the addition of 8 g/L agar and autoclaving at 121 °C for 20 min. Finally, the hypocotyls with needles were inoculated on callus induction medium, [31] (Table 1) under 14/10 h (light/dark) photoperiod at 23 ± 2 °C. After approximately 40 days, yellow or green granular calli were transferred to fresh proliferation medium (Table 1). The calli were subcultured every 2–3 weeks on fresh callus proliferation medium. After four subcultures in the dark, yellow and soft calli were harvested as explants for transformation.

**Plasmid construction and Agrobacterium culture**

The plant binary vector pBH121 plasmid (Fig. 1) was induced into *A. tumefaciens* LBA4404 and GV3101 [32] via the freeze/thaw method. We selected a single colony of *Agrobacterium* cultured overnight in liquid YEB medium containing 50 mg/L kanamycin and 50 mg/L rifampicin (Sigma-Aldrich), followed by incubation with shaking at 180–220 rpm at 28 °C. Then the Agrobacterium cells were harvested by centrifugation at 5000 rpm for 8 min and the cells were rinsed twice in suspension solution containing 10 mM MES (pH 5.6), 10 mM MgCl₂, and 0.005% Tween-20. Two transformation approaches were used; I, cells were suspended in the above liquid medium with same 150 μM AS to optical density at 600 nm (OD₆₀₀) of 0.2, 0.4, 0.6, 0.8, and 1.0, respectively; II, cells were suspended to OD₆₀₀ of 0.8 and add 50, 100, 150, and 200 μM AS, respectively. Finally, the suspension was placed at 23 °C for 3 h for transformation.

### Table 1 Composition of different media used in the study

| Media                        | Composition                                                                 |
|------------------------------|-----------------------------------------------------------------------------|
| Induction medium             | 1/2 MS, 1 mg/L dichlorophenoxyacetic acid (2,4-), 3 mg/L 6-benzylaminopurine (6-BA), 30 g/L source (W/V), 8 g/L agar |
| Proliferation medium         | DCR medium, 3% sucrose, 1.13 mg/L 6-BA, 2.21 mg/L 2,4-o and 3 g/L phytagel  |
| Co-cultivation medium        | DCR medium, 2.5% sucrose, 1.13 mg/L 6-BA, 2.21 mg/L 2,4-o and 3 g/L phytagel |
| Suspension medium            | 10 mM MES, 10 mM MgCl₂, 0.005% Tween20 and 50, 100, 150, 200 μM AS          |
Agrobacterium-mediated transient transformation
The pre-cultured calli were immersed in the Agrobacterium suspension and treated as in the following steps:

1. Calli were co-cultured with suspension solution (OD$_{600}$ = 0.2, 0.4, 0.6, 0.8, and 1.0) for 30 min containing 150 µM AS.
2. Calli were then infected by suspension solution for 10, 20, 30, and 40 min at OD$_{600}$ of 0.8 in the presence of 150 µM AS.
3. Calli were treated by suspension solution (50, 100, 150, and 200 µM AS) for 30 min at an OD$_{600}$ of 0.8.
4. At the end, calli were placed in 50 mL sterile tubes containing 20 mL of Agrobacterium suspension for 30 min at OD$_{600}$ of 0.8 in the presence of 150 µM AS. Subsequently, calli were rinsed three times with sterile distilled water, resuspended in sterile water, and placed in a float at the centre of a sonicator bath. The sonicator was controlled by an electronic timer at a power of 100 W. The calli were sonicated for 5, 10, and 15 min and shaken twice at 5 min intervals.
5. Calli suspended in sterile water in 50 mL sterile tubes were sonicated for 5, 10, and 15 min and infected by suspension solution for 30 min at an OD$_{600}$ of 0.8. After treatment, the infected calli were blotted with sterile filter paper to remove excess bacteria and cultured on cocultivation medium with sterile filter paper in the dark at 23 ± 1 °C for 1–5 days. The hypocotyl seedling or needles were cut transversely into 2 cm long longitudinal fragments for absorption by Agrobacterium and were infected as in step 3, followed by hypocotyls and needles were placed on two layers of wet cheesecloth in Petri dishes in the dark at 23 ± 1 °C for 3 days [8]. The calli were subjected to β-glucuronidase (GUS) staining daily.

GUS staining assays
The transgenic calli, needles, and hypocotyls were submerged in GUS staining solution containing 50 mM sodium phosphate (pH 7.0), 0.5 mg/L 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 0.1% Triton X-100, 0.5 mM K$_4$[Fe(CN)$_6$], and 0.5 mM K$_3$[Fe(CN)$_6$] overnight in the dark at 37 °C. The samples were washed several times in 75% ethanol to remove chlorophyll [33]. A blue colour in tissue was regarded as indicative of positive transgenic explants.

Statistical analysis
Statistical analysis of the callus growth rate and GUS strain frequency of calli, needles, and hypocotyls was performed by Student’s t-test (p < 0.01). Infected calli were transferred to three Petri dishes (replication) per treatment, at nine calli per plate. The transformation efficiency (%) (number of positive calli, hypocotyls, and needles/total number of infected calli, hypocotyls, and needles x 100%) was calculated. Each experiment was repeated three times independently.

Results
Callus induction and proliferation in P. tabuliformis
The mature seeds were germinated in a growth chamber under ambient environmental conditions; 16/8 h (light/dark) photoperiod at 23 ± 2 °C. After 20 days of seed germination, hypocotyls of P. tabuliformis seedlings were cultured on callus induction medium (Table 1). Green callus tissues were developed on hypocotyl segments, followed by 4–5 weeks under light/dark (16/8 h) conditions (Fig. 2a, b). The calli were transferred into proliferation medium (Table 1) and subcultured on fresh medium subsequently every 3 weeks and kept under dark condition. A rapidly proliferating callus was obtained after several subcultures and hypocotyl-derived calli with a texture of pale yellow, smooth surface, a loose structure, and rapid growth were generated after 18–25 days (Fig. 2c, d). The average weight of calli was Three-twofold higher than the initial value after sub-culturing (Fig. 2e). The total weight increased significantly than callus induction weight (Fig. 2f). Sufficient materials for transient transformation were obtained at 18 days post-subculture.

Agrobacterium-mediated transient transformation protocol for callus
In the present study we evaluated the effect on transformation efficiency of Agrobacterium density, treatment
time, AS concentration, co-cultivation duration, and sonication. The highest transformation efficiency was obtained with *Agrobacterium* strains GV3101 and LBA4404 at OD600 of 0.6 and 0.8, respectively (Fig. 3a). The largest number of positive GUS calli (77.7%) was obtained while calli were infected with *Agrobacterium* strain GV3101 at OD600 of 0.6 and with LBA4404 at OD600 of 0.8. The transformation efficiency was decreased at higher or lower concentration of OD600. The highest transformation efficiency recorded from infection with *Agrobacterium* GV3101 and LBA4404 for 30 min (Fig. 3b). A shorter infection time reduced transformation efficiency, and longer infection time might be damage calli. Therefore, we used an infection time of 30 min in subsequent experiments. AS induced the expression of virulence genes and enhances *Agrobacterium* infection of wound segments. The AS concentration significantly influenced by the transformation level efficiency; the highest efficiency of 75.2% (GV3101) and 72.7% (LBA4404) were obtained at 150 µM AS (Fig. 3c). Co-cultivation for 3 days post-infection resulted in the highest transformation efficiencies of 70.1% (GV3101) and 67.7% (LBA4404) (Fig. 3d). The transformation efficiency were decreased with increasing co-culture duration was triggered by overgrowth of *Agrobacterium*, leading to callus browning. We assayed the effect of sonication time by infecting calli with *Agrobacterium* for 30 min followed by sonication (IFS), and by sonicating calli followed by infection for 30 min (SFI) (Fig. 4). Sonication did not significantly influence the transformation efficiency using *A. tumefaciens* LBA4404 and GV3101.

**Application of the transient transformation protocol to other conifer species**

The transient transformation system in needles of *P. tabuliformis* and in seedlings and hypocotyls of *P. tabuliformis*, *P. yunnanensis*, and *Picea crassifolia* Kom were tested (Fig. 6). The tissues were immersed in suspensions of *Agrobacterium* GV3101 and LBA4404 as described above. First, we assessed the transformation efficiency in needles and hypocotyls of *P. tabuliformis* seedlings (Fig. 5) using the IFS and SFI approaches. Sonication under both IFS and SFI conditions enhanced the staining efficiency. However, in case of needles, there was no significant difference between IFS and SFI. The transformation efficiency was observed greater while using single than double ends; the highest efficiency was 71.66% (single end) using LBA4404 by SFI. In hypocotyls, IFS exhibited a higher transformation efficiency than SFI when explants were infected and sonicated for 5–10 min.
(Fig. 5c, d). The highest transformation efficiency was recorded with *Agrobacterium* LBA4404 after sonication for 10 min for hypocotyls (91% [double ends]) (Fig. 5c). These results indicated that *Agrobacterium* was more readily infected hypocotyls than needles and that was not the same case as in callus. GUS staining showed that the optimised protocol is also appropriate for the hypocotyls and needles of other conifer species. Particularly, GUS activity in the hypocotyls of *P. tabuliformis* seedlings was detected 24 h post-infection.
(Fig. 6b). Therefore, we tested the GUS activity of explants at 24, 48, and 72 h post-infection. The results suggested that the agroinfiltration system could be used for other conifer species (P. tabuliformis, P. yunnanensis, and Picea crassifolia) and most hypocotyls showed GUS activity at 24 h post-infection (Fig. 6c, d). Explants were infected and sonicated for 5–10 min for transformation of hypocotyls and needles of P.
tabuliformis seedlings. The optimised transformation system was also suitable for other conifer species.

Discussion

Agrobacterium-mediated transient transformation was used in many angiosperm plants to get transient and high-level expression of target genes, such as in Arabidopsis, rice, wheat, Nicotiana benthamiana, strawberry, and soybean [7, 34–37]. The first successful Agrobacterium-mediated transformation of conifer was in Sugar pine has the longest cones of any conifer. [38]. The method has since been extensively used in other conifer species, including Norway spruce, Loblolly pine [15], White pine [8], P. massoniana [11], Slash pine [13], P. patula [39], and P. radiata [40]. Use of embryos, cotyledons, hypocotyls, and female gametophytes as explants reportedly induces non-embryogenic or embryogenic calli and adventitious buds [41–44]. Mature or immature zygotic embryos were thought to be ideal transformation materials for generating embryonic tissues. However, the process was complex and was influenced by genotype, explant type, developmental stage, and medium composition [45]. Therefore, the production and regeneration of conifers by somatic embryogenesis is difficult and slow as compared to other plant species [45, 46], which hamper the development of transformation systems for conifer species. In this study, hypocotyls with needles from P. tabuliformis seedlings were used as explants to induce calli. Callus from P. tabuliformis seedlings didn’t affected by seasonal variation and can be frequently stable. The hypocotyl-derived calli enabled more rapid and efficient transformation than the embryogenic calli. The calli showed a high growth rate at 25 days post-subculture, and thus Agrobacterium-mediated transient transformation of P. tabuliformis could be completed in < 1 month. Furthermore, hypocotyl-derived calli maintained rapid growth for > 2 years, suggesting the method to be suitable for routine large-scale transformation using Agrobacterium. Moreover, there are many factors contribute to affect the transformation efficiency of plants. Agrobacterium LBA4404 and GV3101 have been used for transformation of Slash pine, P. radiata, P. patula, Loblolly pine, and Norway spruce [13, 15, 26, 28, 39, 40, 47]. In this study, GV3101 showed a higher transformation efficiency than LBA4404 (Fig. 5). Consistently, transient transformation of P. tabuliformis hypocotyl and needles using GV3101 resulted in stronger GUS activity (Fig. 6). An excessively high Agrobacterium concentration damages explants, leading to browning and death of calli. By contrast, an insufficient Agrobacterium concentration results in a low rate of infection [48]. The highest transformation efficiency using Agrobacterium GV3101 and LBA4404 was OD600 of 0.6 (77.7%, GV3101) and 0.8 (77.73%, LBA4404), respectively, as reported previously [13, 49]. Therefore, we used an Agrobacterium suspension with OD600 of 0.8 and an infection duration of 30 min (Fig. 2b). Regarding the AS concentration, the highest transformation efficiency was detected at 150 µM AS. Because we did not add antibiotics to the medium, Agrobacterium overgrowth with increasing co-cultivation duration caused browning and death of calli, which was consistent with the observed lower transformation efficiency. The efficiency of T-DNA delivery into the host cell affects Agrobacterium-mediated transient transformation, and sonication reportedly enhances transformation of calli of several plant species, such as Slash pine [13], this might be due to the cell membrane permeability. However, sonication for 5, 10, and 15 min did not significantly improve transformation efficiency. This result is not in agreement with a prior report on Slash pine [13], likely because prolonged sonication caused damaged to the calli. Furthermore, strong GUS expression was observed in needles

Fig. 6 Histochemical assay of GUS expression in organs of P. tabuliformis, P. yunnanensis, and Picea crassifolia transformed by agroinfiltration. GUS expression was examined in organ cross-sections at 24, 48, and 72 h after infection. a Callus and b hypocotyl and needles of P. tabuliformis. c Hypocotyl of Picea crassifolia. d Hypocotyl of P. yunnanensis. Negative controls (without pBI121). Each treatment comprised 30 explants and was performed in triplicate.
and hypocotyls of *P. tabuliformis* and hypocotyls of *P. tabuliformis*, *P. yunnanensis*, and *Picea crassifolia*. The transformation efficiency was higher in hypocotyls than needles was recorded. Therefore, the efficiency of *Agrobacterium*-mediated transformation in pine was dependent on the explants used. We established and optimised an *Agrobacterium*-mediated transformation system, which will enable studies of functional genes in *P. tabuliformis* and in other conifer species.

**Conclusion**

The *P. tabuliformis* genome has not been sequenced due to large size (~25.6 Gb) and generating transgenic *P. tabuliformis* plants is problematic. Thus, to identify functional genes in *P. tabuliformis*, a rapid and efficient *Agrobacterium*-mediated system was developed. We transformed hypocotyl-derived calli to enable transient expression in *P. tabuliformis*. The transformation process includes callus proliferation required only 2–3 weeks and also suitable/recommended for other conifer species. This protocol enables rapid establishment of transgenic calli and provides the materials needed to study gene functions in *P. tabuliformis* calli.

**Acknowledgements**

We would like to express our gratitude to the national key base for improved forest varieties, Qigou State-owned Forest Farm, Pinquan city, Hebei province, PR. China, for their kind help. Special thanks to Dr. Pervaiz Tanq for helping us to revise the language of manuscript.

**Authors' contributions**

SWL performed the experiments and drafted the manuscript, WL and SHN participated in the experiments design and coordination. JMJ, HML, and YTG participated in the sample preparation. All authors read and approved the final manuscript.

**Funding**

This work was supported by the Fundamental Research Funds for the Central Universities (2015ZCQ-SW-02) and the National Natural Science Foundation of China (31770713, 31860221, and 31870651).

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

All authors agreed to publish this manuscript.

**Competing interests**

The authors declare that they have no competing interests.

**Received:** 16 December 2019  **Accepted:** 3 April 2020  **Published online:** 10 April 2020

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