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Research paper

Agrobacterium tumefaciens-mediated genetic transformation of *Salix matsudana* Koidz. using mature seeds

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An *Agrobacterium tumefaciens*-mediated transformation method was developed for *Salix matsudana* Koidz. using mature seeds as starting material. Multiple shoots were induced directly from embryonic shoot apices of germinating seeds. Although thidiazuron, 6-benzylaminopurine and zeatin induced multiple shoot induction with high frequency, zeatin (4.5 μM) was more effective for elongation of shoots and roots. The binary vector pCAMBIA1303, which contained neomycin phosphotransferase as a selectable marker gene and β-glucuronidase as a reporter gene, was used for transformation. Factors affecting transformation efficiency were examined for optimization of the procedure. Up to 35 of 180 seeds regenerated kanamycin-resistant shoots under optimal transformation conditions as follows: seeds were precultured for 4 days, apices of embryonic shoots were removed and infected with *A. tumefaciens* strain LBA4404 grown to a cell density equivalent (OD_{600}) of 0.6, and then the infected explants were cultivated at 21 °C for 4 days. Storage of seeds at –20 °C for as long as 3 years had no significant effect on the induction of kanamycin-resistant shoots. Using this method, transgenic plants were obtained within ~5 months with a transformation frequency of 7.2%. Analysis by polymerase chain reaction (PCR) showed that 36.4–93.8% of plants from all 13 tested kanamycin-resistant lines were PCR positive. Several 'escapes' were eliminated by a second round of selection. PCR, Southern blot and reverse transcriptase–PCR analyses of selected transgenic individuals 2 years after cutting propagation confirmed the successful generation of stable transformants. Our method, which minimizes the duration of axenic culture, may provide an alternative procedure for transformation of other recalcitrant *Salix* species.

Keywords: chimaeric, embryonic shoot apex, mature seed, multiple shoot, willow, woody plant transformation.

Introduction

The genus *Salix* (Salicaceae) comprises ~470 species, which are mostly found in the temperate and arctic zones of the Northern Hemisphere (Karp et al. 2011). Many *Salix* species are characterized by their considerable capacity to adapt to adverse conditions, high biomass, rapid growth, tolerance of flooding, and high capacity to accumulate heavy metals, organic pollutants and pollutants caused by eutrophication (Greger and Landberg 1999, Smart and Cameron 2008). The increasing severity of global crises caused by reduced availability of energy resources and by severe environmental pollution has increased the economic importance of *Salix* species, given their utility for a wide array of practical applications such as phytoremediation (Greger and Landberg 1999) and bioenergy production (Volk et al. 2006, Smart and Cameron 2008). However, limitations in the use of *Salix* species for phytoremediation of contaminated soils include their failure to hyperaccumulate toxins, the unacceptably slow time-frame needed for remediation, and the fact that heavy metal accumulation is restricted mainly to roots, which complicates the

collection and elimination of toxins (Dowling and Doty 2009). Given these limitations, efficient genetic engineering of *Salix* species is highly desirable for their genetic improvement (Bradshaw and Strauss 2001).

Agrobacterium-mediated transformation is a widely used method for introducing foreign DNA into plant species. However, we are unaware of any reports of the regeneration of transgenic *Salix* species with sufficient efficiency to be of practical value. Vahala et al. (1989) were the first to use *Agrobacterium tumefaciens* to transform stems of *Salix* species and obtain transgenic callus. Vahala et al. (1993) used the same method to express the *ipt* gene in *Salix viminalis* L. callus. No shoots regenerated from transformed callus in these studies.

Salix species are, in general, considered to be recalcitrant to both regeneration and transformation techniques. Only three published reports describe in vitro regeneration of *Salix* plants. Stoehr et al. (1989) induced adventitious shoots from leaf explants of mature *Salix exigua* Nutt. trees indirectly via intervening callus. However, indirect regeneration methods were unsuccessful for transformation of all *Salix* species (Vahala et al. 1989, 1993). Grönroos et al. (1989) induced somatic embryos from callus derived from pistils of *S. viminalis*, although the somatic embryos rarely germinated. More recently, Lyyra et al. (2006) reported the induction of adventitious shoots directly from inflorescences of black willow (*Salix nigra* Marsh.). Although 92% of the inflorescences induced adventitious shoots, few of the shoots elongated. As a consequence, the absence of a high-efficiency system for regeneration and *A. tumefaciens*-mediated transformation remains a key obstacle to the extensive use of willow for a range of biotechnologically important applications. *Agrobacterium*-mediated transformation of apical meristematic explants has enhanced the rapidity and efficiency of shoot regeneration for several plant species recalcitrant to transformation (Sticklen and Oraby 2005). The main advantage of transforming apical tissues is that the rapidity with which they can be regenerated into mature plants can minimize or eliminate the time needed for axenic culture in vitro (Medford 1992). Apical meristematic explants were used for the genetic transformation of some recalcitrant cereal crops (Sticklen and Oraby 2005) and woody plants such as *Vitis vinifera* L. (Dutt et al. 2007).

Salix matsudana Koidz. is a large, deciduous, rapidly growing tree that is native to northeastern China and inhabits a wide range of climatic conditions. The species is one of the most widely distributed and commonly cultivated willow species in China. The species has a high capacity to accumulate heavy metals, making it potentially suitable for phytoremediation (Dos Santos Utmazian et al. 2007). In the present study, we developed an *A. tumefaciens*-mediated transformation system for *S. matsudana* that uses the embryonic apical region as an explant. Use of the embryonic shoot apex for direct shoot regeneration minimizes the duration of axenic culture and may provide an alternative target for transformation of other *Salix* species. To the best of our knowledge, this is the first report of the simultaneous transformation and regeneration of *Salix* species with experimental evidence for stable integration of transfer DNA (T-DNA).

Materials and methods

Materials

Open-pollinated mature seeds of *Salix matsudana* Koidz. var. *matsudana* were collected during late May from 60-year-old trees grown on the campus of Northeast Forestry University, Harbin, China. The seeds were collected using the method reported by Maroder et al. (2000) and stored in sealed plastic bags at −20 °C for use. In preparation for in vitro culture, thawed seeds were agitated in 70% ethanol for 1 min and surface sterilized in 1% sodium hypochlorite solution for 10 min with gentle agitation, before being rinsed five times with sterile distilled water.

The following *A. tumefaciens* strains were used: EHA101, EHA105, LBA4404, C58 and GV3101. The binary plasmid pBI121 (Clontech, Palo Alto, CA, USA), which contains a reporter gene that encodes β-glucuronidase (GUS) and is under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter, as well as a neomycin phosphotransferase II (*npt II*) gene under the control of a nopaline synthase (*nos*) promoter (Chen et al. 2003) were transformed to these individual strains.

Development of the regeneration protocol

The sterilized seeds were cultured on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with either 2.2 or 4.4 μM of 6-benzylaminopurine (BA) (Duchefa, Haarlem, The Netherlands), 2.25 or 4.5 μM of thidiazuron (TDZ; Duchefa), and 2.25 or 4.5 μM of zeatin (Z; Duchefa) alone or combined with 0.54 μM naphthaleneacetic acid (NAA; Duchefa). Each flask contained 20 seeds, and four replicates were performed for each treatment.

To evaluate the effect of wounding on induction of multiple shoots from the apices of embryonic shoots, seeds were precultured on selective shoot induction medium (SIM), which consisted of MS medium with 3% sucrose supplemented with 4.5 μM Z and 0.54 μM NAA, for 2, 4, 6 or 8 days. The base of the emergent apical buds was excised using a scalpel blade and cultured on SIM for 4 weeks. Multiple shoot tips were transferred to shoot elongation medium (EM), which consisted of half-strength MS medium containing 1.5% sucrose without plant growth regulators (PGRs). After 4 weeks, elongated shoots were excised and transferred to rooting medium (RM), which comprised one-third-strength MS medium containing 1.0% sucrose without PGRs, for 4 weeks to induce rooting. Each flask contained five explants, and eight replicates were

performed for each treatment. The cultures were performed in a 250-ml Erlenmeyer flask containing 50 ml medium. The pH of the medium was adjusted to 5.8 before adding 0.8% of plant agar (Duchefa), and then sterilized by autoclaving at 1.1 kg cm^{-2} (121 °C) for 15 min. All cultures were incubated in a culture room at 24 °C with a 16-h photoperiod and a light intensity of 45 μmol m^{-2} s^{-1}.

Determination of the optimal concentration of kanamycin for selection of transformants

The apical buds that developed from seeds precultured on SIM for 4 days were placed on SIM containing 250 mg l^{-1} cefotaxime supplemented with 0, 10, 20, 30, 50 or 70 mg l^{-1} kanamycin. For shoot elongation, multiple shoots were placed on EM supplemented with 0, 10, 30, 50 or 70 mg l^{-1} kanamycin. For root selection, elongated individual shoots were separated and cultured on EM supplemented with 0, 30, 50 or 70 mg l^{-1} kanamycin. After 8 weeks of culture, multiple shoot induction, shoot growth and root growth were examined. All experiments were repeated with five independent replicates.

Evaluation of parameters influencing transformation efficiency

The effect of the following parameters on transformation frequency was evaluated: the length of time that seeds were precultured on SIM (0, 2, 4, 6 or 8 days), the *A. tumefaciens* strain used (EHA101, EHA105, LBA4404, C58 or GV3101), the optical density (OD_{600}) of the *A. tumefaciens* cell culture (0.2, 0.4, 0.6, 0.8, 1.0 or 1.2; see below), the duration of co-cultivation (0, 1, 2, 3, 4 or 5 days), the duration for which seeds were stored at –20 °C (12 months, 1, 2, 3, 4 or 5 years), the temperature during co-cultivation (18, 21 or 24 °C), and the year when the seeds were collected (from 2006 to 2011). The results from assays of transient GUS expression (data not shown) were used to generate the following standard procedure to optimize these parameters that influence the frequency of transformation. The embryo apical region of mature seeds that had been precultured for 4 days was excised and infected with *A. tumefaciens* strain LBA4404 by immersing the explants in a solution of cultured cells grown to an OD_{600} of 0.6, and then co-cultivating the explants with the *A. tumefaciens* strain on SIM medium at 21 °C for 4 days. After co-cultivation, the explants were placed on selection medium to induce the formation of transgenic shoots. This procedure was used in all optimization assays, with the only variable being the parameter to be optimized. Each variant of each parameter was tested using five independent replicates, each of which comprised ~20 seeds.

Preparation and transformation of A. tumefaciens

A single colony of *A. tumefaciens* was inoculated in Luria–Bertani medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl) supplemented with 50 mg l^{-1} kanamycin. The bacterial cultures were grown to an OD_{600} of 0.2–1.2 at 28 °C on a shaker at 180 rpm. A 6-ml aliquot from each culture was pelleted immediately before infection by centrifugation at 6000 *g* for 5 min. The pellet was resuspended in 6 ml of liquid half-strength MS medium containing 4.5 μM Z, 0.54 μM NAA and 3% sucrose at pH 5.8 to generate the same OD_{600} value as the original culture. Explants were submerged in the *A. tumefaciens* cell suspension and shaken (50 rpm) at 25 °C for 30 min (infection step). The explants were blotted dry on sterilized filter paper to remove excess bacterial suspensions. The explants were placed in Petri dishes containing 30 ml SIM that covered two layers of filter paper for co-cultivation. After co-cultivation, the explants were washed four times with sterile water containing 250 mg l^{-1} cefotaxime to decontaminate the agrobacterium. The explants were blotted dry on sterilized filter paper and then transferred to SIM supplemented with 30 mg l^{-1} kanamycin and 250 mg l^{-1} cefotaxime for induction and multiplication of transgenic shoots. Multiple shoots that formed 12 weeks after selection were transferred to EM containing 50 mg l^{-1} kanamycin and 250 mg l^{-1} cefotaxime. After 4 weeks, elongated shoots were excised and transferred to EM containing 50 mg l^{-1} kanamycin and 250 mg l^{-1} cefotaxime to induce rooting.

Molecular analysis and selection of transgenic plants

Genomic DNA was extracted from leaves of 66 plants belonging to 13 putative transformed lines (named G1–G9, G12 and G14–G16) and control plants using a modified cetyltrimethylammonium bromide method (Jaakola et al. 2001). Shoot tips of polymerase chain reaction (PCR)-positive plants (each ~1.5 cm long) were excised and cultured on EM containing 50 mg l^{-1} kanamycin. After 8 weeks, a second round of PCR analysis was conducted. Selected PCR-positive plants were transferred to pots that contained an autoclaved 1 : 3 mixture of sand and soil. The pots were covered with polythene bags, which were removed after 3 weeks when new leaves appeared on the plants. Selected PCR-positive plant lines were maintained by propagation from cuttings in a greenhouse.

After 2 years, the efficiency of transgenesis was evaluated by PCR and Southern blot analyses. The PCR was carried out using a primer pair specific for the *GUS* gene (F: 5′-AATCCATCGCAGCGTAATGCTCT-3′; R: 5′-GCTGGCCTGCCCAACCTTTCGGTAT-3′). The PCR product was expected to be a 1.0-kb fragment. The PCR conditions were as follows: 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min, and a final 7 min extension at 72 °C.

For Southern blot analysis, 20 μg genomic DNA isolated from the leaves of PCR-positive plants were digested with HindIII and electrophoresed in a 1.0% agarose gel before subsequent transfer to a nylon membrane (Hybond-N + ; Millipore Co., Billerica, MA, USA). The probe for a 700-bp fragment was

generated from the coding region of the *GUS* gene using the PCR DIG Probe Synthesis Kit (Roche Diagnostics, Mannheim, Germany) and a pair of primers specific for the *GUS* gene (F: 5′-ATGGTAGATCTGACTAGTTTACGTC-3′; R: 5′-TGGCTGTGA-CGCACAGTTCATAGAG-3′). The thermal cycling conditions were identical to those specified above. The subsequent hybridization steps were performed according to the instruction manual for the DIG labelling and detection system (Roche Diagnostics).

For reverse transcriptase–polymerase chain reaction (RT–PCR) analysis, total RNA was extracted from randomly sampled leaves of transgenic plants using the Plant RNA Purification Reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA synthesized from 0.5 μg purified RNA was reverse-transcribed with a Reverse Transcriptase kit (TaKaRa Biotech, Dalian, China). The PCR conditions were identical to those mentioned above.

Histochemical GUS assay

Histochemical assays of GUS activity were performed using standard procedures (Jefferson et al. 1987) with the modifications described by Matsunaga et al. (2012).

Scanning electron microscopy and histological observation

For scanning electron microscopy (SEM), samples were glued onto aluminium stubs and placed on a chamber stage that had been precooled to –120 °C. The samples were viewed using a VP-SEM instrument (S-3500N, Hitachi, Tokyo, Japan). Histological observations were performed using the procedure described by Yang et al. (2010*a*, 2010*b*).

Statistical analysis

Analysis of variance was performed with SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). Means that differed significantly were compared using Duncan's multiple range test at the 5% probability level.

Results

Development of the regeneration protocol

Mature seeds of *S. matsudana* were used as the source of explants for shoot induction (Figure 1a). All combinations of PGRs tested induced the formation of multiple shoots (Table 1). In the presence of TDZ, many multiple shoots formed but few elongated (data not shown). When combined with NAA, both Z and BA induced multiple shoots with almost the same frequency. The difference between Z and BA treatments was that shoots induced on Z were more intensely green and grew faster after transfer to EM (data not shown). Therefore, Z was used subsequently to induce multiple shoots (Figure 1b). The highest induction frequency was observed for medium

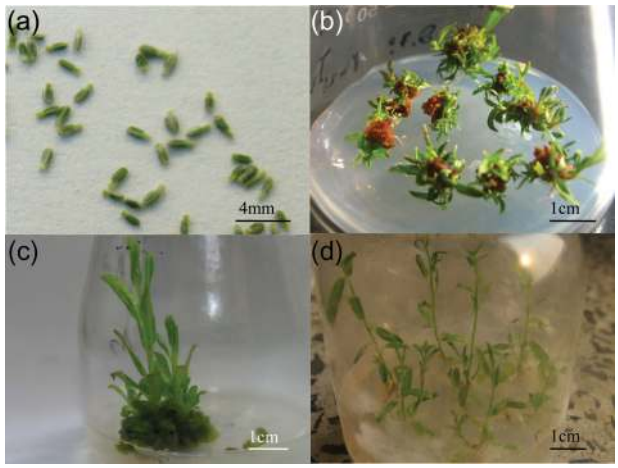

Figure 1. Plant regeneration from mature seeds of *S. matsudana*. (a) Mature seeds, (b) multiple shoots induced from seeds, (c) elongated shoots and (d) regenerated plantlets.

Table 1. Effect of TDZ, BA, Z and NAA on multiple shoot induction frequency from mature seed explants after 4 weeks of culture.

Plant growth regulators (μM)				Multiple shoot induction frequency (%)[1]
TDZ	BA	Z	NAA	
2.25				81.4b
2.25			0.54	86.1ab
4.5				82.6b
4.5			0.54	90.7a
	2.2			60.2f
	2.2		0.54	74.6cd
	4.4			68.2e
	4.4		0.54	80.7bc
		2.25		61.6f
		2.25	0.54	75.0c
		4.5		68.7de
		4.5	0.54	81.4b

[1]Means followed by the same letters are not significantly different using Duncan's multiple range test at $P = 0.05$.

supplemented with 4.5 μM Z and 0.54 μM NAA (Table 1). When the multiple shoots that were induced in the presence of Z and NAA were transferred to EM, multiple shoots elongated to a length of >20 mm within 4 weeks (Figure 1c). All of these shoots produced roots within 4 weeks, at which point elongated shoots were separated and transferred to RM (Figure 1d).

When cultured on medium supplemented with 4.5 μM Z and 0.54 μM NAA, seeds (Figure 2a) began to germinate after 2 days (Figure 2b), and the apical dome emerged after an additional 2 days (Figure 2c). By the sixth day, after transfer of the seeds to the medium, the apical dome had further developed into primary leaves (Figure 2d). By the eighth day, further development of the apical buds was apparent, along with callus formation for many of the seed explants (Figure 2e). Multiple shoot induction was observed from apical regions within 2 weeks (Figure 2f). Observation by SEM showed that

multiple shoots originated directly from the regions at the junction between the hypocotyl and cotyledons (see Figure S1a available as Supplementary Data at *Tree Physiology* Online), whereas no shoot induction was observed for seeds cultured on medium that lacked PGRs (see Figure S1b available as Supplementary Data at *Tree Physiology* Online).

The ability of *A. tumefaciens* to infect plant explants at sites of wounding and to incorporate exogenous genes into the plants is well documented. To evaluate the effect of wounding on the induction of multiple shoots, seeds were incubated on preculture medium for 2, 4, 6 or 8 days, the base of the emergent apical buds was excised using a scalpel blade (Figures 3a–c), and the apical-bud explants were cultured on SIM for 4 weeks. Seeds precultured for 4 days (76.4%) showed a significantly higher frequency of multiple-shoot regeneration than that observed for seeds precultured for either 6 days (64.7%) or 8 days (57.0%) ($P < 0.001$). Although seeds began to germinate after the second day of incubation on SIM, it was not, however, easy to excise the almost-invisible apical dome (Figure 2c). Multiple shoots were induced mainly from the cut surface of the seed explants (Figure 3d). Histological observation confirmed that the site of direct induction of multiple shoot formation was a multicellular region at the base of the apical bud meristem of seed explants (Figure 3e). Control explants had no capacity to form multiple shoots (Figure 3b). Observation by SEM revealed that all of the multiple shoots originated from the cut surface (Figure 3f).

Determination of the optimal concentration of kanamycin for transformation selection

We first cultured the seeds on SIM supplemented with different concentrations of kanamycin. The induction of multiple shoots was completely inhibited by 30 mg l^{-1} kanamycin (data not shown). As a consequence, a concentration of 30 mg l^{-1} kanamycin was selected to screen putative transformants. Given that 50 mg l^{-1} kanamycin completely prevented new growth of multiple shoots when cultured on EM (data not shown), this concentration of kanamycin was used to select for transgenic shoots. For root selection on EM, kanamycin at 30, 40 and 50 mg l^{-1} significantly decreased the percentage of single shoots that produced roots (26.5, 12.6 and 0%, respectively; $P < 0.001$). Thus, 50 mg l^{-1} kanamycin was chosen to select for transgenic roots because it completely prevented non-transformed shoots from producing roots.

Optimization of the transformation procedure

After seed explants were precultured for 2–8 days and co-cultivated with *A. tumefaciens* for 2 weeks, kanamycin-resistant shoots began to emerge from the cut surface (Figure 4a). Staining for GUS activity provided no evidence for blue staining in control shoots (Figure 4b), whereas the *GUS* gene was expressed in kanamycin-resistant shoots (Figure 4c). The preculture period significantly affected the transformation efficiency ($P < 0.001$). Preculture of mature seeds for 4 days on SIM provided the highest frequencies of formation of kanamycin-resistant shoots and GUS-positive shoots (Figure 5a). Therefore, a 4-day preculture period was used to optimize the remaining parameters evaluated.

The strain and density of *A. tumefaciens*, co-cultivation duration and co-cultivation temperature significantly affected the transformation efficiency (Figure 5b–e; $P < 0.001$). The best results in terms of induction of GUS-positive kanamycin-resistant shoots were obtained using the LBA4404 strain and involved dipping explants into a bacterial suspension ($OD_{600} = 0.6$) for 30 min and then co-cultivating the explants in darkness for 4 days at 21 °C. Storage of seeds at –20 °C for as long as 3 years had no significant effect ($P > 0.05$) on induction of kanamycin-resistant shoots. Although

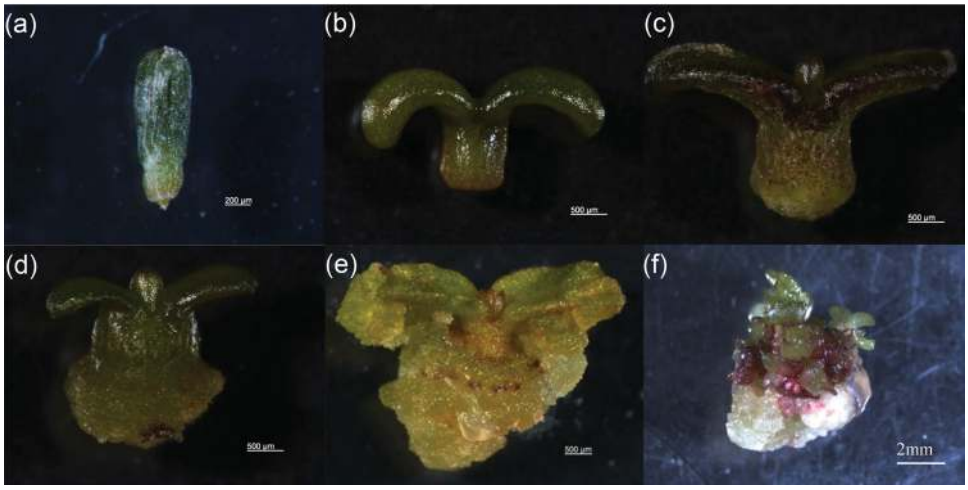

Figure 2. Morphological changes in mature seeds of *S. matsudana* cultured on MS medium containing 4.5 μM Z and 0.54 μM NAA. (a) Mature seed, and seeds cultured for (b) 2 days, (c) 4 days, (d) 6 days, (e) 8 days and (f) 14 days.

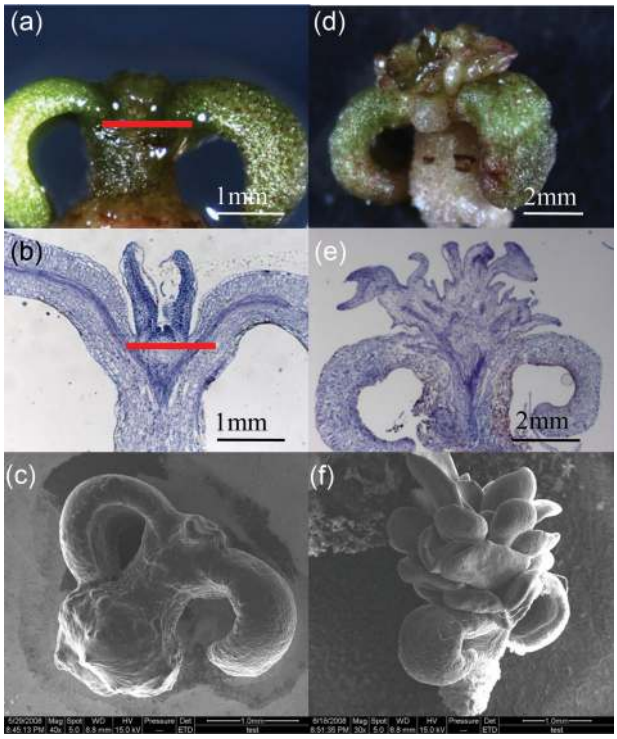

Figure 3. Microscopic and histological observations of multiple shoot induction from the cut surface of mature seed explants. (a) The emergent apical buds from seeds precultured for 4 days were excised at the base using a scalpel blade. A line indicates the excision site. (b) Histological observation of a control seed germinated on medium lacking PGRs. A line indicates the excision site. (c) Scanning electron micrograph of emergent apical buds excised from seeds precultured for 4 days. (d) Multiple shoots induced from the cut surface of the excised apical bud seed explants. (e) Histological observation of multiple shoots originating from the cut surface of seed explants. (f) Scanning electron micrograph of multiple shoots originating from the cut surface of seed explants.

transformants were obtained from seeds stored for at least 5 years, the transformation frequency for these seeds was lower than that for seeds stored for no more than 3 years (Figure 5f). The optimal conditions for transformation of *S. matsudana* (*A. tumefaciens* strain LBA4404 and 4 days co-cultivation at 21 °C) were used to infect explants from mature seeds that had been precultured for 4 days.

Development of transgenic plants

From a total of 180 seeds cultured on SIM medium for 4 weeks, kanamycin-resistant shoots were regenerated from as many as 35 seeds. After explants with kanamycin-resistant shoots were cultured on the same medium for a further 8 weeks, rapidly proliferating kanamycin-resistant shoots were present on 13 explant lines (Figure 4d); all other explant lines had become chlorotic, grew slowly or died because of excessive bacterial growth. Staining for GUS activity indicated that GUS was expressed in the kanamycin-resistant shoots (Figure 4e). After each of the multiple shoots had grown to a length of ~2.0 cm, the shoot clumps were divided into small pieces (~five shoots per explant) and cultured on EM for 4 weeks. Of these excised shoots, 74.2% developed healthy lateral roots and had elongated (>4.0 cm long) on EM containing 50 mg l^{-1} kanamycin after 4 weeks of culture.

Histochemical GUS analysis showed homogeneous blue staining in the roots, stems and leaves of all of the regenerating transgenic plants, whereas no GUS expression was observed in non-transformed tissues (Figure 4f).

Confirmation of transgene insertion by PCR, Southern blot and RT–PCR analysis and isolation of transgenic plants

To confirm that *GUS* was inserted into the *S. matsudana* genome, the transgene was amplified using the *GUS*-specific primer pair, yielding a band of the expected size (1.0 kb). Analysis of the 13 kanamycin-resistant plant lines indicated that the highest frequencies with which PCR-positive transformants were generated ranged from 36.4 to 93.8% (Table 2). To isolate the transgenic plants, PCR-positive shoot tips that were ~1.5 cm long were excised and cultured on EM containing 50 mg l^{-1} kanamycin. Approximately 79.4% of shoots rooted and grew vigorously after 8 weeks of culture in vitro. The second round of PCR analysis of selected plants identified more PCR-positive lines than during the first round, with four lines showing 100% transgenesis (Table 2). The in vitro generation of 13 lines of transgenic plants equates to a transformation frequency of 7.2%. The transformation cycle from inoculation to plantlet regeneration took ~5 months.

All PCR-positive plants were transplanted into soil. Acclimated plants showed 96% survival after transplantation and grew normally, reaching a height of >1.2 m after 6 months (Figure 6a). The PCR analysis of transgenic plants maintained for 2 years after propagation by cuttings showed that 73.3–100% of the plants generated from the 13 putatively transformed lines and transferred to the field were PCR positive (Table 2). Nine putatively transformed lines showed no signs of chimaerism because all plants of each line were PCR positive (Figure 7). Strong staining for GUS activity was observed in the young leaves (Figure 6b) and roots (Figure 6c) of selected transformed plantlets, whereas no GUS expression was observed in non-transformed tissues (data not shown).

For Southern blot analysis, genomic DNA was digested with HindIII. The membrane was hybridized with a 700-bp fragment from *GUS*. No hybridization signal was detected using control DNA, whereas the nine independent lines analysed showed several hybridization bands, with the number of inserted transgene copies ranging from one to two as determined by digestion with HindIII (Figure 8). RT–PCR analysis further confirmed the expression of the *GUS* gene in transgenic plants (see Figure S2 available as Supplementary Data at *Tree Physiology* Online).

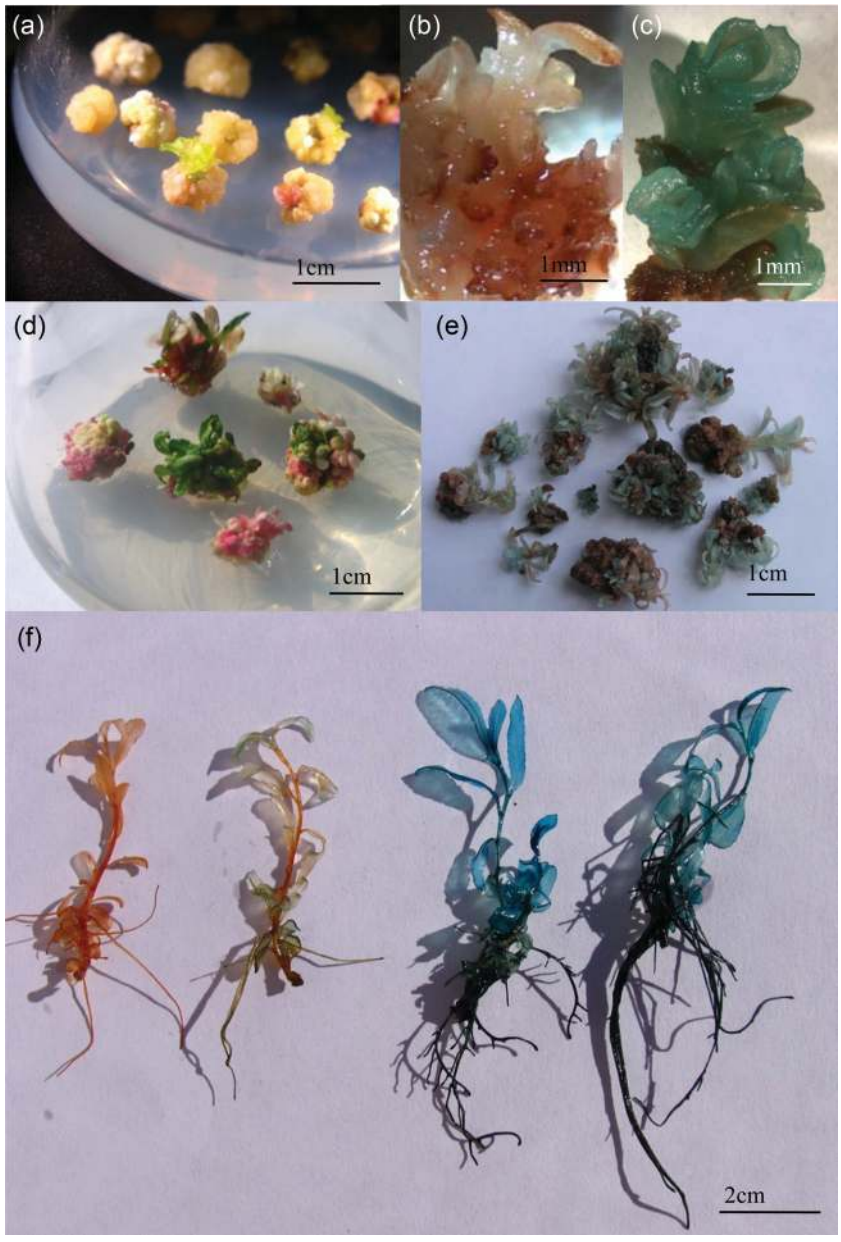

Figure 4. Regeneration of transgenic *S. matusudana* plants and histological GUS assay. (a) Kanamycin-resistant shoots formed after co-cultivation with *A. tumefaciens* LBA4404 harbouring the binary vector pBI121. (b) Histochemical staining for GUS activity in control shoots. (c) Histochemical staining for GUS activity in kanamycin-resistant shoots. (d) Multiplied kanamycin-resistant shoots. (e) Histochemical staining for GUS activity in the multiplied kanamycin-resistant shoots. (f) Histochemical staining for GUS activity in regenerating transgenic plants (the two right-hand plants) and non-transformed plants (the two left-hand plants).

Discussion

The application of genetic engineering to *Salix* species requires the availability of efficient methods for gene transfer, selection and plant regeneration. *Agrobacterium*-mediated transformation methods using vegetative tissues, such as leaf and stem explants, have been reported for willows (Vahala et al. 1989, 1993). Although in these previous reports transgenic callus was obtained, regeneration of shoots was unsuccessful in both studies. In the present study, we established a simple and reliable *A. tumefaciens*-mediated transformation system for *S. matsudana* using the embryonic apical region of germinated seeds. The overall duration of the transformation and plant regeneration process was very short (5 months), and hence attractive for willow breeders. Moreover, this study is the first report of regeneration of transgenic willow plants and the proposed protocol may be applicable for transformation of other recalcitrant *Salix* species.

Shoot regeneration is an essential prerequisite for *Agrobacterium*-mediated transformation experiments. Shoot apical meristem tissues have been the explants of choice for

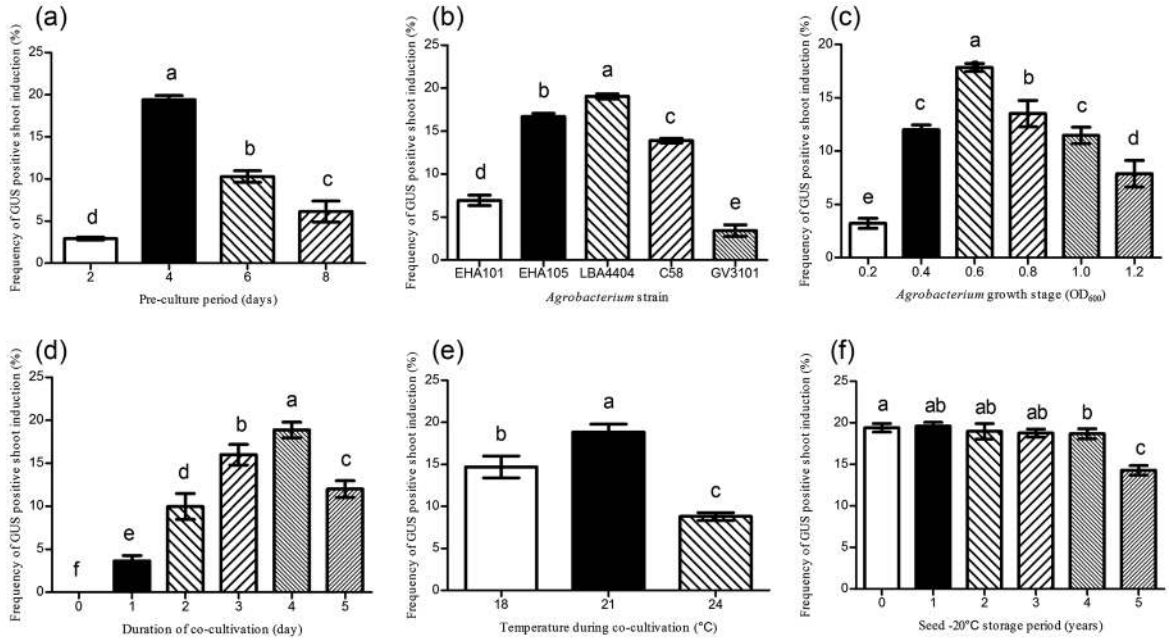

Figure 5. Factors that affect the frequency of GUS-positive kanamycin-resistant shoot induction in *S. matsudana*. (a) Preculture period. (b) *Agrobacterium* strain. (c) *Agrobacterium* growth stage. (d) Duration of co-cultivation. (e) Temperature during co-cultivation. (f) Duration of seed storage at –20 °C. Results are presented as the means and standard errors from three independent experiments. Means followed by the same letters are not significantly different using Duncan's multiple range test at $P = 0.05$.

Table 2. PCR-positive frequencies of kanamycin-resistant plant lines of *S. matsudana* after three rounds of PCR analysis of the *GUS* gene.

Putatively transformed lines	PCR analysis								
	First round of PCR[1]			Second round of PCR[2]			Third round of PCR[3]		
	No. of plants	No. of PCR-positive plants	PCR positive (%)	No. of plants	No. of PCR-positive plants	PCR positive (%)	No. of plants	No. of PCR-positive plants	PCR positive (%)
G1	19	11	57.9	17	14	82.4	24	18	75.0
G2	27	22	81.5	22	20	90.9	29	29	100
G3	22	8	36.4	7	5	71.4	15	11	73.3
G4	15	9	60.0	18	15	83.3	30	30	100
G5	20	11	55.0	10	7	70.0	15	13	86.7
G6	25	20	80.0	17	15	92.0	30	30	100
G7	18	15	83.3	13	13	100	25	25	100
G8	24	17	70.8	14	12	85.7	24	24	100
G9	25	18	72.0	17	17	100	34	34	100
G12	18	11	61.1	9	6	66.7	24	20	83.3
G14	16	15	93.8	12	12	100	24	24	100
G15	26	23	88.5	18	18	100	36	36	100
G16	27	21	77.8	17	15	88.2	30	30	100

[1]Kanamycin-resistant in vitro plants were analysed.
[2]PCR-positive plants further selected on kanamycin containing medium for 8 weeks were analysed.
[3]PCR-positive plants 2 years after transplantation were analysed.

regeneration and transformation studies of recalcitrant crops (Sticklen and Oraby 2005). They have also been used to transform woody plants such as *V. vinifera* (Dutt et al. 2007) and loblolly pine (Gould et al. 2002). In the present study, embryonic apical regions of germinating seeds efficiently produced adventitious shoots directly, which were further rapidly multiplied and developed roots. Willows are, in general, considered to be recalcitrant to adventitious bud regeneration via callus (Vahala et al. 1989, 1993). Lyyra et al. (2006) reported direct adventitious bud induction from the inflorescence of black willow without tissue browning, but few of the buds elongated. Although the underlying mechanisms remain to be determined, these observations suggest that the embryonic shoot apical region is the best tissue source for bud induction in willows.

Figure 6. Transplantation of PCR-positive plants. (a) Surviving plants after acclimation for 6 months. From left to right are PCR-positive plant lines G2, 4, 6, 7, 8, 9, 14, 15, 16, respectively. Histochemical staining for GUS activity in the young leaves (b) and roots (c) of selected transformed plantlets.

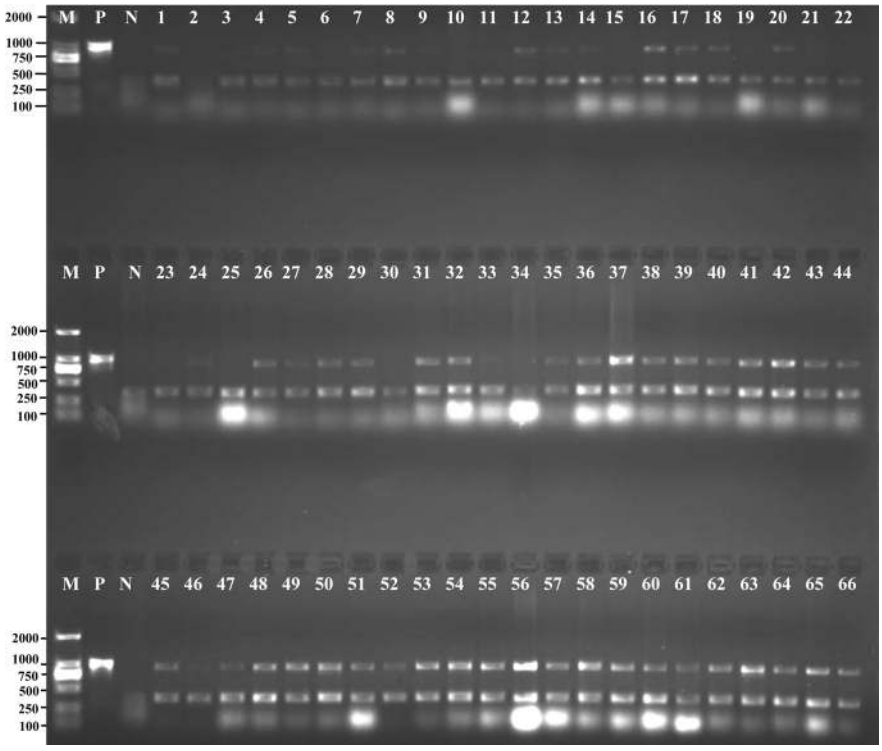

Figure 7. PCR amplification of the *GUS* gene (1.0 kb) in randomly selected transformed plantlets maintained for 2 years after propagation from cuttings. Lane M: 2000 bp plus DNA marker; lane P: pBI121 plasmid DNA as a positive control; lane N: genomic DNA from a non-transformed plantlet as a negative control; lanes 1–66: genomic DNA from plants of 13 putatively transformed lines G1 (lanes 1–4), G2 (lanes 5–8), G3 (lanes 9–15), G4 (lanes 16–18), G5 (lanes 19–25), G6 (lanes 26–29), G12 (lanes 30–34), G7 (lanes 35–40), G8 (lanes 41–45), G9 (lanes 46–50), G14 (lanes 51–55), G15 (lanes 56–61) and G16 (lanes 62–66) carrying the *GUS* gene.

In the current study, the elongation and regeneration of buds induced by Z was superior to that by BA or TDZ. Moreover, adventitious buds induced on medium containing Z originated mainly from the cut surface of the seed explants (i.e., from cells that were well exposed to *Agrobacterium* for T-DNA transfer), which is beneficial for transgenic bud regeneration and selection (Matsunaga et al. 2012). The superiority of Z over either BA or TDZ for promotion of shoot regeneration was reported by Matsunaga et al. (2012) for *Eucalyptus globulus* Labill. and by Yevtushenko and Misra (2010) for *Populus nigra* L. × *P. maximowiczii* A. Henry. We noted that TDZ and BA induce multiple buds, but the buds ceased growth in *S. matsudana*. Similar results were reported by Lyyra et al. (2006) for black willow and by Khan et al. (2011) for Indian willow (*Salix*

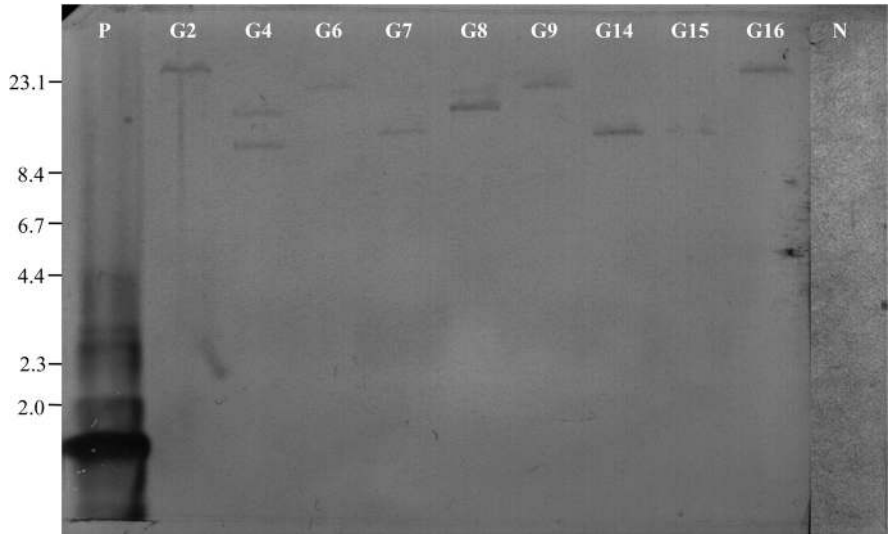

Figure 8. Southern blot analysis of transgenic plants. Lane P: pBI121 plasmid as a positive control; lanes 1–9: genomic DNA isolated from transgenic plant lines (G2, G4, G6, G7, G8, G9, G14, G15 and G16); lane N: genomic DNA isolated from a non-transformed plant as a negative control. DNA samples were digested with HindIII, and the PCR-amplified product of the *GUS* gene was used as a probe.

tetrasperma Roxb.). The developmental phase of source plants is also important. It is known that increasingly juvenile plant tissues are more efficient for regeneration (Peña and Séguin 2001). Ghorbel et al. (2000) suggested that the presence of newly synthesized cell walls is required for successful attachment of *Agrobacterium* for transformation to proceed. Our experiments showed that a visible apical dome emerged after the fourth day of seed preculture. Accordingly, the frequency of multiple bud induction was highest when the apical dome region of seeds was excised at this time. The highest frequency of kanamycin-resistant buds was also observed when mature seeds were precultured for 4 days prior to infection with *A. tumefaciens*.

The *Agrobacterium* strain is among the most important factors that affect transformation efficiency. Octopine strain LBA4404 is reported to be more efficient than other strains in tree species including poplars (Fladung et al. 1997) and *Eucalyptus* (Prakash and Gurumurthi 2009). However, an agropine/succinamopine-type strain such as EHA105 is reportedly more successful than LBA4404 for transformation of *Populus trichocarpa* Torr. & Gray × *P. deltoides* Bartr. ex. Marsh. (Han et al. 2000) and *Phellodendron amurense* Rupr. (Yang et al. 2013). In the present study, LBA4404 induced the highest frequency of kanamycin-resistant buds. We noted that a super-virulent strain such as EHA105 was less effective than the less-virulent strain LBA4404. It is proposed that increased *Agrobacterium* virulence could decrease the activity of the embryo meristematic cells, and this could be the cause of the lower transformation efficiency. A similar result was reported by Ghorbel et al. (2000) for sour orange.

The co-culture duration and co-culture temperature affect *Agrobacterium*–plant cell interactions. Longer periods of co-culture seem to be effective for transfer of the T-DNA into plant cells. However, prolonged co-culture results in excessive bacterial growth and decreases the transformation efficiency in plant species (Yang et al. 2010*a*, 2010*b*). Many attempts to overcome this problem have been undertaken. Ribas et al. (2011) reported that inoculation of the infected explants on filter paper in the co-cultivation medium was effective to reduce overgrowth of *Agrobacterium* and extended the co-cultivation time in *Coffea arabica* (L.). In *P. amurense*, reduction of the co-cultivation temperature prevented bacterial overgrowth and simplified the decontamination process, which resulted in increased transformation efficiency (Yang et al. 2013). On the basis of our present results, at a co-cultivation temperature of 21 °C, the bacterium grew more slowly than at 24 °C or a higher temperature (data not shown), and almost no necrosis was apparent until the fourth day of preculture. In contrast, the kanamycin-resistant bud induction efficiency was not increased at 18 °C, which might be because a low temperature decreases *Agrobacterium* virulence. Co-cultivation on filter paper for 4 days at 21 °C was optimal for transformation.

Transgenic plants regenerated directly from shoot apical meristem tissues that were targeted for transformation are always chimaeric in corn (Sairam et al. 2003), rice (Yookongkaew et al. 2007), jujube (Gu et al. 2008), Kentucky bluegrass (Zhang et al. 2010) and poplar (Yevtushenko and Misra 2010). One way to overcome this technological barrier for trees might be to screen each regenerated plant to confirm transgenic integration (see the review by Hansen and Wright 1999). In the present study, PCR analysis showed that 7.2–63.6% of kanamycin-resistant plants derived from multiple buds from all tested 13 lines were PCR negative. Several 'escapes' were eliminated by a second round of selection. A

similar benefit of second-round selection for the elimination of transgene loss in tree species was reported for *P. nigra* × *P. maximowiczii* hybrid poplar (Yevtushenko and Misra 2010). The results of the present study also underscore the importance of selecting transgenic lines shortly after transformation.

In conclusion, we report the development of a highly efficient short-term in vitro regeneration system for gene transfer into *S. matsudana* mediated by *A. tumefaciens*. Our method for direct shoot regeneration from the apical region of the embryonic shoot minimizes the duration of axenic culture and provides an alternative procedure for transformation of other recalcitrant *Salix* species. This rapid and efficient system for regeneration of transformants from mature seeds will enable genes for desirable traits to be introduced into commercially important *Salix* species. Such traits might increase the feasibility of using *Salix* species for phytoremediation and bioenergy applications.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared.

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