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ORIGINAL PAPER

An efficient *Agrobacterium*-mediated transformation and regeneration system for leaf explants of two elite aspen hybrid clones *Populus alba* × *P. berolinensis* and *Populus davidiana* × *P. bolleana*

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Abstract Transgenic technology has been successfully used for gene function analyses and trait improvement in cereal plants. However, its usage is limited in woody plants, especially in the difficult-to-transform but commercially viable hybrid poplar. In this work, an efficient regeneration and transformation system was established for the production of two hybrid aspen clones: *Populus alba* × *P. berolinensis* and *Populus davidiana* × *P. bolleana*. A plant transformation vector designed to express the reporter gene *uidA*, encoding *β*-glucuronidase (GUS), driven by the cauliflower mosaic virus 35S promoter, was used to detect transformation event at early stages of plant regeneration, and to optimize the parameters that may affect poplar transformation efficiency. Bacterium strain and age of leaf explant are two major factors that affect transformation efficiency. Addition of thidiazuron (TDZ) improved both regeneration and transformation efficiency. The transformation efficiency is approximately 9.3% for *P. alba* × *P. berolinensis* and 16.4% for *P. davidiana* × *P. bolleana*. Using this system, transgenic plants were usually produced in less than 1 month after co-cultivation. The growth characteristics and morphology of transgenic plants were identical to the untransformed wild type plants, and the transgenes could be inherited by vegetative propagation, as confirmed by PCR, Southern blotting, RT–PCR and *β*-glucuronidase staining analyses. The establishment of this system will help to facilitate the studies of gene functions in tree growth and development at a genome level, and as well as the introduction of some valuable traits in aspen breeding.

Keywords *Agrobacterium tumefaciens* · Aspen · *Populus* · Regeneration · Transformation

Abbreviations

AS	Acetosyringone
6-BA	6-Benzyladenine
GUS	*β*-Glucuronidase
MS	Murashige and Skoog (1962)
NAA	Naphthalene-acetic acid
nptII	Neomycin phosphotransferase gene
TDZ	Thidiazuron

Introduction

Recent advances in molecular genetics and biotechnology have made plant transformation and regeneration a powerful tool for crop improvement and gene function analyses. Although transgenic plants have been obtained by *Agrobacterium tumefaciens*-mediated transformation in many species (Han et al. 1996; Jouanin et al. 1993; Kim et al. 1997), plant transformation and regeneration are still difficult and time-consuming in many economically important species, especially in forest tree species (Birch 1997; Han et al. 2000).

Modern methods of genetic engineering can help breeders to overcome limitations of the conventional techniques for tree improvement (Confalonieri et al. 2003). *Populus* is one of the major renewable resources that are

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highly valued by the pulp and paper industry for their fast growth and high quality fiber (Stettler et al. 1996). They are also extensively used for land reforestation and contaminated soil phytoremediation (Balatinecz et al. 2001; Rishi et al. 2001). However, due to the long generation period and callus/shoot initiation on different media, *Agrobacterium*-mediated poplar transformation was reported only for a limited number of model genotypes (Confalonieri et al. 2000; Dai et al. 2003; De Block 1990; Delledonne et al. 2001; Eriksson et al. 2000; Fillatti et al. 1987; Gallardo et al. 1999; Han et al. 1997, 2000; Howe et al. 1994; Hu et al. 1999; Pilate et al. 2002; Sepanen et al. 2004; Song et al. 2006; Tsai et al. 1994; Wei et al. 2006).

Recently, Yevtushenko and Misra (2010) reported the successful transformation of a hybrid poplar *P. nigra* L. × *P. maximowiczii* A. Henry (genotype NM6), with a mean transformation efficiency of 36.3%. A novel approach for in situ bud transformation of *P. cathayana Rehd* by *Agrobacterium* was also established (Yang et al. 2010). We have focused on improving salt resistance in commercially valuable hybrid poplars. *P. alba* × *P. berolinensis* and *P. davidiana* × *P. bolleana* are two salt-sensitive aspen hybrid clones widely grown in the north part of China. In the present work, we describe a rapid and efficient protocol which does not require a callus induction phases for producing transgenic hybrid poplars.

Materials and methods

Plant material and growth conditions

Two aspen hybrid clones commercially grown in the north part of China, Yinzhong (*P. alba* × *P. berolinensis*) and Shanxin yang (*P. davidiana* × *P. bolleana*), were used in this study. Generally, in vitro grown plants were sub-cultured monthly by aseptically transferring shoot apices to the fresh MS medium (Murashige and Skoog, 1962) supplemented with 0.1 mg/l NAA (MS1) in glass cultivation bottles (Table 1). Plantlets were grown in the culture room with cool white fluorescent light (~200 μmol m^{-2} s^{-1}) under short day condition (12 h light/12 h dark). The temperature was kept at about 21–25°C in the daytime and 15–18°C at night.

Table 1 Composition of media used in *Agrobacterium*-mediated transformation of Yinzhong (*Populus alba* × *P. berolinensis*) and Shanxin yang (*Populus davidiana* × *P. bolleana*)

Medium	Composition
MS1	MS with 0.1 mg/l NAA + 500 mg/l cefotaxime
MS2	MS with 0.1 mg/l NAA + 0.2 mg/l 6-BA + 50 mg/l AS
MS3	MS with 0.1 mg/l NAA + 0.2 mg/l 6-BA + 0.01 mg/l TDZ + 500 mg/l cefotaxime
MS4	MS3 + 50 mg/l kanamycin
MS5	MS1 + 25 mg/l kanamycin

Vector and *Agrobacterium* strains

The binary vector, pCAMBIA2301 (Huang et al. 2009), which contains a β-glucuronidase (GUS)-encoding gene (*uidA*), driven by the cauliflower mosaic virus 35S promoter, and a selection marker gene (*nptII*) (Fig. 1), was introduced into *A. tumefaciens* strain LBA4404, GV3101 and EHA105, respectively, as described previously (Holsters et al. 1978). Growth of pCAMBIA2301-containing *Agrobacterium* culture was carried out as described previously (Ho et al. 1998).

GUS expression analysis

To optimize the transformation procedure, factors that may affect the regeneration and transformation frequencies, including *Agrobacterium* strain, explant source, physiological condition for regeneration, and phytohormone type and concentration, were examined by GUS transient expression assay. For each treatment, 120 leaf explants were used and each experiment was repeated at least three times. Juvenile, adult and senescent leaf explants (2–3 cm^2) collected from in vitro 21-day-old poplar plants of Yinzhong (*P. alba* × *P. berolinensis*) grown on MS1, were co-cultivated on MS2 (Table 1) for 2 days with different *A. tumefaciens* strains (LBA4404, GV3101 and EHA105) harboring pCAMBIA2301 under different conditions. The explants were then transferred to *A. tumefaciens* inhibition medium MS3 (Table 1) for GUS expression analysis. Histochemical GUS staining was conducted as described previously (Gallagher 1992). After infection and co-cultivation, leaf explants were incubated overnight at 37°C in a reagent mix containing 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc), 0.1 M sodium phosphate buffer (pH 7.0), 0.5 mM each potassium ferri- and ferrocyanide, 10 mM EDTA (pH 7.0), and 0.1% Triton X-100. After staining, the tissues were cleared of chlorophyll using 95% ethanol. GUS transformation efficiency was expressed as the percentage of leaf explants showing GUS activity relative to the total number of co-cultivated leaf explants. For GUS detection in transgenic plants, whole leaves from the regenerated kanamycin-resistant plants were used.

Plant transformation

Leaf explants excised from 3-week-old plantlets were inoculated by swirling in the *Agrobacterium* culture for 8–10 min. The inoculated explants were placed

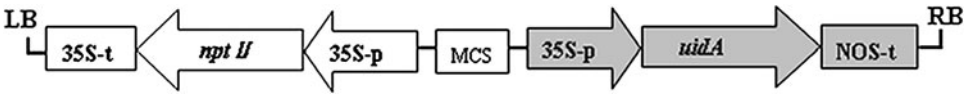

Fig. 1 Schematic representation of T-DNA region in the plasmid vector pCAMBIA2301. *LB* T-DNA left border repeat, *35S-t* CaMV35S terminator, *npt-II* neomycin phosphotransferase II gene, *35S-p* CaMV35S promoter, *MCS* multiple cloning sites, *uidA* β-glucuronidase gene, *NOS-t* NOS terminator, *RB* T-DNA right border repeat

horizontally and co-cultivated on MS2 (Table 1) at 24 ± 2°C in the dark for 2 days. The co-cultivated explants were washed four times in sterilized distilled water for 5 min each, briefly blotted dry on a sterilized filter paper, and cultured on MS3 (Table 1) at 24 ± 2°C, 16 h light/8 h dark cycle with supplemental lighting (Philips, 600 W) of 200 $\mu Em^{-2}\ s^{-1}$, for 7 days. Then, the explants were transferred onto MS4 (Table 1), followed by sub-culturing on fresh MS4 every 14 days until the transgenic shoots were formed. This usually takes about 21–30 days after co-cultivation. Transgenic shoots (1 cm in length) were excised from the explants and rooted on MS5 (Table 1). Transgenic plants were transplanted into soil and maintained in a greenhouse. Transformation frequency was defined as the percentage of co-cultivated leaf explants that produced transgenic shoots. For each leaf explant, only one shoot was selected and further analyzed.

Statistical analysis

For statistical analyses, the Student's *t* test was used to generate every *P* value. The alpha level was 0.01. The tests were one-tailed. The data were normalized and all samples were normally distributed with homogeneity of variance. Differences were considered to be statistically significant when the confidence intervals showed no overlap for the mean values with an error of 0.01.

Plant genomic DNA isolation and PCR analysis

Genomic DNA was isolated from leaves (0.5 g) of transgenic and control plant as described previously (Kang et al. 2010). To ensure that samples were free from *Agrobacterium* contamination, each sample was taken from shoots growing on rooting medium with 25 mg/l kanamycin for 4 weeks. For PCR analyses of *uidA*, gene-specific primers (forward 5′-GCCGGAATCCATCGCAGCGTA-3′ and reverse 5′-CCCGCTTCGAAACCAATGCCT-3′) were used to amplify a 602-bp fragment.

Southern blot analysis of transgenic plants

For Southern blot analysis, four micrograms of genomic DNA isolated from the leaves of transformed and control poplar plants was digested with *Eco*RI, electrophoresed on 0.8% agarose gels, and transferred onto Hybond N^+ nylon membranes by vacuum blotting system. The 602-bp fragment of *uidA*-coding region was used as a hybridization probe. Standard procedures for Southern blot analysis and probe labeling were conducted with DIG DNA Labeling and Detection Kit1 (Roche, Germany) following the manufacturer's instruction.

Reverse transcriptase-PCR analyses

Total RNA was isolated from leaves of 3-week-old poplar plants with the TRIZOL Reagent (Invitrogen, Shanghai, China) following the manufacturer's instruction. After treated with DNase I (Promega), 2 μg of total RNA was subjected to reverse transcription reaction using the reverse transcriptase ReverTra Ace (TOYOBO, Japan) at 42°C for 1 h. Reverse transcriptase-mediated PCR (RT–PCR) was performed using *uidA* gene specific primers to amplify a PCR product of 602 bp. Expression level of *PtEF1β* was also determined with forward (5′-GACAAGAAGGCA GCGGAGGAGA G-3′) and reverse (5′-CAATGAGGGA ATCCACTGACACAA G-3′) primers (to serve as a quantitative control).

Kanamycin-resistance stability evaluation

Leaf explants excised from PCR, RT–PCR and GUS staining confirmed plantlets (3-week-old) were directly cultured on MS4 (Table 1) at 24 ± 2°C with cool white fluorescent light (~200 $\mu mol\ m^{-2}\ s^{-1}$) on a 12 h light/12 h dark photoperiod. Regenerated kanamycin-resistant shoots (1 cm in length) were excised from the explants and rooted on MS5 (Table 1). Transgenic plants with well-developed root systems were obtained usually after 2–3 weeks on MS5.

Results

Optimization of the transformation procedure

The commercial aspen hybrid clone Yinzhong (*P. alba* × *P. berolinensis*) was used for the preliminary GUS expression experiments. After infection with *A.*

tumefaciens EHA 105 harboring pCAMBIA2301 (Fig. 1), GUS expression were detected in 3 days on MS3 (Figs. 2a, 3d), with the best staining 6 days after transfer (Fig. 2a). *Agrobacterium* strain is one of the major factors affecting transformation efficiency. EHA105 produced the highest transformation frequency while GV3101 showed little or not virulence (Fig. 2b). Therefore, EHA105 harboring pCAMBIA2301 was chosen for the following examination of other factors. Addition of acetosyringone (Fig. 2c), age of leaf (Fig. 2d), growth stage of bacterium (Fig. 2e) and dark period during co-cultivation (Fig. 2f) all significantly affect the expression of *uidA* gene. The best results in terms of GUS expression were obtained using EHA105 pCAMBIA2301 *A. tumefaciens* strain, dipping juvenile leaf explants into a bacterial suspension ($OD_{600} = 0.6$) for 8–10 min, and co-cultivating them in dark conditions on MS2 medium supplemented with 50 mg/l acetosyringone.

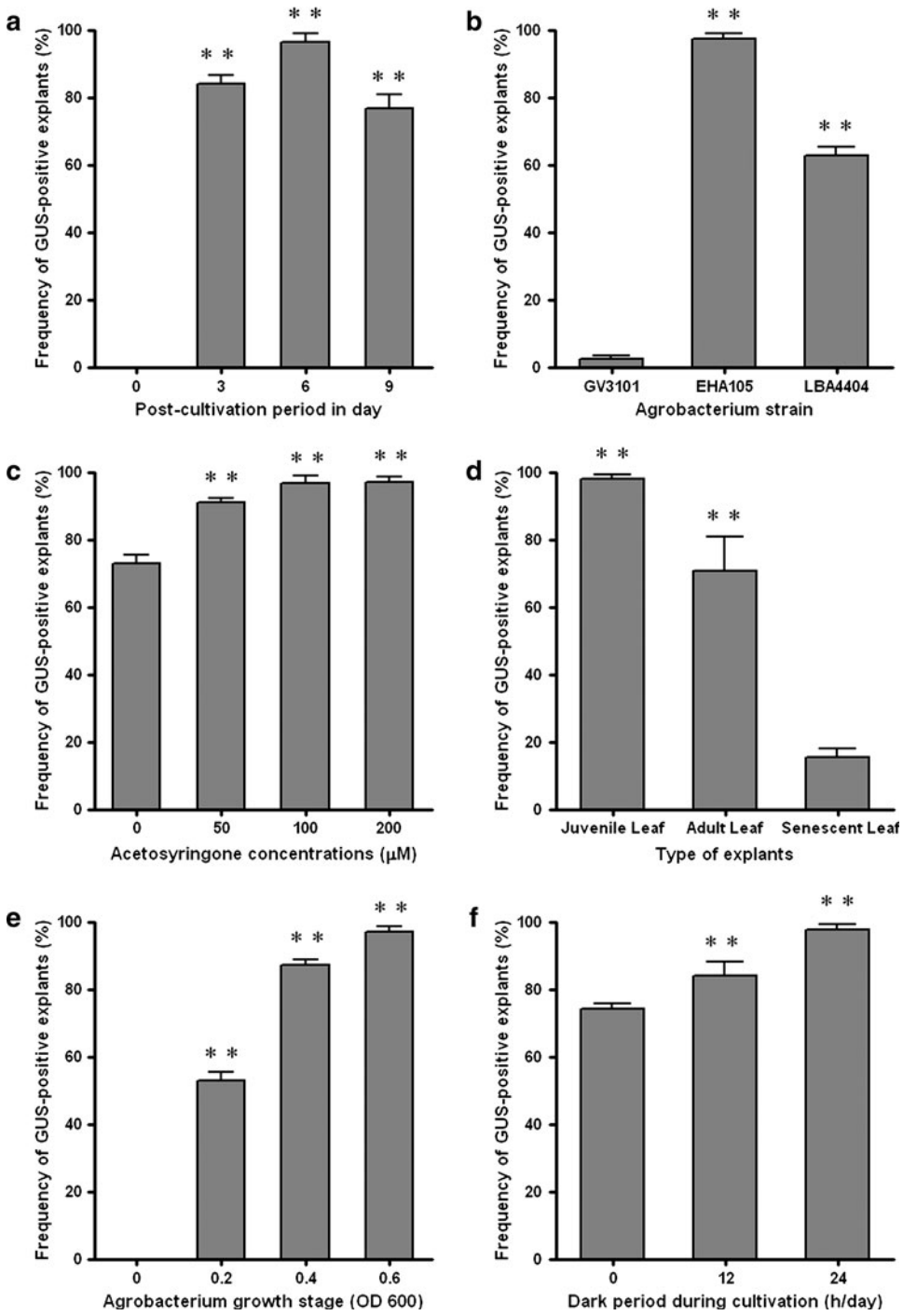

Fig. 2 Factors that affect the transient expression of *uidA* gene in leaf segments of Yinzhong (*Populus alba* × *P. berolinensis*). **a** Post-cultivation periods with *Agrobacterium* strain EHA105 harboring pCAMBIA2301. **b** *Agrobacterium* strains. **c** Acetosyringone (AS) concentrations. **d** Ages of leaves. **e** Growth stages of *Agrobacterium* bacteria. **f** Dark periods during co-cultivation. Results are presented as means and standard errors from three independent experiments. **Significant difference in comparison to the controls at $P < 0.01$ (Student's *t* test)

Stable transformation experiments

Based on the preliminary experiments, a total of 1,136 infected leaf segments of Yinzhong (*P. alba* × *P. berolinensis*) were transferred onto MS4 after 7 days pre-cultivation on MS3 (Table 1). Kanamycin-resistant shoots emerged in 2 weeks (Fig. 3a) and were ready for rooting in 4 weeks (Fig. 3b). For whole plant regeneration, regenerated kanamycin-resistant shoots were separated from the leaf explants when they were about 1 cm long, and transferred onto MS5 (Table 1) to induce roots (Fig. 3c). After 2 weeks, most of them had rooted (Fig. 3h). The rooting percentages were approximately 95%. In total, 106 independent kanamycin-resistant lines were obtained with a transformation frequency of 9.3%. Rooted shoots were then propagated on MS5.

To determinate the stability of kanamycin-resistance in regenerated shoots, leaf explants of wild type grown on MS1 and 3-week-old regenerated plants grown on MS5 were subjected to a second round regeneration on MS4. After 40 days, shoots were regenerated from the leaf explants of regenerated kanamycin-resistant shoot, but all the wild type leaf explants died (data now shown). The expression of *uidA* gene of the kanamycin-resistant shoots propagated on MS5 was further confirmed by GUS expression analysis (Fig. 3i–k).

The efficiency of the above-described transformation system was tested also in *P. davidiana* × *P. bolleana* (Shanxin yang) and a detailed regeneration protocol is shown in Fig. 4. Among the 420 co-cultivated leaf explants, 69 of them produced kanamycin-resistant shoots in 3 weeks (Fig. 4d, f). As expected, control leaf explant did not regenerate shoots (Fig. 4e). Expression of the *uidA* gene was detected by histochemical GUS analysis in transgenic shoots (Fig. 4d). The kanamycin-resistant shoots rooted on MS5 in 2 weeks (Fig. 4g, h; plant in the right) while wild-type shoots did not produce roots (Fig. 4g, h; plant in the left). For both varieties, regenerated plants were propagated on MS5 (Fig. 4i) and grown in soil in the greenhouse for further confirmation of GUS expression (Fig. 4j).

Confirmation of transgenic plants by PCR, Southern blot, RT–PCR, and GUS histochemical analyses

To confirm that the *uidA* gene was inserted into the *populus* genome, *uidA* gene was PCR-amplified using specific primer pair, yielding the expected band size of 602 bp (Fig. 5a, b). Southern blot analyses of selected transgenic plants confirmed that most transgenic plants had 1–2 copies of the transgene (Fig. 5a, b). Transcripts of *uidA* gene were detected in all the PCR-positive lines (Fig. 6a, b). GUS staining further confirmed the expression of *uidA* gene in transgenic plants (Fig. 6c).

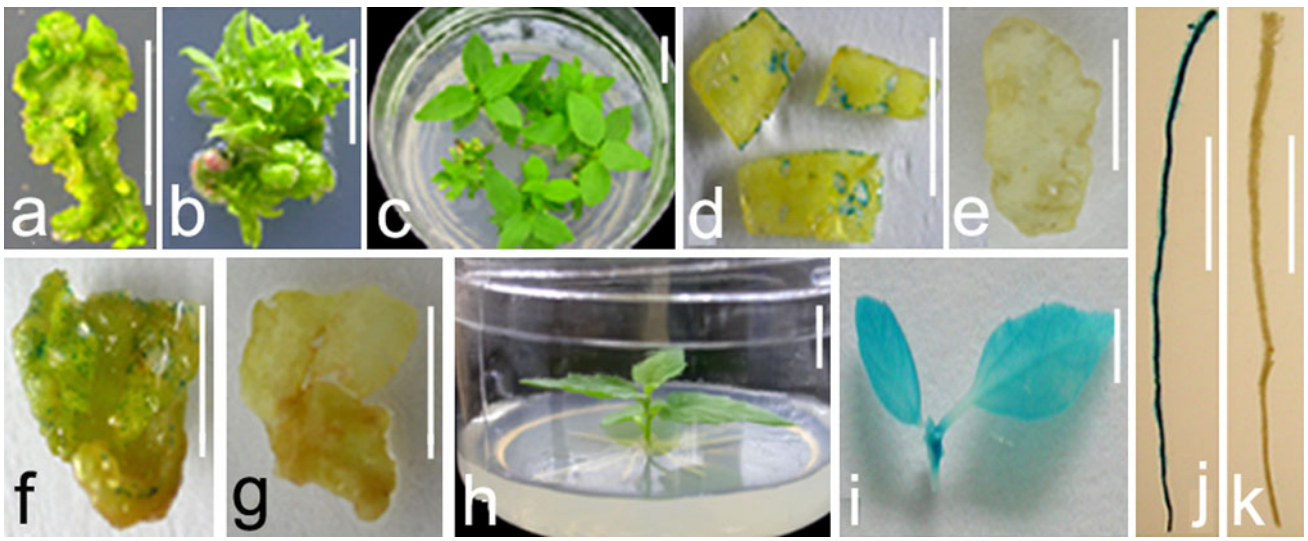

Fig. 3 Regeneration of transgenic Yinzhong (*Populus alba* × *P. berolinensis*) plants. **a** An explant on MS4 for 2 weeks after infection. **b** An explant on MS4 for 4 weeks after infection. **c** Regenerated shoots excised from explants and cultured on MS5 for shoot elongation. **d** GUS staining of explants 3 days on MS3 after co-cultivation with *Agrobacterium* strain EHA105 harboring pCAMBIA2301. **e** GUS staining of an uninfected explant 3 days on MS3 after co-cultivation (negative control). **f** GUS staining of an explant 2 weeks on MS4 after co-cultivation. **g** GUS staining of an uninfected explant 2 weeks on MS4 after co-cultivation (negative control). **h** A regenerated shoot rooted on MS1 for 2 weeks. **i** GUS staining of a transgenic shoot. **j** GUS staining of a transgenic root. **k** GUS staining of a wild type root (*bar* 1 cm)

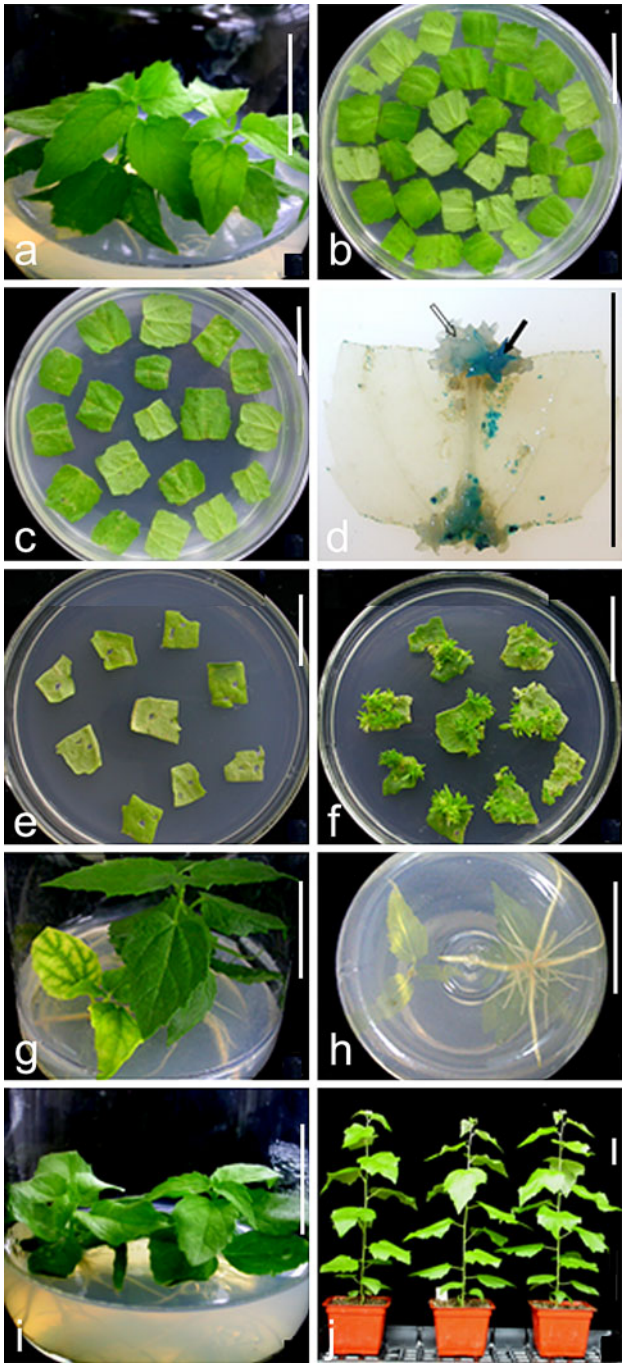

Fig. 4 A flowchart of the *Agrobacterium*-mediated transformation system for Shanxin yang (*Populus davidiana* × *P. bolleana*). **a** 3-week-old plants grown on MS1 ready for leaf explant preparation. **b** Leaf explants were co-cultivated with EHA105 harboring pCAMBIA2301 on MS2 for 2 days. **c** Leaf explants were cultured on MS3 for 7 days to inhibit the growth of EHA105. **d** GUS staining of an explant 3 weeks on MS4 after co-cultivation. Transgenic (*solid arrow*) and non-transgenic (*empty arrow*) shoots are shown. **e** Uninfected explants on MS4 3 weeks after co-cultivation (negative control). **f** Infected explants on MS4 3 weeks after co-cultivation. **g** Root induction of regenerated shoots on MS5. *Left* non-transgenic plant, *right* transgenic plant. **h** Bottom views of **g**. **i** Transgenic shoots propagated on MS5. **j** 7-week-old transgenic plants grown in a greenhouse (*bar* 2 cm)

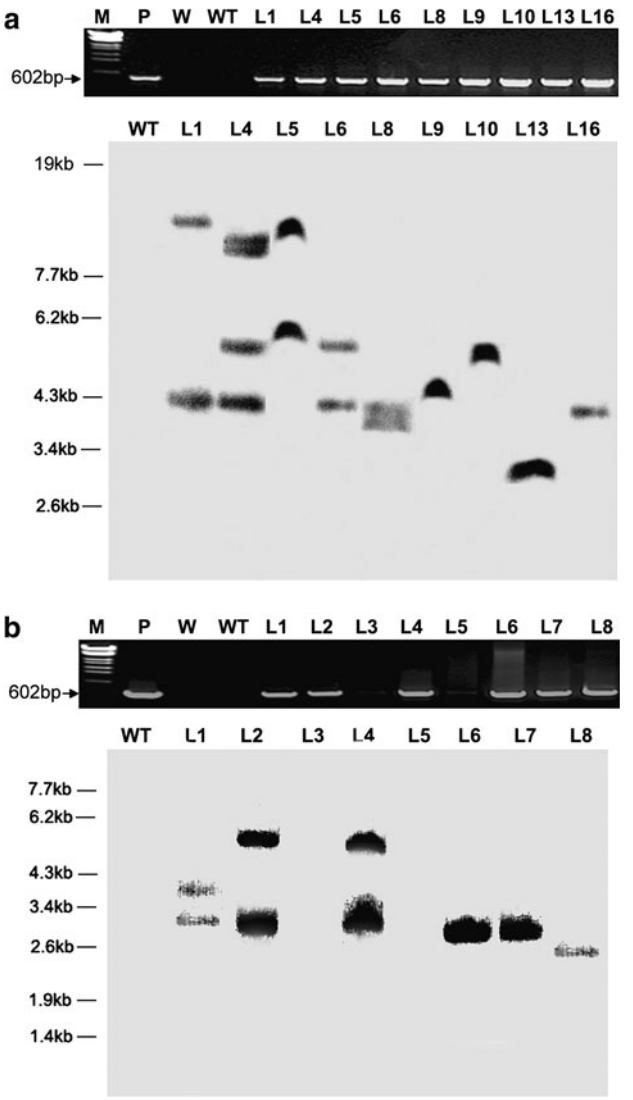

Fig. 5 PCR and Southern blot analyses of transgenic plants. **a** Yinzhong plants. **b** Shanxin yang plants. A 602-bp PCR product was detected in most transgenic lines. *M* λ-EcoT14 I digest DNA markers, *P* PCR product with pCAMBIA2301 plasmid DNA as template, *W* PCR product with double distilled water as template, *WT* PCR product with wild type plant genomic DNA as template, *L1*-16 and L1-*8* PCR products with genomic DNA from regenerated Yinzhong and Shanxin yang as template, respectively. For Southern blot analysis of transgenic plants, genomic DNA was digested with *Eco*RI, electrophoresed, and probed with a DIG-labeled 602-bp PCR product of *uidA* gene as above. The number of bands reflects the number of transgene insertions. Molecular weight DNA markers are shown on the *left*. *Lane 1* wild type plant (WT), *lanes 2–10* regenerated poplar lines. *Lane 4* (*L3*) and *Lane 6* (*L5*) in **b** presented non-transformed plants

Discussion

A major problem in the production of transgenic *populus* is regeneration of shoots from transformed cells. To date, although poplars have been transformed for research purposes far more than any other species of forest tree, many

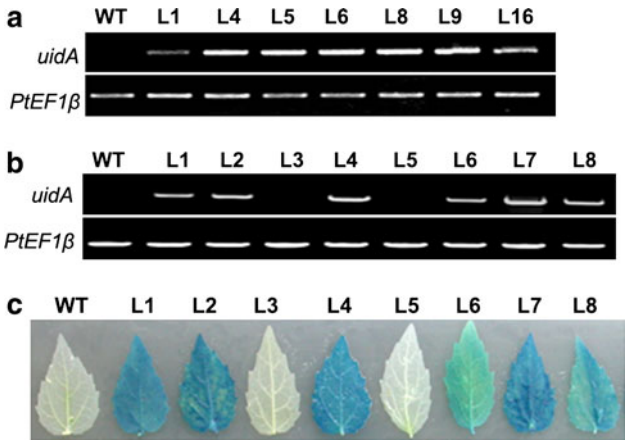

Fig. 6 RT–PCR and histochemical GUS staining analyses of transgenic plants. **a**, **b** RT–PCR analysis of wild type and transgenic lines of Yinzhong (**a**) and Shanxin yang (**b**). Total RNA was isolated from wild type (WT) and different transgenic lines (L1–L16). RT–PCR was performed with *uidA*-specific primers (30 cycles) or *PtEF1β*-specific primers (25 cycles). The *PtEF1β* gene was used as an internal control. **c** Histochemical GUS staining of wild type and transgenic (L1–L8) leaves of Shanxin yang

commercially important genotypes and species still remain difficult to transform (Han et al. 1996, 2000). Several laboratories have reported highly efficient transformation systems for poplars (Fladung et al. 1997; Leple et al. 1992; Tsai et al. 1994; Tzifra et al. 1996, 1997), however, most of the work are restricted to a few model hybrids and species of section Leuce (Han et al. 2000; Song et al. 2006), and the transformation efficiencies are low, ranging mostly from 2 to 13%. Recently, an efficient transformation system was established for a commercial hybrid poplar *P. nigra* L. × *P. maximowiczii* A. Henry (Yevtushenko and Misra 2010). Here, we report the establishment of a transformation system for another two hybrid clones commercially grown in China, *P. alba* × *P. berolinensis* and *P. davidiana* × *P. bolleana*.

A large number of factors that affect the efficiency of DNA delivery into the targeted cells and the regeneration of transgenic plants are involved in *Agrobacterium* transformation, including *Agrobacterium* strain, explant source, genotype, physiological condition for regeneration, and phytohormone type and combination. We observed that bacterium strain and the age of leaf explant are the two major factors that affect the transformation efficiency for these two hybrid clones. Addition of TDZ improved the regeneration and transformation frequency (data now shown). The 1 week pre-cultivation on MS3 is also a key step to improve transformation efficiency. Unlike the system reported for *Populus trichocarpa* Genotype Nisqually-1 (Song et al. 2006), in which the entire process from co-cultivation to whole plant regeneration takes about 5–8 months, shoots can be directly regenerated without

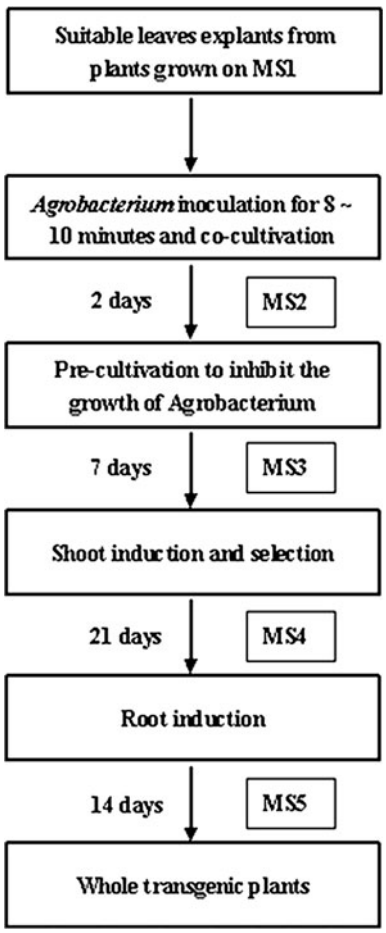

Fig. 7 Stepwise protocol for transforming Yinzhong (*Populus alba* × *P. berolinensis*) and Shanxin yang (*Populus davidiana* × *P. bolleana*) using leaf disc as explants. Medium MS1 to MS5 are shown in Table 1

callus induction in this system. Figure 7 illustrates the stepwise process using leaf discs as explants. As set out in Fig. 7, the entire process from co-cultivation to whole plant regeneration takes less than 2 months.

Asexual transformation methods provide a means for introducing new traits that are difficult to obtain via traditional breeding, and allow modification of valuable clones without the genetic recombination that occurs during sexual reproduction (Han et al. 2000). With the availability of a rapid and efficient transformation-regeneration system from leaf explants, it is now feasible to introduce genes for desirable traits, such as disease and abiotic stress resistance, into these two economically important aspen hybrid clones.

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