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ORIGINAL PAPER

Genetic transformation of *Populus tomentosa* to improve salt tolerance

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Abstract Soil salinity can be a limiting factor for productivity in agriculture and forestry. In order to fully utilize saline lands productively in plantation forestry for pulp production, the genetic modification of tree species for salt-tolerance may be required. The *AhDREB1* gene, a DREB-like transcription factor gene, was transferred into *Populus tomentosa* by *Agrobacterium*-mediated transformation. Transgenic plants were regenerated and selected using a two-step process; first on Murashige and Skoog (MS) basal medium containing 4.44 μM 6-benzyladenine (BA), 1.61 μM α-naphthaleneacetic acid (NAA), 30 mg l^{-1} kanamycin, and 250 mg l^{-1} ceftomine, and then enhanced selection on medium with 50 mg l^{-1} kanamycin. The putative transformants were confirmed by polymerase chain reaction (PCR) and Southern hybridization for the A*hDREB1* gene. Transgenic plants were rooted on half-strength MS medium containing 5.71 μM indole-3-acetic acid (IAA), 1.61 μM NAA, 2 g l^{-1} sucrose, and 5 g l^{-1} agar. The salt tolerance of transgenic plants in pots in the greenhouse showed a survival rate of 100, 100, 84.4, and 44.4% after watering with a solution of 34.2, 68.4, 102.7, and 136.9 mM NaCl, respectively.

Keywords AhDREB1 · Chinese white poplar · Genetic engineering · Transgenic

Abbreviations

BA	6-Benzyladenine
IAA	Indole-3-acetic acid
MS	Murashige and Skoog
NAA	Napthaleneacetic acid
*npt*II	Neomycin phosphotransferase
PCR	Polymerase chain reaction
qPCR	Quantitative real-time polymerase chain reaction

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Introduction

Populus tomentosa Carr. (Chinese white poplar; Salicaceae) is a tree species native to and grown in China for pulp (Zhu and Zhang 1997). It is mainly distributed in ten provinces in the northern part of China (Zhu 1988). Chinese white poplar exhibits many excellent characteristics, such as broad adaptability, short-rotation, and rapid-growth, but it does not grow well on saline soils limiting its use and distribution in other areas. It is known that differences in salt tolerance exist among different poplar species and ecotypes, with *P. euphratica* being the most tolerant (Chen and Polle 2010). Soil salinity is a major abiotic stress factor that disrupts homeostasis and ion distribution in the plant cell, thus affecting plant growth and

development. In 1995, it was reported that approximately 20% of irrigated agricultural land was adversely affected by salinity (Flowers and Yeo 1995). With the increased use of irrigation and fertilization, salt stress is increasing in many areas of the world. Therefore, it will be important to develop salt-tolerant agronomic crop or tree species to exploit and utilize broad areas of saline land (Hasthanasombut et al. 2011; Qiao et al. 2010). Plant genetic engineering provides an approach to improve abiotic and biotic tolerance in *Populus*, and several studies have focused on the development of techniques for the genetic improvement of *P. tomentosa* (Deng et al. 2009; Du et al. 2002; Hu et al. 2005; Lin et al. 2006; Liu et al. 2008a, b; Zhang et al. 2005, 2008a).

Over the last 15 years, many salt tolerance-related genes have been identified and transferred into various plant species (Hayashi et al. 1997; Kavi Kishor et al. 1995; Oraby et al. 2005; Xu et al. 1996). Although salt tolerance characteristics may be a result of several genes with genetic and physiological complexity, transgenic plants have shown enhanced tolerance to saline conditions when a single or a few salt tolerance-related genes were transferred into plants (Fan et al. 2002; Hu et al. 2005; Liu et al. 2006; Li et al. 2010a, b; Liu et al. 2008b; Wang et al. 2010; Yang et al. 2001, 2009; Zou et al. 2006). Hu et al. (2005) transferred the *mtl*D gene (encoding mannitol-1-phosphate dehydrogenase) into *P. tomentosa*, and an increased salt tolerance of transgenic plants was observed both in hydroponic culture and in vitro. Liu et al. (2008a) transferred the apple *SPDS* gene (spermidine synthase) into *P. tomentosa*, and four transgenic lines were confirmed by polymerase chain reaction (PCR), one copy confirmed integrated by Southern blotting, and the relative expression levels (one to three) analyzed by quantitative real-time PCR (qPCR). Liu et al. (2008b) transferred the *PLD/AtNHX1* gene (phospholipase D/*Arabidopsis thaliana* Na^+/H^+ antiporter gene) into *P. tomentosa*, and transgenic lines were reported to have improved salt tolerance. Zhang et al. (2008b) overexpressed the *AtPLDα* gene in *P. tomentosa*, and found enhanced drought and salt tolerance in transgenic plants.

A promising strategy to improve salt tolerance is the characterization and cloning of transcription factor genes that regulate a number of downstream or upstream genes, thus activating a cascade of genes that act together in enhancing tolerance to multiple stresses (Sharma and Lavanya 2002). Yang et al. (2009) introduced the *DREB1C* gene (a dehydration-responsive element binding transcription factor) into an elite hybrid clone of *Populus* × *euramericana* cv. Nanlin 895. The results showed that the transgenic plants had a greater resistance to drought and salt than non-transgenic plants. *AhDREB1* is a DREB-like transcription factor gene isolated from the halophyte *Atriplex hortensis*, and overexpression of *AhDREB1* in transgenic tobacco led to the accumulation of putative downstream genes (Shen et al. 2003). The transgenic lines were also tested under salt-stressed conditions, and two lines were found to be stress-tolerant (Shen et al. 2003). The *AhDREB1* gene was also transformed into black locust (*Robinia pseudoacacia*), transgenic plants were identified by PCR, but further molecular analysis and salt tolerance of PCR-positive lines were not reported (Shen et al. 2008).

In this study, our main objective was to transfer the transcription factor gene *AhDREB1* into *P. tomentosa* by *Agrobacterium*-mediated transformation, in order to improve its salt tolerance. The development of transgenic *P. tomentosa* with salt-tolerant properties will largely extend cultivated areas for planting *P. tomentosa*, and improve the utilization efficiency of saline soils.

Materials and methods

Plant materials

Adventitious shoot regeneration and rooting of *P. tomentosa* were achieved as described by Du et al. (2002). Shoot cultures of *P. tomentosa* were maintained in 250 ml flasks with 30 ml of Murashige and Skoog (MS) basal medium (1962) supplemented with 1.33 μM 6-benzyladenine (BA), 0.54 μM α-naphthaleneacetic acid (NAA), 30 g l^{-1} sucrose, and 5 g l^{-1} agar. Cultures were maintained at 25 ± 1°C under a 16-h photoperiod (80 μmol m^{-2} s^{-1}) provided by cool-white fluorescent lights, and cultures were transferred to fresh medium every 4 weeks. The first three youngest leaves from the top were excised from shoot cultures, the leaves were cut transversely from the edge to midrib, and placed adaxial side down on regeneration medium (Du et al. 2002). Explants treated in this manner were used to determine explant sensitivity to the antibiotics and for transformation experiments.

Effects of kanamycin and ceftomine on regeneration from leaf tissues

Wounded leaves were placed on regeneration medium [MS basal medium supplemented with 4.44 μM BA, 1.61 μM NAA, kanamycin (0, 10, 20, 30, 50, or 100 mg l^{-1}), or ceftomine (0, 100, 200, 400, or 600 mg l^{-1}; Beijing BioDee Biotechnology Co. Ltd., China) in glass Petri plates (100 × 25 mm; 45 ml medium). Kanamycin and ceftomine were dissolved in sterile, deionized water, filter-sterilized (0.22 μm), and added to the medium after autoclaving. Three replicates with six leaf explants each were used. The leaf regeneration response was recorded after 4 weeks of in vitro culture. All media included 30 g l^{-1} sucrose and 5 g l^{-1} agar, and the pH of the medium was adjusted to 5.8 prior to

autoclaving. All cultures were incubated at 25 ± 1°C under a 16-h photoperiod (80 μmol m^{-2} s^{-1}).

Transformation vector and bacterial strain

Agrobacterium tumefaciens strain EHA101 harboring a binary vector pBI121 was used for plant transformation experiments. The binary vector consisted of the *AhDREB1* gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter, and the neomycin phosphotransferase gene (*npt*II) under the control of the nopaline synthase (NOS) promoter. (Fig. 1). The *Agrobacterium* culture was grown overnight in 50 ml YEP medium (10 g l^{-1} yeast extract, 10 g l^{-1} bacto peptone, 5 g l^{-1} NaCl, pH 7.0) containing 50 mg l^{-1} kanamycin on a rotary shaker (180 rpm) at 28°C. Overnight cultures of *Agrobacterium*-pBI121 (OD_{600} = 0.6- 0.8) were used for co-cultivation.

Plant transformation

Wounded leaf explants were placed on regeneration medium in the light for 2 days pre-culture, and then immersed in an overnight culture of *A. tumefaciens* for 10 min at 28°C with gentle shaking. Explants were blotted on sterile filter paper to remove excess bacterial solution before transfer to regeneration medium and incubated in the dark at 25 ± 1°C for 2 days. After 2 days co-cultivation, leaf explants were rinsed (1 min) three times with sterile, distilled water to remove excess bacteria, blotted on sterile filter paper, and transferred to selection medium. For initial selection, leaf explants were cultured on regeneration medium containing 30 mg l^{-1} kanamycin and 250 mg l^{-1} ceftomine for 3 weeks. Adventitious shoots were excised when approximately 2- to -3-cm in length, and transferred to glass jars (50 ml medium) containing MS basal medium with 1.33 μM BA, 0.54 μM NAA, 50 mg l^{-1} kanamycin, and 250 mg l^{-1} ceftomine, in order to make the second selection more stringent and elongate shoots. After an additional 3 weeks for shoot elongation on selection medium containing 50 mg l^{-1} kanamycin, the kanamycin-resistant shoots were subsequently rooted. Kanamycin-resistant shoots were transferred to glass jars with root induction medium consisting of half-strength MS medium supplemented with 5.71 μM indole-3-acetic acid (IAA), 1.61 μM NAA, 20 g l^{-1} sucrose, 50 mg l^{-1} kanamycin, 250 mg l^{-1} ceftomine, and 5 g l^{-1} agar for 6 weeks under a 16-h photoperiod (80 μmol m^{-2} s^{-1}).

Molecular analysis of transgenic plant lines

Total genomic DNA was isolated from young leaves of six kanamycin-resistant transgenic lines and a non-transformed control plant using a modified cetyltrimethylammonium bromide (CTAB) method as described by Saghai-Maroof et al. (1984). PCR was performed to amplify a specific DNA sequence in transgenic plants corresponding to the *AhDREB1* gene. A primer set (forward primer 5′- GAA GAA AGA TGT TGC TAA TAA TAA C and reverse primer 5′-AAT AAT AAT ACT TCA CTA AAA ATG ATC-3′) was designed to amplify a 666 bp PCR product for the *AhDREB1* gene. A 25 μl PCR reaction was prepared containing 2.5 μl of 10× PCR buffer, 1 μl 10 mM dNTP, 0.8 μl 50 mM Mg^{2+}, 1 μl of 10 μM *AhDREB1*-F and *AhDREB1*-R primers, 1 μl of 200 ng μl^{-1} DNA template, and 0.2 μl 5 U μl^{-1} Taq polymerase. Plasmid DNA served as a positive control. DNA from a non-transformed control plant and distilled water were used as negative controls. The PCR reaction included a denaturing step of 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 40 s, 72°C for 1 min, and a final cycle at 72°C for 10 min. Amplified PCR products were visualized and photographed under ultra-violet light after electrophoresis on a 1% (w/v) agarose gel containing 0.5 μg ml^{-1} ethidium bromide.

For Southern blot analysis, *P. tomentosa* genomic DNA (10 μg) from leaves of five transgenic lines was digested with *Dra*I, fractionated on a 0.8% (w/v) agarose gel, and transferred to a Hybond N^+ membrane. The membrane was hybridized with PCR-generated probe for the *AhDREB1* gene (666 bp) labeled with [α-^{32}P] dCTP by the random prime labeling system. Purified PCR product (1 ng) of *AhDREB1* (666 bp) served as a positive control. Hybridization was carried out according to the method of Sambrook et al. (1989). DNA from a non-transformed control plant was used as a negative control. Pre-hybridization and

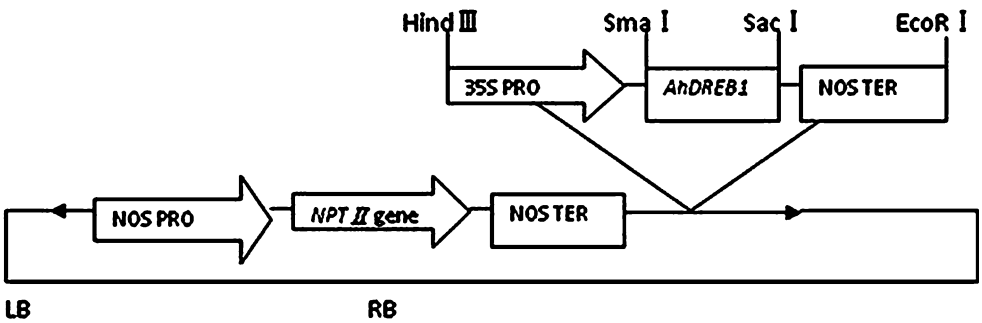

Fig. 1 Schematic diagram of T-DNA region of vector pBI121. *LB* left border, *RB* right border, *NOS PRO* nopaline synthase promoter, *NPT II* neomycin phosphotransferase II, *NOS TER* nopaline synthase gene terminator, *35S PRO* cauliflower mosaic virus 35S promoter

hybridization were carried out at 65°C overnight in buffer solution containing 6× SSC containing 0.9 M NaCl and 0.09 M sodium citrate, 5× Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and 100 mg ml^{-1} denatured salmon sperm DNA. The membrane was then washed at 65°C twice for 20 min in 2× SSC, 0.1% SDS, and once for 10 min in 0.5× SSC, 0.1% SDS. The membrane was exposed overnight to a Phosphor Imaging Plate and developed in a related scanner system.

Salt tolerance of transgenic plants and analysis of proline content

Salt tolerance of five transgenic lines and one randomly chosen non-transformed control plant were conducted in the greenhouse. In vitro rooted plants were acclimatized in soil to the greenhouse for several weeks, re-potted in a fine sand mix (0.5–1 mm diameter) when approximately 15 cm in height, and then watered daily for 15 days with 0, 34.2, 68.4, 102.7, or 136.9 mM NaCl solution. Five plants per treatment per line were used, with each treatment duplicated twice. One plastic pad was laid under each pot to avoid salt drainage during the watering process (Lutts et al. 2001). Salt concentration in the soil (via EM-38 conductivity meter; Geonics Ltd., Mississauga, Ontario, Canada), corresponding survival rate of transgenic plants and the control, and the proline content were determined after the 15 day treatment. Proline concentration was determined according to the method of Bates et al. (1973) and Zhang et al. (1990). Fresh fully-expanded leaf samples were homogenized in 3% sulfosalicylic acid and extracts were incubated in acid ninhydrin solution for 30 min at 100°C. Absorbance (520 nm) of the samples was determined and proline concentrations calculated from a standard curve according to Li (2000).

Statistical analyses

Data were analyzed by analysis of variance (ANOVA) using the GLM procedure of SAS, version 8 (SAS institute 1999). When the ANOVA indicated statistical significance, a Tukey's comparison test was used to distinguish differences between treatments at the 5% probability level.

Results

Effects of kanamycin and ceftomine on regeneration from leaf tissues

Kanamycin is widely used for selecting transformed cells and plants in genetic transformation studies. It is critical to investigate the appropriate concentration of kanamycin to screen for putative transformants. In this study, the dose–response of leaf explants of *P. tomentosa* was evaluated by the morphological appearance and percent adventitious shoot regeneration under the selection conditions (Table 1). Shoots regenerated from leaf explants were severely affected by kanamycin. Leaf explants became medium green in color and no adventitious shoots were regenerated at 20 mg l^{-1} kanamycin. Higher concentrations of kanamycin (30, 50, or 100 mg l^{-1}) produced increased leaf chlorosis and no adventitious shoots were regenerated. Therefore, 30 mg l^{-1} kanamycin was chosen as the minimal concentration for initial selection. After 3 weeks, kanamycin-resistant shoots were transferred to a second selection medium with 50 mg l^{-1} kanamycin to increase selection stringency and to avoid false positive plants. Ceftomine was used to inhibit *Agrobacterium* overgrowth. Our results indicated that ceftomine at 0–600 mg l^{-1} did not significantly affect adventitious shoot regeneration from wounded leaf explants (Table 2). Shoot regeneration decreased to 88.9% at 600 mg l^{-1} ceftomine. Therefore, 250 mg l^{-1} ceftomine was chosen to inhibit *Agrobacterium* overgrowth based on the *P. tomentosa* study by Hao et al. (1999) and our results.

Plant transformation

The study by Hao et al. (1999) on the optimization of *P. tomentosa* transformation provided a foundation for the present study. After 2 days co-culture the explants were transferred to the first selection medium composed of regeneration medium with 30 mg l^{-1} kanamycin and 250 mg l^{-1} ceftomine for 3 weeks. Adventitious shoots were induced from the wounded leaves via direct organogenesis (Fig. 2a). The adventitious shoots together with the

Table 1 Effect of kanamycin on regeneration percentage of adventitious shoots from *Populus tomentosa* leaf explants

Kanamycin (mg l^{-1})	Leaf characteristics	Adventitious shoot regeneration (%)
0	Dark green, shoots regenerated	100a
10	Dark green, shoots regenerated	83.3a
20	Medium green, no shoots regenerated	0b
30	Light green, no shoots regenerated	0b
50	Light yellow, no shoots regenerated	0b
100	Dead, no shoots regenerated	0b

Wounded leaf explants were cultured on Murashige and Skoog medium supplemented with 4.44 μM 6-Benzyladenine, 1.61 μM Napthaleneacetic acid, 3% sucrose, 0.5% agar with different concentrations of kanamycin. Data were collected after 6 weeks of culture. Values represent means for six explants per treatment, replicated three times. Means in each column followed by the same letter are not significantly different according to Tukey's multiple comparison test

Table 2 Effect of ceftomine on regeneration percentage of adventitious shoots from *Populus tomentosa* leaf explants

Ceftomine (mg l^{-1})	Adventitious shoot regeneration (%)
0	100a
100	100a
200	100a
400	100a
600	88.9a

Wounded leaf explants were cultured on Murashige and Skoog medium supplemented with 4.44 μM 6-Benzyladenine, 1.61 μM Napthaleneacetic acid, 3% sucrose, 0.5% agar with different concentrations of ceftomine. Data were collected after 6 weeks of culture. Values represent means for six explants per treatment, replicated three times. Means in each column followed by the same letter are not significantly different according to Tukey's multiple comparison test

initial leaf explant were transferred to the second selection and elongation medium for 3 weeks. Shoots that survived on the first selection medium, either died or elongated on the second selection medium (Fig. 2b). All kanamycin-resistant shoots were transferred to rooting medium (Fig. 2c), but some kanamycin-resistant shoots failed to root in rooting medium containing kanamycin. Therefore, rooting putative transgenic shoots of *P. tomentosa* on medium containing kanamycin was used to further eliminate false-positive plants. We also found that leaves from the kanamycin-resistant shoots placed on medium containing 50 mg l^{-1} kanamycin regenerated many adventitious shoots, whereas leaves from the wild-type (control) plants wilted (Fig. 2d).

Molecular analysis of transgenic plant lines

Genomic DNA was extracted from six independently regenerated transgenic *P. tomentosa* lines (T12, L26, L63, L73, L83, and T46) and a non-transformed control plant, and used in PCR amplification to verify transgene insertion. A 666 bp fragment corresponding to the *AhDREB1* gene was present in all six transgenic lines (Fig. 3, Lanes 4–9) and the positive control plasmid (Fig. 3, Lane 2), and was absent in the non-transformed control plant (Fig. 3, Lane 3). For Southern blot analysis, genomic DNA from five of these six transgenic lines and the non-transformed control plant was digested with *Dra*I. The membrane was hybridized with a 666 bp fragment corresponding to the *AhDREB1* gene to analyze transgene integration. No

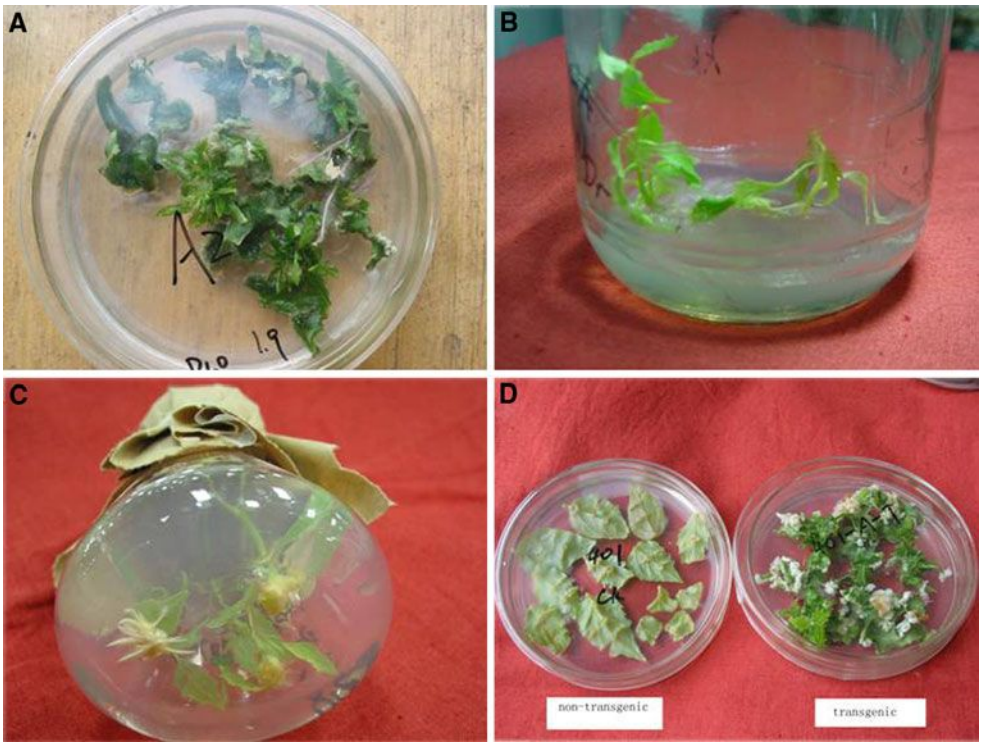

Fig. 2 Adventitious shoot formation and rooting of *Populus tomentosa* plantlets from transformed leaves. **a** Adventitious shoot regeneration, **b** Shoots that survived on initial selection medium containing 30 mg l^{-1} kanamycin elongated or died on second selection medium with 50 mg l^{-1} kanamycin, **c** Rooting of elongated transgenic shoots on half-strength Murashige and Skoog medium supplemented with 5.71 μM Indole-3-acetic acid and 1.61 μM Napthaleneacetic acid after 6 weeks, **d** Leaf explants from kanamycin-resistant shoots regenerated adventitious shoots (*right*), leaves from wild-type control plants wilted or died on the second selection medium (*left*)

hybridization was detected in the non-transformed control plant (Fig. 4, Lane 2). One or two copies (hybridization bands) were detected in transgenic lines T12, L26, L63, and L73 (Fig. 4, Lanes 3–6) confirming that the regenerated lines were stably transformed, but no hybridization band was detected in line L83 (Fig. 4, Lane 7).

Salt tolerance of transgenic plants and analysis of proline content

Large fluctuations in salt concentration within the soil may occur with daily watering. In order to try to better reflect the salt concentration in the soil more accurately, the salt concentration in the soil at the end of the experiment was measured, and the average electrical conductivity value was considered as the total salt concentration exposed to the plants (Table 3). Salt concentration (electrical conductivity) in the soil in the pots increased, and survival rates of transgenic plants and the control both decreased with increasing salt concentration from 0 to 136.9 mM (Table 3). There was no difference in survival rate (100%) between the control and transgenic plants for 0 or 34.2 mM NaCl treatment. However, transgenic plants had a 100% survival rate and the control a 40% survival rate when plants were exposed to 68.4 mM NaCl. When the NaCl concentration was increased to 102.7 mM or 136.9 mM NaCl, the survival rate of the transgenic plants versus the controls decreased to 84.4 and 44.4%, respectively. At these high concentrations all control plants died. From these results, we can project that the transgenic plants containing the *AhDREB1* gene had improved salt tolerance over the controls. Among the many physiological parameters affected by salt stress tolerance is the accumulation of proline. Several studies have shown that enhanced proline accumulation occurs in transgenic plants under salt stress, and this may play a role in counteracting the negative effect of salt stress (Kumar et al. 2010). In our study, the proline content of transgenic plants exposed to 0, 68.4, or 102.7 mM NaCl was determined (Fig. 5). The proline accumulation in transgenic plants exposed to 68.4 mM NaCl was greater than 4-fold of that of the control plants. The proline accumulation in transgenic plants exposed to 102.7 mM NaCl was 2½ times greater than of the control plants. The multiple comparative analyses indicated a significant difference between the transgenic lines and the control plants. Transgenic plants were adapted to this salt stress environment, accumulated proline, and the overall salt tolerance was improved considerably.

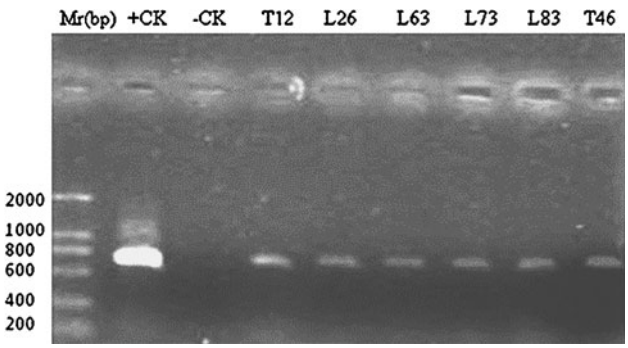

Fig. 3 Polymerase chain reaction analysis of genomic DNA isolated from leaves of non-transformed control and transgenic *Populus tomentosa* plants using primer pairs specific for amplification of 666 bp *AhDREB1* gene. *Mr* molecular marker, *+CK* positive control pBI121, *−CK* negative control non-transformed *P. tomentosa*, *Lanes 4–9 (T12, L26, L63, L73, L83, and T46)* putative transgenic *P. tomentosa* plants

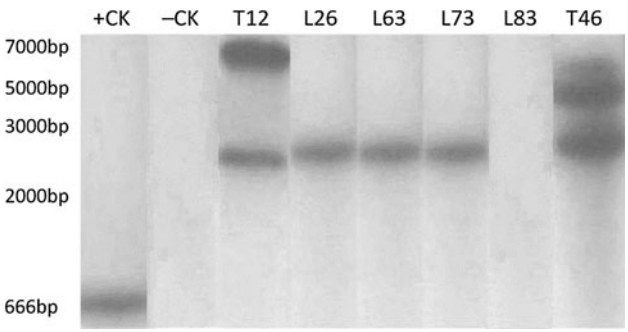

Fig. 4 Southern blot analysis of five transgenic plants of *Populus tomentosa* for *AhDREB1* gene. DNA samples (10 μg) were digested with *Dra*I. *+CK* positive control, *−CK* negative control (non-transformed plant), *Lanes 3–7 (T12, L26, L63, L73, and L83)* putative transgenic plants

Discussion

To our knowledge, this is the first report of successful transformation and insertion of the transcription factor gene *AhDREB1* into *P. tomentosa*. Based on PCR, Southern blot analysis, preliminary salt tolerance results, and analysis of proline content of transgenic plants, we have shown that the expression of the *AhDREB1* gene in *P. tomentosa* improved its salt tolerance. Overexpression of the *AhDREB1* gene in transgenic tobacco resulted in tobacco plants expressing increased salt tolerance (Shen et al. 2003). The *AhDREB1* gene has also been transferred into black locust and insertion of the gene in transgenic plants verified by PCR (Shen et al. 2008). A soybean DREB orthologue, *GmDREB1*, expressed in transgenic alfalfa (*Medicago sativa* L.) plants increased the level of salt tolerance as measured by ion leakage, chlorophyll fluorescence, free proline, and total soluble sugars (Jin et al. 2010). Hu et al. (2005) over-expressed the *mtlD* gene in *P. tomentosa* and improved its salt tolerance through the direct or indirect accumulation of mannitol. In vitro plants

Table 3 Salt concentration in the soil (pot experiment) and survival rate of control and transgenic plants of *Populus tomentosa* exposed to saline conditions

Salt concentration					
Beginning	0	34.2 mM	68.4 mM	102.7 mM	136.9 mM
Ending[a]	0	1.66 dS m^{-1}	2.11 dS m^{-1}	4.96 dS m^{-1}	6.85 dS m^{-1}
Average[a]	0	1.83 dS m^{-1}	3.06 dS m^{-1}	5.48 dS m^{-1}	7.43 dS m^{-1}
Survival rate (%)					
Control	100	100	40	0	0
Transgenics	100	100	100	84.4	44.4

[a] Electrical conductivity measured using a EM-38 conductivity meter

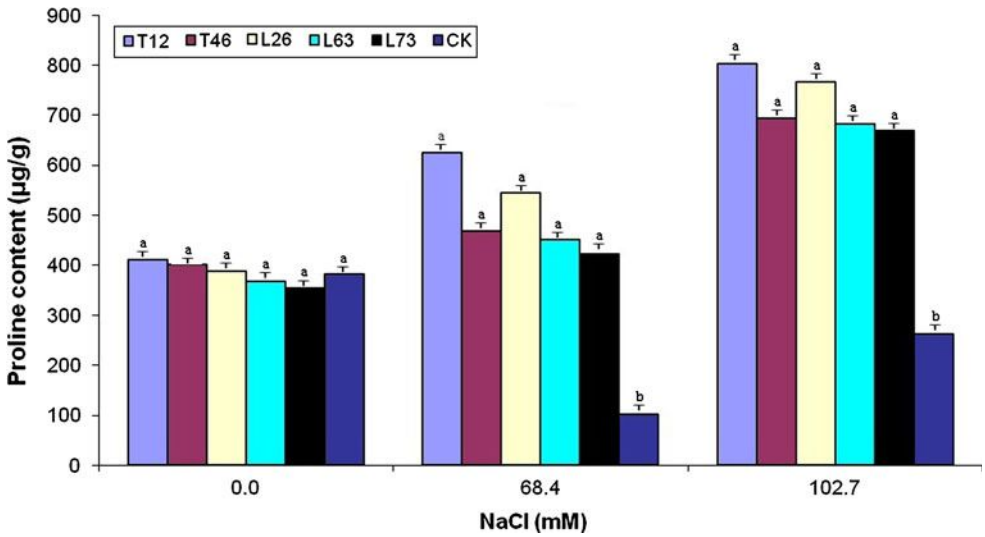

Fig. 5 The effect of 0, 68.4, and 102.7 mM NaCl on leaf proline accumulation for five transgenic plants and a non-transformed control plant. *Bars* with the same *letters* are not statistically different ($P < 0.05$)

were rooted on medium containing 50 mM NaCl, and transgenic plants grown in hydroponic culture survived a 75 mM NaCl stress treatment. Liu et al. (2008a) transformed *P. tomentosa* with the *SPDS* gene from apple, and four transgenic plants were confirmed. Zhang et al. (2008b) over-expressed the *AtPLDα* in *P. tomentosa*, and results showed that the rooting rate and root length of transgenic lines were significantly higher than wild-type plants, as the concentration of NaCl was increased in the medium. Liu et al. (2008b) reported that transgenic plants of *P. tomentosa* containing the *PLD/AtNHX1* gene showed increased salt tolerance. Jiang et al. (2010) over-expressed the *AtNHX1* gene in *P.* x *euramericana* 'Neva', and reported higher dry weights, chlorophyll, and carotenoid content in transgenic plants versus wild-type plants in the presence of NaCl. The *AtNHX1* gene was also transformed into *Liquidambar formosana* (a commercially valuable hardwood species), and transgenic plants showed improved growth, higher soluble protein, and increased superoxide dismutase (SOD) activity in response to salt treatment (Qiao et al. 2010). Overexpression of the *Malus MdNHX1* in a commercially important apple dwarfing rootstock (M.26) conferred high tolerance to salt stress (Li et al. 2010a, b). Wang et al. (2010) reported enhanced salt tolerance of transgenic poplar (*P. davidiana* × *P. bolleana*) expressing the *MnSOD* gene (manganese superoxide dismutase). The relative weight gain of the transgenic plants was 8- to 23-fold that of the wild-type plants after NaCl stress. Few other species and hybrids have been utilized for genetic modification of poplar for enhancing salt tolerance (see Chen and Polle 2010).

Although transgenic poplar plants have been obtained with degrees of improved salt tolerance, to date no commercial lines have been developed. Further analyses of these salt-tolerant lines needs to be conducted. Since salt tolerance may be a result of several genes with significant complexity, future research should focus on transformation with multiple genes for enhancing stress to saline soil.

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