Journal of Forestry Research (2014) 25(4): 813–818
DOI 10.1007/s11676-014-0529-z

ORIGINAL PAPER

Improved salt tolerance of *Populus davidiana* × *P. bolleana* overexpressed LEA from *Tamarix androssowii*

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Received: 2013-12-30; Accepted: 2014-01-23

Abstract: Development of transgenic plants with tolerance to environmental stress is an important goal of plant biotechnology. Late-embryogenesis-abundant (LEA) proteins accumulate in seeds during late embryogenesis, where they protect cellular membranes and macromolecules against drought. In this work, we transferred the *Tamarix androssowii* LEA gene into hybrids of *Populus davidiana*×*P. bolleana*. We compared relative rates of height growth, chlorophyll fluorescence kinetic parameters, and leaf Na+ levels of six TaLEA-containing lines with non-transferred plants (NT), all grown under 0.8% NaCl stress condition. Survival percentages of transgenic lines were all higher than for NT controls after rehydration and the survival percentage of SL2 was five-fold higher than for NT controls. Seedling height increased 48.7% in SL2 (from the onset of induced stress to the end of the growing season), 31% more than for the NT controls. Chlorophyll fluorescence kinetic parameters showed a marked increase in photosynthetic capacity in SL2 and SL5. Na+ levels in young leaves of transgenic lines were lower than in control NT leaves, but higher in yellow and withered leaves, indicating improved salt tolerance in transgenic lines.

Keywords: *Populus davidiana* × *P. bolleana*, LEA gene, transformation, salt tolerance

Abbreviations: SOD: Superoxide Dismutase; POD: Peroxidase; MS: Murashige and Skoog; 6-BA: 6-Benzylaminopurine; NAA: Naphthyl acetic acid; IBA: 3-Indolebutyric acid; CTAB: Cetyl trimethyl ammonium bromide

Project founding: This work was supported by the National High-Tech Research and Development Program of China, 863 Program (Molecular Breeding and New Species Creating of Poplar) (Grant No. 2011AA100201).

The online version is available at http://www.springerlink.com

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Corresponding editor: Chai Ruihai

Introduction

Plants are subject to adverse environmental conditions during growth and development, such as drought, salinity, aciditiy and alkalinity, elevated temperature and extreme cold. In response to stress, plants express stress-response genes and modify their physiology in order to tolerate and adapt to adverse conditions (Kasuga et al. 1999). Using genetic engineering and breeding technologies to improve stress tolerance in woody plants through manipulation of stress-response genes is an important goal of biotechnology. Poplar is one of the most economicaly important tree species in temperate regions of the world (Zhao et al. 2013) and one that has proven responsive to improvement through biotechnology.

Late-embryogenesis-abundant (*LEA*) genes are expressed in plant seeds during maturation and development, and in dehydrated nutritional tissues in response to stress stimuli. The resulting LEA proteins accumulate and help to improve stress tolerance (Ingram and Bartels 1996; Thomashow 1999). *LEA* genes have no specificity when expressed in other tissues such as cotyledons, flowers, roots, stems and leaves (Shao et al. 2005). Many studies have focused on *LEA* associated tolerance against stresses such as drought, salt and cold (Vivekananda et al. 1992; Zhao et al. 2010; Xiao et al. 2007; Kader et al. 2012; Xu et al. 1996; Dalal et al. 2009; Wu et al. 2011; Park et al. 2005；Yuan et al. 2012).

TaLEA genes (EST sequence No. CV791266, CV791413, CV791679, CV79196s, CV792397) were previously cloned from *Tamarix androssowii* cDNA libraries and transferred into *Nicotiana tabacum L.* and *Vaccinium uliginosum L.* in our laboratory. *TaLEA*-transferred lines showed higher POD and SOD activities, significantly improved growth and rooting capabilities, and enhanced drought and cold tolerance (Gao et al. 2012; Zhao et al. 2011;Wang et al.2006). Based on these findings,

we transferred *TaLEA* into the *Populus davidiana* × *P. bolleana* hybrid in order to enhance its tolerance under abiotic stresses.

Material and methods

Populus davidiana × *P. bolleana* plantlets stored in our laboratory were selected as transgenic receptors and cultured in MS media supplemented with 0.1 mg/l 6-BA, and 0.05 mg/l NAA. A subculture medium of half-strength MS containing 0.3 mg/l IBA was used as a rooting medium around day 25. MS containing 0.5 mg/l 6-BA and 0.05 mg/l NAA was used as a differentiation medium. *Agrobacterium tumefaciens* strain EHA105 that carried the constitutive expression vector pLR driven by the CaMV35S promoter and harbouring the *TaLEA* genes was used for the transformations.

Agrobacterium tumefaciens-mediated genetic transformation

The tissue culture leaves were infected using *Agrobacterium tumefaciens* at an OD600 = 0.2 for 10 min, cultured in the dark at 25°C for 2 days, then transferred to medium containing 50 mg/l kanamycin and 300 mg/l cephalosporin. Calluses on leaves after about 20 days were cut for continuous culture for 20 days. Calluses were then transferred to the rooting medium for rooting.

PCR determination

Total DNA was extracted using the CTAB method and the positive control *pRLEA* plasmid was extracted using the fast extraction tiny-plasmid purification kit (Shanghai HuaShun Biological Engineering Co., Ltd.). *TaLEA*-specific forward (CTAGAGGTACCATGGCTCGCTGCTCTTACTCTAAT) and reverse
(TCTAGCTCGAGTCAGTGAGAGGATCGTTGAACTTG)
primers were synthesized and used to amplify the gene. The *pRLEA* plasmid containing a fragment of the target gene was used as a positive control, and total DNA in non-transgenic plants was used as a negative control. Total DNA samples from transgenic plants were amplified using PCR, and the amplified products were detected using 0.8% agarose gel electrophoresis.

Northern blot analysis

Total RNA samples in transgenic and non-transgenic lines were extracted using the SDS method. Samples were then transferred to nylon membranes after electrophoresis on 1% formaldehyde agarose gels, hybridized and determined using standard Northern blot procedures.

Survey of percentage height growth and survival percentage of experimental lines under NaCl stress.

Six transgenic lines (SL1-6) and non-transgenic (NT) controls were propagated by cottage technology in early May 2012. A single stem annual branch (0.5 cm diameter and 15 cm in length) was used as a shoot for cuttings, and rooted in pots filled with 2.5 kg of turfy soil, riversand and black soil in a 4:2:2 ratio (v/v). A plastic tray was placed under each pot. One plant was rooted in each pot, and 15 plants were rooted for each line, yielding 105 pots in total including control plants. The cuttings were managed in plastic greenhouses using standard procedures. The NaCl stress test was performed when the shoots grew to 50 cm in height (mid-June 2012). Before stress test, tree height was measured and the initial value was recorded. The seedlings were irrigated with 500 ml 0.8% NaCl every 3 days. NaCl stress was stopped after 12 days before commencing rehydration. After the seedlings ceased growing (end of September 2012), tree height was measured and relative rate of height growth and survival rate were calculated as follows:

Percentage height growth = [(final height - initial height) / initial height] × 100%.

Survival percentage = [number of surviving plants / total number of plants] × 100%

Determination of chlorophyll fluorescence kinetic parameters

The chlorophyll fluorescence kinetic parameters were determined at 0, 3, 6, 9, and 12 days after completion of NaCl stress using a PAM-2500 (WALZ, Germany) fluorescence detector. Three healthy leaves of three plants in each NaCl stress group were selected for determination. Maximum photochemical efficiency (F_v/F_m), photochemical quenching (qP) and actual photochemical quantum yield (Φ_{PSII}) were determined at room temperature after 20 min of dark adaptation.

Determination of leaf Na^+ level

Yellow, withered and green leaves were picked at 12 days after NaCl stress and dried at 105°C for 20 min. Na^+ levels were determined using a TAS-990 atomic absorption spectrophotometer (Purkinje General Instrument Ltd., Beijing, China) for 0.2 g samples, three times in parallel. The mean of Na^+ level was obtained.

Statistical Analyses

The data analyses were performed using SPSS version 16.0. Statistical difference was compared based on the Ducan test, at significance level $p < 0.05$.

Results

Transgenic lines

Six *TaLEA* kanamycin-resistant lines were obtained and named SL1-6. PCR showed a single band was amplified at 309 bp in all six *TaLEA* transgenic lines, while no bands were amplified with NT lines or water controls (Fig. 1A), which demonstrates *TaLEA*

integration into the genome of transgenic lines. Northern blotting showed hybridization bands in the six *TaLEA*-transferred lines that were absent in NT lines (Fig. 1B), indicating *TaLEA* mRNA successfully expressed in all six transgenic lines.

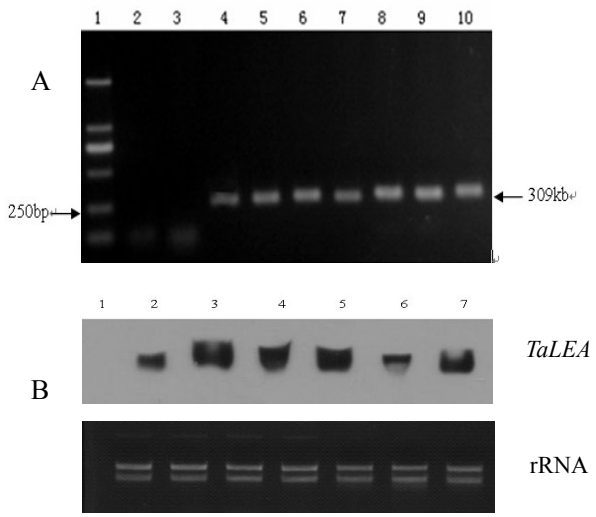

Fig. 1: Molecular analysis of transgenic lines to confirm successful *TaLEA* introduction and expression. (A) PCR of transgenic lines; lane 1, DL2000 Marker; lane 2, water control group; lane 3, negative control group; lane 4, positive control group; lanes 5-10, transgenic lines SL1-6. (B) Northern Blot of transgenic lines; lane 1, negative control group; lanes 2-7, transgenic lines SL1-6.

Salt tolerance – percentage height growth and survival percentage

Before NaCl stress, all experimental seedlings were of similar height (Table 1). After NaCl stress, the mean percentage height increments of SL1, SL2, SL4 and SL6 were approximately 26% greater than for NT lines. The height increments of SL3 and SL5 were less than those for NT lines. Survival percentages after rehydration were higher in all transgenic lines than in NT lines. SL2 and SL3 exhibited highest survival at 68% and 53%, respectively. Survival of the other transgenic lines ranged from 20−27%.

Table 1: Height increments of transgenic and NT seedlings following NaCl stress, and survival after post-stress rehydration

Line	Average height (cm)		Percentage height growth (%)	Survival percentage after rehydration (%)
	Height before stress	Height after stress		
NT	48.91 ± 2.84	67.09 ± 6.44b	37.17	13.33
SL1	48.40 ± 3.86	69.20 ± 5.43b	42.97	26.67
SL2	51.11 ± 6.90	76.00 ± 8.09a	48.70	66.67
SL3	50.91 ± 6.12	67.45 ± 5.26b	32.49	53.33
SL4	46.40 ± 2.80	67.20 ± 4.85b	44.83	26.67
SL5	49.00 ± 4.15	60.56 ± 5.68c	23.59	26.67
SL6	46.18 ± 5.65	69.82 ± 5.76b	51.19	20.00

Salt tolerance - chlorophyll fluorescence kinetic parameters

The chlorophyll fluorescence kinetic parameters F_v/F_m, qP, andΦ_{PSII} were determined at 0, 3, 6, 9, and 12 days after termination of NaCl stress. Analysis of variance showed that the chlorophyll fluorescence kinetic parameters of transgenic lines were all significantly greater than NT lines at 12 days (Table 2). F_v/F_m (maximal photochemical efficiency of Photosystem (PS)II under dark adaptation) was between 0.75 and 0.84 in higher plants under normal condition, but declined under stress conditions. F_v/F_m reflects the degree of damage to the PSII photosynthetic reaction centre. F_v/F_m values were slightly higher at 3 days than at 0, 6 and 9 days after NaCl stress, and exhibited a significant decline at 12 days in all experimental lines (Fig. 2A). F_v/F_m in SL1, SL2 and SL5 declined on average by only 3%, which was significantly lower than the 24% decline recorded for NT lines. This suggested enhanced photosynthetic capabilities in these transgenic lines. F_v/F_m in the other transgenic lines was comparable to NT lines (Table 2).

Table 2: Chlorophyll fluorescence kinetic parameters of transgenic lines and NT following NaCl stress

Line	*F*v / *F*m			Line	*q*P			Line	Φ_{PSII}		
	Before stress	12 days after stress	Decline Ratio %		Before stress	12 days after stress	Decline ratio %		Before stress	12 days after stress	Decline ratio %
NT	0.766 ± 0.013	0.58 ± 0.01d	24.28	NT	0.914 ± 0.018	0.496 ± 0.055c	45.73	NT	0.655 ± 0.005	0.184 ± 0.001e	71.91
SL1	0.766 ± 0.008	0.734 ± 0.023ab	4.18	SL1	0.920 ± 0.008	0.729 ± 0.012ab	20.76	SL1	0.66 ± 0.014	0.412 ± 0.008b	37.58
SL2	0.771 ± 0.008	0.753 ± 0.023a	2.33	SL2	0.899 ± 0.015	0.796 ± 0.059a	11.46	SL2	0.64 ± 0.027	0.508 ± 0.016a	20.62
SL3	0.768 ± 0.006	0.650 ± 0.038c	15.36	SL3	0.912 ± 0.028	0.7 ± 0.019b	23.24	SL3	0.655 ± 0.005	0.3 ± 0.020d	54.20
SL4	0.739 ± 0.018	0.690 ± 0.038bc	6.63	SL4	0.929 ± 0.009	0.559 ± 0.033c	39.83	SL4	0.629 ± 0.02	0.265 ± 0.003d	57.87
SL5	0.775 ± 0.011	0.752 ± 0.024a	2.97	SL5	0.898 ± 0.024	0.788 ± 0.026a	12.25	SL5	0.653 ± 0.017	0.472 ± 0.026a	27.72
SL6	0.752 ± 0.038	0.687 ± 0.018bc	8.64	SL6	0.932 ± 0.004	0.75 ± 0.019ab	19.53	SL6	0.655 ± 0.006	0.356 ± 0.040c	45.65
Mean	0.762 ± 0.014	0.692 ± 0.025	9.19	Mean	0.915 ± 0.015	0.688 ± 0.032	24.81	Mean	0.650 ± 0.012	0.357 ± 0.016	45.07

*q*P is a measure of PSII primary quinone acceptor (QA) oxidation state, and reflects the proportion of light energy captured by the PSII antenna pigments for photochemical electron transport. Typically, stress reduces *q*P. This indicates

that QA re-oxidation capacity is reduced because stress damages the PSII electron transport activity. We recorded a marked reduction in *q*P after NaCl stress in all experimental lines (Fig. 2B). Between 0 and 9 days after termination of NaCl stress, *q*P declined slightly, whereas after 12 days the decline was more significant (Table 2). *q*P for NT lines decreased by approximately 46%, whilst the decline was only 11% and 12% for SL2 and SL5, respectively. This is further evidence that these two transgenic lines possessed a more robust and salt-tolerant photosynthetic machinery.

Φ_{PSII} is the actual quantum yield or photosynthetic capacity of PSII under any light condition. The PSII photosynthetic reaction center partially closes under stress, and Φ_{PSII} reflects the actual photosynthetic capacity under stress. In all experimental lines, Φ_{PSII} exhibited a slight rise between days 0-9, and a sharp decline 12 days after termination of NaCl stress (Fig. 2C; Table 2). SL2 and SL5 again exhibited the smallest declines in photosynthetic efficiency, 21% and 28%, respectively, while NT lines declined by 72%.

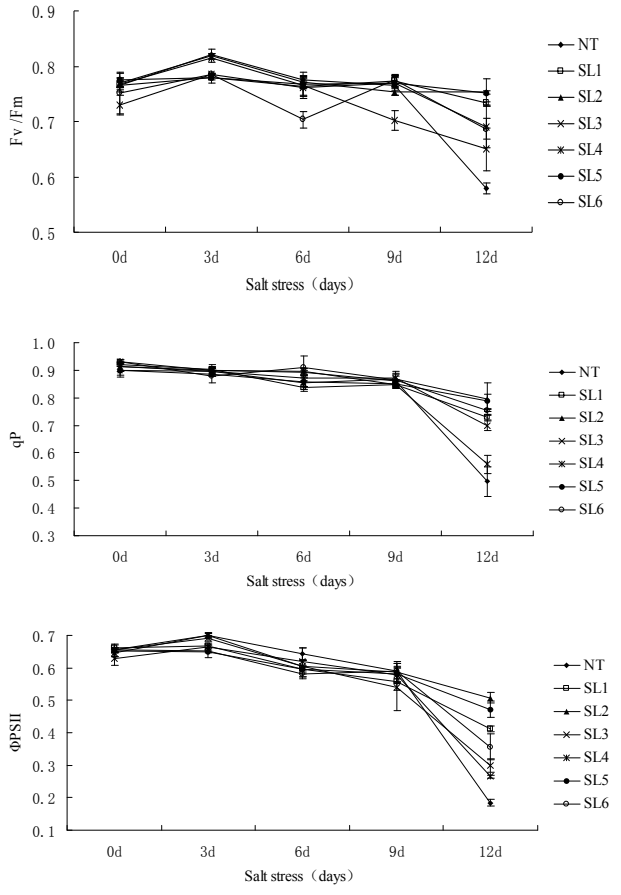

Fig. 2: Fluorescence kinetic parameters reflecting changes in photosynthetic capacity after salt stress. (A) F_v/F_m is the maximal photochemical efficiency of PSII under dark adaptation (B) *q*P is a measure of PSII primary quinone acceptor (QA) oxidation state, and reflects the proportion of light energy captured by the PSII antenna pigments for photochemical electron transport (C) Φ_{PSII} is the actual quantum yield or photosynthetic capacity of PSII.

Salt tolerance - leaf Na^+ levels

Differences were apparent in the experimental lines 12 days after termination of NaCl stress (Fig. 3). Some of the lower leaves turned yellow in the seedlings of most transgenic lines, whilst the upper leaves were less damaged. Upper leaves turned yellow and became withered in NT lines. Na^+ levels in withered leaves averaged 8.75 mg/g, 4.95 mg/g in yellow leaves, and 3.22 mg/g in green leaves. SL3 exhibited lower Na^+ levels in withered leaves than did NT lines, but the other transgenic lines had similar or higher levels. Withered leaf Na^+ levels in SL5 and SL1 were 30% and 16% higher than in NT lines, respectively. In yellow leaves, SL5 displayed lower Na^+ levels than did NT lines, while levels were similar or higher than in NT lines for the other transgenic lines. In green leaves, Na^+ levels were significantly lower in the transgenic lines with the exception of SL1 (Table 3).

Fig. 3: Photograph of transgenic lines and NT lines 12 days after NaCl stress. The two plants on the left are non-transgenic lines and the two plants on the right are two of the six transgenic lines.

Table 3: Leaf Na^+ levels in transgenic lines and NT following NaCl stress

Line	Withered leaves (mg/g)	Yellow leaves (mg/g)	Green leaves (mg/g)
NT	8.73 ± 0.9cd	4.43 ± 0.99b	4.34 ± 0.28b
SL1	10.13 ± 0.5b	5.91 ± 0.37a	5.10 ± 0.08a
SL2	8.86 ± 0.15c	4.54 ± 0.72b	2.70 ± 0.25cd
SL3	6.45 ± 0.32e	6.24 ± 0.40a	2.50 ± 0.27cd
SL4	7.76 ± 0.23d	6.21 ± 0.66a	3.07 ± 0.8c
SL5	11.38 ± 0.56a	2.90 ± 0.06c	2.06 ± 0.32d
SL6	7.97 ± 0.21d	4.45 ± 0.04b	2.76 ± 0.92cd

Discussion

The LEA protein is rich in hydrophilic amino acids such as glycine and lysine, while hydrophobic amino acids are relatively scarce. This distinctive primary structure bestows on LEA some prominent physical and chemical properties; namely high hydrophilicity, high thermal stability, and high aqueous solubility even at very high temperatures (Close et al. 1989; Battaglia et al.

2008). LEA has a powerful osmotic regulation capability, is important for plant tolerance to abiotic stress, and is widely investigated for possible utilisation in transgenic plants to enhance stress tolerance. However, most previous studies have focussed on herbaceous transgenic plants (Vivekananda et al. 1992; Zhao et al.2010; Xiao et al. 2007; Kader et al. 2012; Xu et al.1996; Dalal et al.2009; Wu et al.2011; Park et al. 2005). To investigate a possible stress tolerance role in ligneous plants, we transformed *Populus davidiana* × *P. bolleana* hybrids with *Tamarix androssowii TaLEA*. The resulting transgenic lines showed significantly improved salt tolerance. This further demonstrates the involvement of *LEA* in tolerance to stress and suggests a possble role for LEA-containing transgenic plants in stress-tolerant forests.

Salt stress affects plants in many ways, including slowing the rate of growth considerably as the plant diverts resources away from growth and towards stress response (Zhu 2001). In previous studies, heights of *TaLEA*-transferred tobacco lines increased by 205−341%, 57 days after termination of abiotic stress, while heights of non-transgenic tobacco plants increased by 170% (Wang et al. 2006). Gao et al. (2013) reported that growth increments of transgenic lines ranged from 2.2% to 20.5%, much higher than for non-transgenic plants (1.2%). In this work, height increments and survival of six *TaLEA*-transferred lines were measured after 12 days of salt stress and a 2-month rehydration regime. Survival percentages of all six transgenic lines were higher for NT lines, with SL2 exhibiting the highest survival of 67%, five-fold higher than for NT controls. Four of the six transgenic lines exhibited significantly greater height increments over NT lines. Transgenic rice lines expressing *LEA* have similarly enhanced survival and tolerance to salt stress (Xu et al. 1996).

Light energy absorbed by plant leaves is typically used in three changeable pathways: photosynthetic electron transport, chlorophyll fluorescence emission, and thermal dissipation. Changes in photosynthesis and heat dissipation can cause changes in fluorescence (Roháček 2002). Plant stress tolerance capability can be evaluated according to chlorophyll fluorescence parameters because the influence of stress conditions on photosynthesis can be reflected by *in vivo* chlorophyll fluorescence induction kinetics. Therefore, plants must maintain higher *q*P and PSII linear electron transport efficiency to effectively resist stress. Our results show that three different measures of photosynthetic capacity (F_v/F_m, *q*P and Φ_{PSII}) were significantly reduced after 12 days of salt stress, suggesting salt-induced damage to chloroplast membrane structure inhibited electron transfer in the PSII photosynthetic reaction center, and ultimately hindered photosynthesis. In transgenic lines, photosynthetic capacity was impeded far less than in NT lines. This was most pronounced for the SL2 and SL5 *TaLEA* transgenic lines.

Under salt stress, plants absorb and transport large quantities of ions from soils to leaves, resulting in leaf withering, necrosis and shedding (Parvaiz and Satyawati 2008). We found that in the initial stages of salt stress, older leaves on lower parts of transgenic plants turned yellow, whereas newer leaves on upper parts of the plants remained green without obvious salt damage. In contrast, older leaves at lower parts of NT plants remained green whilst younger leaves at upper parts withered (Fig. 3). As Na^+ accumulated over time, upper leaves of transgenic lines withered and lower leaves of NT plants turned yellow. Although seemingly puzzling, these observations are consistent with previous studies that demonstrated ion regionalization phenomena in halophytes and non-halophytes; non-halophytes minimally absorb, transport and store harmful salt ions to the older tissues to protect the young tissues (Cheeseman 1988). Plants will be damaged by salt stress once the salt concentration exceeds levels that can be tolerated. In this study, Na^+ levels were highest in withered leaves, moderate in yellow leaves, and lowest in green leaves. This suggests that the expression of exogenous *TaLEA* enhances Na^+ transport from new leaves to old leaves that subsequently turn yellow, become withered and finally fall, ridding the plant of the excess Na^+. In a recent study, *P. simonii* × *P. nigra* hybrid plants under NaCl stress were reported to upregulate the expression of genes associated with ion transport (Chen et al. 2012). This indicates that poplar trees have an inherant respose to, and tolerance of, salt stress. This current work demonstrates that further improvements to the salt tolerance of another poplar hybrid, *Populus davidiana* × *P. bolleana*, can be made by transferring the *Tamarix androssowii TaLEA* gene.

Plant responses to salt stress are complex processes that involve salt sensing, signal transduction, transcriptional regulation of stress response genes and the expression of functional genes. This study investigated the phenotypic traits of transgenic lines by measuring survival, growth rates, chlorophyll fluorescence kinetic parameters and leaf Na^+ levels. In future studies, we intend to investigate the molecular mechanisms of constitutive expression of LEA and other exogenous genes involved in improving the salt tolerance in *Populus davidiana* × *P. bolleana.*

Acknowledgements

This work was supported by the National High-Tech Research and Development Program of China, 863 Program (Molecular Breeding and New Species Creating of Poplar) (Grant No. 2011AA100201).

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