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RESEARCH PAPER

Growth adaptability and foreign gene stability of *TaLEA* transgenic *Populus simonii* × *nigra*

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Abstract

• ***Key message*** **Late-embryogenesis-abundant proteins (LEA) typically accumulate to high levels during seed dehydration and have an important role in the plant response to abiotic stresses. Transgenic hybrid poplars of 9-year-old *TaLEA* lines showed adaptation to drought and saline–alkali conditions. The exogenous gene was expressed normally, and the T-DNA insertion sites were identified on different chromosomes.**

• ***Context*** Improving the survival rate of afforested species is essential for forest production in saline–alkali areas. Transgenic hybrid poplars could provide potential genetic materials for adaptation to semi-arid areas.

• ***Aims*** We investigated the growth stability and adaptability of 9-year-old *TaLEA* transgenic lines of *Populus simonii* × *nigra*, as well as the position of T-DNA insertion and the stability of exogenous genes.

• ***Methods*** Transgenic poplar field trials were carried out using a multi-site joint analysis. Molecular characteristics and the T-DNA insertion sites were identified by genome resequencing.

• ***Results*** The XL-9, XL-7, and XL-13 preferred lines consistently showed the highest and most stable growth across three testing sites. The exogenous gene was integrated into the genome chromosome and was expressed normally in the XL-1, XL-7, and XL-9 lines after 9 years.

• ***Conclusion*** XL-9, XL-7, and XL-13 are the preferred lines for subsequent production tests. Transgenic hybrid poplar lines can be selected for adaptability 2 years after field experiments. The results of this study will provide further guidance for testing of transgenic trees for future forest production.

Keywords *Populus simonii* × *nigra* · Late-embryogenesis-abundant proteins · Abiotic stress · Field experiment

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1 Introduction

Late-embryogenesis-abundant (LEA) proteins are widely expressed in higher plants, and typically accumulate to high levels during seed dehydration at the last stage of embryogenesis in plant seeds. LEA proteins have strong hydrophilicity and a relevant role in the plant response to abiotic stresses such as low temperature, osmotic stress, and exogenous abscisic acid (ABA) (Manfre et al. 2006; Zegzouti et al. 1997). It has been proven that the number of LEA proteins are increased under abiotic stress as a mechanism to improve the stress resistance of plants by maintaining the osmotic pressure of cells, protecting the cell membrane system and other biological macromolecules from destruction (Liang et al. 2004; Lu et al. 2013; Wang and Hua 2018; Wang et al. 2015). It was found that a large amount of LEA protein accumulated in LEA transgenic rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), and tobacco (*Nicotiana tabacum* L.), which showed resistance to drought, low temperature, and saline–alkali conditions under stress (Sivamani et al. 2000; Wang et al. 2006; Xu et al. 1996).

Populus simonii × *nigra* is a hybrid of *Populus simonii* and *Populus nigra*. It is an important afforestation and ornamental tree species in Northeast China. In order to improve the survival rate of afforested species in saline–alkali and arid areas, our team previously introduced *TaLEA* from *Tamarix* (*Tamarix androssowii*) to transgenic plants of *P. simonii* × *nigra*. Overexpression of *TaLEA* at the seedling stage showed that the 11 transgenic lines were resistant to abiotic stress to varying degrees. Intermediate common garden plantations were established at six sites in mild saline–alkali land and semi-arid areas. The tree height and diameter at breast height (DBH) were recorded for 2-year-old plants. XL1, XL9, and XL14 lines were selected based on their genetic stability using the Tai model (Tai 1971). All three lines showed fast growth and strong stability at the six experimental sites. They were the best-growing lines in the early stage of afforestation (Li et al. 2013). The genetic stability of the ten 3-year-old transgenic lines in four afforestation test sites was analyzed by combining with 1-year-old growth data, and the XL-1 was thus selected as a fast-growing and resistant line (Liu et al., 2015). However, due to a strong genotype × environment × year interaction, early-age results of these salt-tolerant *TaLEA* transgenic *P. simonii* × *nigra* lines needed to be further verified as the growing transgenic trees reached the age of 10 years. It was found that the foreign genes of most transgenic plants could be inherited and stably expressed after transplantation in the field (He et al. 2008; Liu et al. 2014; Srivastava and Vasil 1996; Wang et al. 2013, 2004). However, there was also a phenomenon of the silencing or inactivation of foreign genes in transgenic plants. For example, transgenic *Petunia* (*Petunia* hybrid) was transferred to the field, and transgenic inactivated plants were found (Meyer and Heidmann 1994). In addition, the phenomenon of foreign gene silencing was observed in caffeic acid O-methyltransferase (COMT) transgenic poplar (*Populus tremula* × *Populus alba*) (Jouanin et al. 2000). It has been nearly 10 years since the establishment of the *TaLEA* transgenic poplars pilot forest by our research team. We hypothesized that the exogenous *TaLEA* gene was stably expressed and transgenic lines could be selected at early growing stage for adaption to saline–alkali areas. Therefore, in this study, we evaluated the growth adaptability of the 9-year-old *TaLEA* transgenic *P. simonii* × *nigra* lines, as well as the stability and molecular characteristics of the foreign genes.

2 Materials and methods

2.1 Materials and experimental design

Ten *TaLEA* transgenic *P. simonii* × *nigra* lines (nos. XL-1, XL-3, XL-4, XL-5, XL-6, XL-7, XL-9, XL-10, XL-13, XL-14) and one wild-type (WT) line were used in this study. Field trials were established in 2009 at three field sites in China: (1) Cuohai Forestry Center, Longjiang County; (2) Daqing City, Heilongjiang Province; and (3) Lanxi County, Suihua City (Table 1). The fields were planted using a randomized complete block design: a total of 1100 trees (11 lines × 20 trees per plot × 5 replications) with a double row arrangement 2 m × 2 m in Daqing City and Lanxi County, and 990 trees (11 lines × 18 trees per plot × 5 repetitions) with row spacing of 2 m × 3.3 m in the Cuohai Forestry Center. A measurement of tree height and DBH was recorded in autumn 2017. In the Cuohai Forestry Center, 10–20 cuttings per line were collected and propagated in a greenhouse, under natural light, with a temperature of 28 ± 2° C during the day and 20 ± 2 °C at night

Table 1 Geographical and climatic conditions of the test sites

Test site	Longitude	Latitude	Altitude (m)	Annual average temperature (°C)	Annual precipitation (mm)	Soil type	pH
Cuohai	124°25′E	47°55′N	340	3.4	450.0	Dark brown forest soil	6.8
Daqing	124°25′E	45°51′N	146	4.2	427. 5	Chestnut soil	8.4
Lanxi	125°54′E	46°25′N	170	2.4	504.0	Chernozem	7.4

for 2 months. The cutting medium was peat soil mixed with vermiculite at a 1:1 ratio. The displayed leaves of rooted cuttings were collected for subsequent identification of the T-DNA insertion sites of the transgenic lines. The leaves from the common garden plantation were collected for subsequent molecular detection of the transgenic lines.

2.2 Molecular detection and T-DNA insertion site identification of transgenic lines

2.2.1 Molecular detection of transgenic lines

Total DNA was extracted from the leaves of the tested lines using the DNAquick Plant System (TIANGEN BIOTECH (BEIJING) CO., LTD.). A pair of primers based on the *TaLEA* gene open reading frame (ORF) (forward primer, 5'ATGGCTCGCTGCTCTTACTCTAATG-3′; and reverse primer, 5' TCAGTGAGAGGATCGATTGAACTTG 3′) were used to confirm the presence of the *TaLEA* gene transgenes in the genome of the transgenic plants. The polymerase chain reaction (PCR) was performed in 20 μl volume consisting of 1.1× T3 Super PCR Mix (PCR premixed solution with concentration of 1.1×, containing DNA polymerase, produced by TsingKe Biological Technology), 50 pmol of each primer, 7 μl deionized water, and 20 ng genome DNA template. The PCR program was run at 94 °C for 3 min for pre-denaturing, followed by 30 cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 2 min, with a final extension at 72 °C for 7 min. Amplified DNA fragments were electrophoresed on 1% agarose gel and visualized under UV light.

RNA was extracted from the leaf tissue of the transgenic lines (XL-1, XL-7, XL-9) and WT lines using the Universal Plant Total RNA Extraction Kit (spin-column) (BioTek Corporation). Reverse transcription was performed with the ReverTra Ace® qPCR RT Kit (TOYOBO CO., LTD.). The reverse transcription system proceeded as follows: RNA was immediately placed on ice after denaturation at 65 °C, and 1 μg iced RNA was added to 4 μl of 4× DN Master Mix (premixed solution with concentration of 4×, containing RNase inhibitor and reaction buffer), which was mixed with gDNA Remover. The volume of deionized water was supplemented to 16 μl, and a reaction was performed at 37 °C for 5 min. Then, 4 μl of 5× RT Master Mix II (premixed solution with concentration of 5×, containing reverse transcriptase, Random Primer, Oligo dT Primer, reaction buffer, and dNTPs) was added, and the reaction procedure was performed as follows: 37 °C for 15 min, followed by 5 min at 50 °C, 5 min at 98 °C, and 4 °C for heat preservation. Reverse transcription products were used as RT-PCR templates, and the primers, amplification systems, and procedures used for RT-PCR amplification were the same as those for the abovementioned PCR detection.

18S was chosen as a reference gene for quantitative real-time PCR (qRT-PCR). qRT-PCR was performed on an ABI 7500 real-time PCR detection system using SYBR Green Real-time PCR Master Mix Plus (TOYOBO, Osaka, Japan). According to the results, the cDNA concentration was adjusted to be consistent. PCR was performed again using cDNA as template, and the number of cycles was changed to 26. The products were electrophoresed on 2% agarose gel, after which the agarose gel was placed into the automatic digital gel image analysis system (Tanon 2500R), and the concentration was read using Tanon Gis software.

2.2.2 Identification of T-DNA insertion sites in transgenic lines

The identification of the T-DNA insertion sites was performed according to a previously published study (Gang et al. 2019a). Fresh young leaves of the transgenic lines XL-1, XL-7, and XL-9 were extracted and sent to Annoroad Gene for genome resequencing. Each sample produced no less than 95% of 2.5 Gb clean data, and the total sequencing amount was no less than 7.5 Gb of clean data. The genome resequencing data of at least 50X were obtained by the Illumina sequencing platform. The sequencing results were compared with the plasmid vector sequences by BWA (Burrows-Wheeler Alignment), and the reads and pairs containing vector sequences were extracted by SAM (Sequence Alignment/Map) tools. The reads were further compared with the reference genome of *Populus trichocarpa* by BWA and BLAST (Basic Local Alignment Search Tool), and the specific insertion sites were obtained according to the genomic annotation information for *Populus trichocarpa* (https://phytozome.jgi.doe.gov/pz/portal.html).

According to the results of the genome resequencing of the XL-1, XL-7, and XL-9 lines, primers were designed based on the flanking sequences of T-DNA insertion sites in the genomes of each line (Wang et al. 2021). The forward primers were designed according to the vector sequence, and the reverse primers were designed according to the genome sequences of *P. simonii* × *nigra* near the insertion sites. Six pairs of primers were designed for the XL-1 line. One pair of primers was designed for the XL-7 line, and one pair of primers was designed for the XL-9 line (Table 2). Primers were synthesized by General Biosystems (Anhui) Co., Ltd. PCR amplification product sequencing was completed by TsingKe Biological Technology (Harbin) Co., Ltd.

2.3 Statistical analysis

2.3.1 Analysis of variance

Data were analyzed for three test sites combined using analysis of variance (ANOVA) according to the following linear model in Eq. 1:

Table 2 Primers used in this study

Primer name	Primer sequence(5′→3′)	Expected amplification length(bp)	Detected lines
XL-1 Chr01	F: AGCTATGCAATCCCAACAACA R: TCGCAAGACCCTTCCTCTATA	737 bp	XL-1
XL-1 Chr03	F:GCCAAATGTTTGAACGATCGG R:CTTGAAAGTTTTTGCCATTCCCT	751 bp	
XL-1 Chr05	F:GAAGAATTCCATAGCCTCGACA R:GGAACAACACTCAACCCTATCTC	608 bp	
XL-1 Chr09	F:ATGGATTTGGGTACGGTGAAG R:GTCATAGCCGAATAGCCTCTC	729 bp	
XL-1 Chr14	F:TGGAGATATCACATCAATCC ACTTG R:ACGCGCATAGCTAGTCTTTATTC	756 bp	
XL-1 Chr16	F:ATCTCCAGGAAATCAAATAC CTTCC R:CTTTCGTAGATCAGTTGCCTCAT	744 bp	
XL-7 Chr09	F:TTGCTGGTCTTGTTCTGTTCA R:ATTTAGTGCTTTACGGCACCT	723 bp	XL-7
XL-9 Chr05	F:CGGTTAAGAATTGAATTTCA TAAT R:GTTACTAGATCGGGAATTCA CTGG	643 bp	XL-9

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk} \quad (1)$$

where Y_{ijk} is the performance of line i within site j, μ is the overall mean, α_i is the line effect, β_j is the site effect, $(\alpha\beta)_{ij}$ is the random effect of line i within site j, and ε_{ijk} is the random error. Microsoft Excel was used for data processing, and SPSS (Statistical Product and Service Solutions) version 19.0 software was used for variance analysis and multiple comparisons.

Variation among lines within each site was analyzed by ANOVA according to the linear model in Eq. 2:

$$X_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk} \quad (2)$$

where X_{ijk} is the performance of line i within block j, μ is the overall mean, α_i is the line effect, β_j is the block effect, $(\alpha\beta)_{ij}$ is the random effect of line i within block j, and ε_{ijk} is the random error.

2.3.2 Stability analysis

Stability analysis was carried out with DPS (Data Processing System) software, and the effect value was calculated. The Tai model (Eq. 3) was used in the stability analysis model:

$$Y_{ijk} = \mu + g_i + l_j + (gl)_{ij} + b_{jk} + \varepsilon_{ijk} \quad (3)$$

where Y_{ijk} is the performance of line i within site j and block k, μ is the overall mean, g_i is the genetic effect of line i, l_j is the environmental effect of site j, $(gl)_{ij}$ is the random effect of line i within site j, b_{jk} is the random effect of block k within site j, and ε_{ijk} is the random error.

2.3.3 Genetic parameter analysis

The coefficient of phenotypic variation (PCV) was calculated using Eq. 4:

$$PCV = SD/X*100\% \quad (4)$$

where 'X and SD are the phenotypic mean and standard deviation of the trait, respectively.

Repeatability (H2) was calculated using Eq. 5:

$$H2 = 1–1/F \quad (5)$$

where F is the F value in the analysis of variance.

The genetic gain was calculated using Eq. 6:

$$G(\%) = R*S/X*100\% \quad (6)$$

where R is the trait repeatability, S is the difference in the phenotypic mean between the preferred lines and test lines, and 'X is the phenotypic mean.

3 Results

3.1 The variation in transgenic lines in three field trials

Table 3 describes the results of the joint ANOVA of the test lines across three field trials. The site and line effects were significantly different for tree height and DBH ($P < 0.01$). There was a significant interaction between the lines and the sites for DBH ($P < 0.05$). Significant differences were also found for either the same transgenic line growing at different sites or different lines growing in the same site. In addition, the interaction between line and site was not significant for tree height ($P > 0.05$), and the interaction between line and site was significant for DBH ($P < 0.05$). The effect of interaction between genotype and environment on DBH was greater than that on tree height.

The differences in tree height and DBH among the lines were highly significant ($P < 0.01$, Table 4). Among the three test sites, the tested lines grew best at the Daqing test site, and the average tree height and DBH were 8.07 m and 9.35 cm, respectively. Compared with the other two experimental sites, the coefficients of variation of tree height and DBH were the highest at Daqing, 22.8 and 28.0, respectively, while the coefficient of variation of growth at the Lanxi test site was lowest (Table 4). The lowest repeatability of height and DBH in the three test sites was 0.66. The repeatability of height and DBH in Cuohai and Daqing was between 0.78 and 0.94. The high repeatability indicated that growth traits were controlled by genetic factors.

3.2 Comparison of growth traits and preservation rate of test lines

The multiple comparisons of growth traits of the tested lines at the three test sites are presented in Figs. 1, 2, and 3. Because the geographical climate environment and soil pH of the three experimental sites were different, the growth performance for the same tested lines were different among the sites. Using the average volume of the tested lines and 0.5 times the standard deviation as the selection criteria, several preferred lines were identified. The results showed that XL-1 and XL-9 lines were selected at Cuohai. The two selected lines exceeded the population average of 12.4% and 7.59% for height, 15.2% and 17.4% for DBH, and 40.6% and 39.9% for volume, respectively. The afforestation preservation rate of the two selected lines was higher than 74.0% and the death rate was less than 26.0% (Table 5), and the genetic gains in the volume were 37.0% and 36.3%, respectively. The transgenic line selected at the Daqing test site was XL-1, whose tree height, DBH, and volume were 25.6%, 13.1%, and 59.5% higher, respectively, than the population mean, and the genetic gain was 51.7%. Because the afforestation site is alkaline soil, and the terrain is uneven, most of the lines planted in low-lying land did not survive, so the preservation rate of XL-1 was only 43.0%. XL-14 was selected at Lanxi; its tree height, DBH, and volume were 3.82%, 14.1%, and 31.9% higher, respectively, than those of the population; its preservation rate was 54.0%, and the genetic gain in volume was 25.9%.

3.3 Analysis of growth adaptability of tested lines

The interaction between line and site for DBH was significantly different (Table 3). This indicated that the DBH of transgenic lines varied due to the environmental conditions of the site (Fig. 2). Therefore, the Tai model analysis of the tested lines was carried out using DBH data (Table 6). The prediction interval of $\alpha_i = 0$ and the confidence interval of $\lambda_0 \geq 1$ were calculated according to DPS software (Fig. 4). Among the 11 lines tested, all the lines except XL-5 were in the

Table 3 Joint analysis of variance of the test lines

Growth traits	Source of variation	DF	SS	MS	*F*	*P*
Height (m)	Block in site	12	64.2	5.35	7.07	< 0.001
	Site	2	83.3	41.7	55.0	< 0.001
	Line	10	29.9	2.99	3.95	< 0.001
	Line × Site	20	23.8	1.19	1.57	0.0732
	Error	109	82.5	0.757		
	Total	153	284			
Diameter at breast height (cm)	Block in site	12	68.8	5.73	3.92	< 0.001
	Site	2	24.1	12.1	8.25	< 0.001
	Line	10	63.1	6.31	4.32	< 0.001
	Line × Site	20	59.2	2.96	2.03	0.0112
	Error	109	159	1.46		
	Total	153	374			

DF = degrees of freedom, SS = sum of squares, MS = mean square, *F* = statistic value of the *F* test (*F* = MS/MSE), MSE = mean square of error), *P* value < 0.01 = highly significant difference; 0.01 < *P* value < 0.05 = significant difference; *P* value > 0.05 = nonsignificant difference

Table 4 Genetic parameters of the growth traits of the test lines at different sites

Growth traits	Test sites	Standard deviation	*F*	*P*	Generic parameter			
					Mean	Amplitude of variation	Coefficient of variation (%)	Repeatability (H^2)
Height (m)	Cuohai	1.05	9.64	<0.01	6.33	3.5–9.7	16.6	0.90
	Daqing	1.84	17.1	<0.01	8.07	5.0–13.8	22.8	0.94
	Lanxi	1.23	2.91	<0.01	7.67	4.6–11.2	16.0	0.66
Diameter at breast height (cm)	Cuohai	2.37	12.4	<0.01	9.09	3.2–16.6	26.1	0.92
	Daqing	2.62	4.57	<0.01	9.35	5.1–19.0	28.0	0.78
	Lanxi	1.82	6.88	<0.01	8.54	3.8–14.9	21.3	0.85

average stability range; that is, 10 lines (except XL-5) had average stability, and the growth stability and adaptability were good at the three test sites. However, XL-5 was in the range of $\lambda_0 > 1$, which indicates that the reliability of predicting the environmental sensitivity of XL-5 with the α_i value is poor, and the growth performance of DBH at the tested site was unstable.

3.4 Comprehensive evaluation of transgenic lines

Based on the analysis of the Tai model, the interactive effect values of DBH growth at each test point were estimated, and the growth adaptation of the tested lines was further evaluated (Table 7). The results showed that the XL-7, XL-9, and XL-13 lines had higher adaptability than other lines at the three field trials, indicating that the three lines had strong adaptability. The transgenic lines with good growth at only one experimental site were XL-1 and XL-14; that is, XL-1 grew well at Cuohai, and XL-14 grew well at Lanxi.

3.5 Molecular detection of transgenic lines

The results showed that the 10 transgenic lines had specific amplification bands at the expected 309 base pairs (bp). This confirmed that the exogenous *TaLEA* gene was in the genome of the transgenic *P. simonii* × *nigra*, and expressed normally in the selected XL-1, XL-7, and XL-9 lines using semi-quantitative PCR (Fig. 5).

3.6 Analysis and verification of T-DNA insertion sites

The results showed that six T-DNA insertion sites were detected in the XL-1 transgenic line (Table 8), including Chr01 2,759,251 (IS1), Chr03 11,710,668 (IS2), Chr05 10,599,509 (IS3), Chr09 7,653,220 (IS4), Chr14 14,552,526 bp (IS5), and Chr16 9,294,838 (IS6). One T-DNA insertion site was detected in the XL-7 transgenic line, which was Chr09 5,030,092 (IS-XL-7). Another T-DNA insertion site was detected in the XL-9 transgenic line, which was Chr05 6,685,809 (IS-XL-9) (Table 7).

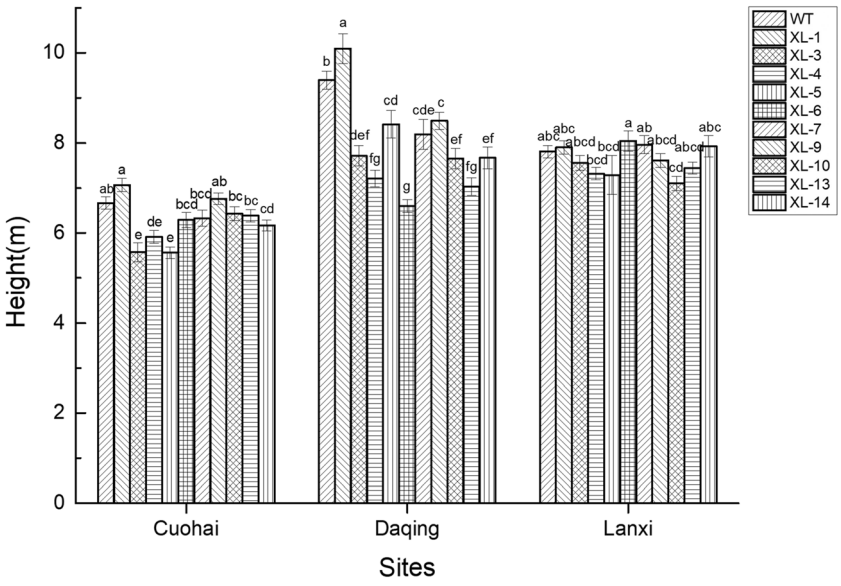

Fig. 1 Height of the test lines in the three sites

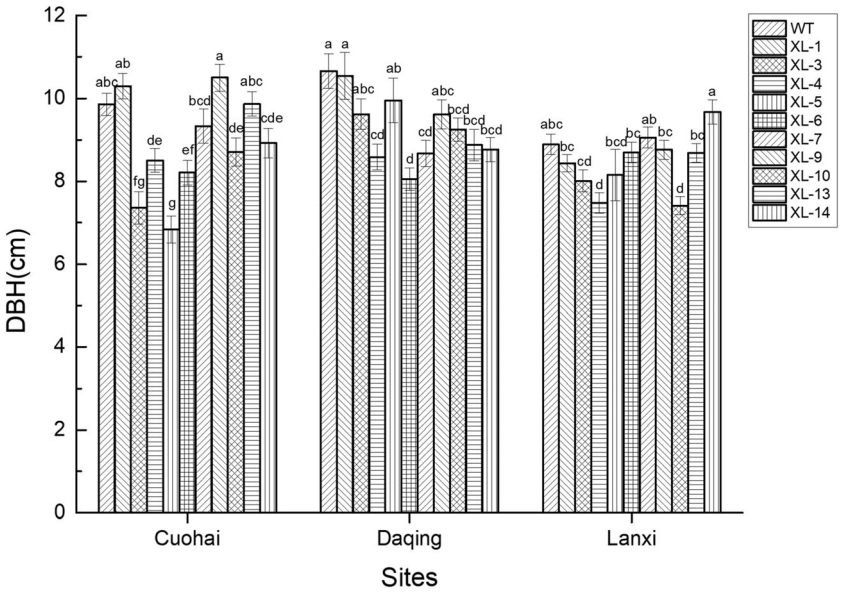

Fig. 2 DBH of the test lines in the three sites

In order to validate the T-DNA insertion sites of the XL-1, XL-7, and XL-9 lines, the above lines were amplified by PCR with specific primers (Table 2). The results showed that specific bands were obtained in XL-1 lines with XL-1 Chr01, XL-1 Chr05, and XL-1 Chr09 primers. The length of each amplified band was consistent with expectations, indicating that XL-1 had a T-DNA insertion on Chr01, Chr05, and Chr09.

The specific bands were amplified by XL-7 Chr09 primers in XL-7 and XL-9 Chr05 primers in XL-9 (Fig. 6), and the PCR product sequencing comparison showed that XL-7 had T-DNA insertion on Chr09 and XL-9 had T-DNA insertion on Chr05.

4 Discussion

4.1 Selection of transgenic lines

Generally, the insertion of T-DNA in transgenic plants is random, and the number of foreign gene insertion sites is also different. Foreign genes may show gene silencing after insertion, and some may cause mutations or deletions of functional genes at the insertion sites (Gang et al. 2019b; Li et al. 2017; Shin et al. 2016). This results in different levels of tolerance of transgenic lines to stress. Therefore, it is often necessary to choose several generations or many years. Only the transgenic lines whose growth and yield are not affected can be used as a

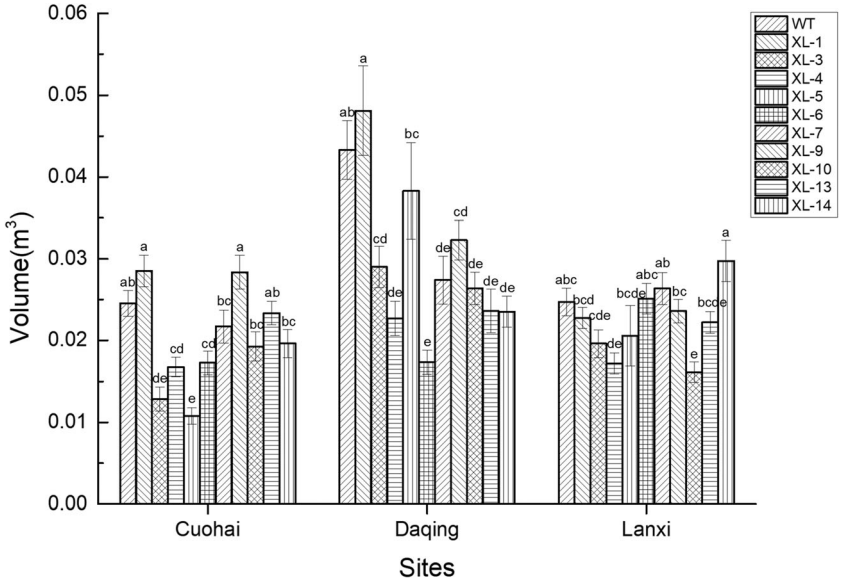

Fig. 3 Volume of the test lines in the three sites

Table 5 Survival rate of the tested lines at the three test sites

Test line	Cuohai		Daqing		Lanxi	
	Survival rate (%)	Mortality rate (%)	Survival rate (%)	Mortality rate (%)	Survival rate (%)	Mortality rate (%)
XL-1	77.8	22.2	43.0	57.0	63.0	37.0
XL-3	46.7	53.3	49.0	51.0	29.0	71.0
XL-4	68.9	31.1	54.0	46.0	63.0	37.0
XL-5	56.7	43.3	46.0	54.0	14.0	86.0
XL-6	61.1	38.9	39.0	61.0	65.0	35.0
XL-7	57.8	42.2	53.0	47.0	59.0	41.0
XL-9	74.4	25.6	64.0	36.0	68.0	32.0
XL-10	46.7	53.3	54.0	46.0	49.0	51.0
XL-13	72.2	27.8	43.0	57.0	66.0	34.0
XL-14	71.1	28.9	32.0	68.0	54.0	46.0
WT	76.7	23.3	53.0	47.0	62.0	38.0

new transgenic variety for commercial production. For example, among the 13 transgenic lines obtained by *betA* transgenic *P. simonii* × *nigra*, four lines grew slowly and showed dwarfism; NaCl stress treatment was then carried out on nine transformed lines with normal phenotypes, and four preferred lines with high salt tolerance were obtained (Liu et al. 2006). These transgenic lines were used to construct the intermediate test forest for the mild saline–alkali land. T1, T6, and T8 lines were selected as preferred salt-tolerant lines at age 5 years (Mu et al. 2009). Under NaCl and $NaHCO_3$ stress, SL2 with the strongest salt and alkali tolerance was selected from six lines of transgenic *Populus davidiana* × *P. bolleana* transformed with the *TaLEA* gene (Sun 2014). In another example, Shuang et al. conducted drought and NaCl stress treatment on seedlings transplanted for 1 month after obtaining *TaLEA* transgenic poplar, and selected T11 (XL-11) as the strongest stress resistance line, followed by T1 (XL-1) and T14 (XL-14) (Gao et al. 2013). However, with the growth of the transgenic poplar, it was found that XL-11 was significantly different from other transgenic lines. It was identified that XL-11 was a T-DNA insertion mutant, mainly characterized by dwarfing, small leaves, and epidermis and palisade ratios were significantly higher than in the other 10 transgenic lines (Liu et al. 2013). The *TaLEA* transgenic *P. simonii* × *nigra* was then used to construct an intermediate test forest in semi-arid and mild saline–alkali soil areas. At the age of 2 years, XL1, XL9, and XL14 lines were selected as the transgenic lines with the best applicability and stability according to their growth traits, compared with other transgenic lines; the height growth of XL-11 was slower (Li et al. 2013). Due to the disadvantage of tree height, its survival cannot compete with other transgenic lines. Therefore, the preservation rate of XL-11 was almost zero in the investigation and analysis of 3-year-old *TaLEA* transgenic poplar common garden plantations (Liu et al. 2015). Therefore, it is necessary to select transgenic trees in the field. This study is a follow-up investigation on the 9-year-old transgenic intermediate common garden plantations with the *TaLEA* gene. The results showed that the growth patterns of the 11 lines tested at different experimental sites were different. XL-1 and XL-9 were the best at the Cuohai test site, XL-1 was the best at the Daqing test site, and XL-14 was the best at the Lanxi test site. The soil of the Daqing and Lanxi experimental sites was weakly alkaline, and the selected XL-1 and XL-14 lines might have strong salt and alkalinity tolerance. It was found that the growth of WT at the Daqing test site was also good, and the reasons for this need to be explored in follow-up studies. In addition, in the afforestation experiment, the three afforestation sites may be affected by human activities to varying degrees.

Pilate et al. evaluated the agronomic and pulping performance of transgenic poplar with CAD or COMT genes

Table 6 Parameters of the DBH growth stability of the test lines. The linear response to environmental effects is represented by α; the deviation of linear response is represented by λ

Test line	Mean of DBH (cm)	α	λ
XL-1	9.61	0.743	1.16
XL-3	8.55	0.503	2.67
XL-4	8.23	0.109	0.264
XL-5	8.26	0.796	5.57
XL-6	8.18	−0.955	0.372
XL-7	9.27	−0.682	0.407
XL-9	9.59	0.165	1.07
XL-10	8.25	0.413	0.439
XL-13	9.12	−0.437	1.17
XL-14	9.12	−1.00	2.25
WT	9.93	1.00	0.501

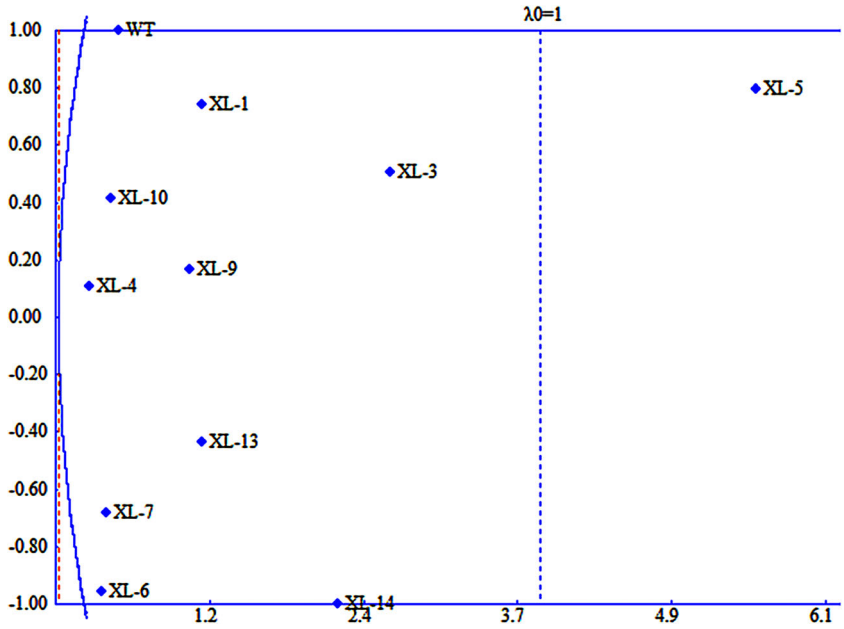

Fig. 4 Distribution map of the DBH growth stability of the test lines. It shows the average stability when the transgenic lines in the area are surrounded by the curve and line

planted in the field in two different countries for 4 years. The results showed that among the four transgenic lines, ascad21 was of commercial value, saving 6% of chemicals and increasing pulp production by 2–3% (Pilate et al. 2002). Pasonen et al. (2008) studied the growth and adaptive traits of chitinase transgenic silver birch (*Betula pendula* Roth) through field experiments. It was found that three lines (1,10,12) of 15 lines had a lower relative growth rate (RGR), late germination, and poor health status compared with the control. The authors believe that these differences are likely explained by the position effect of transgenes, that is, the effects associated with T-DNA integration sites.

Studies on transgenic switchgrass showed that the phenotype of transgenic lines overexpressing the microRNA (mir156) gene was inconsistent between greenhouse and field, and between different growing seasons. One low-expressing transgenic line consistently produced more biomass (25–56%) than the control across all three seasons, which translated to the production of 30% more biofuel per plant during the final season. The other three transgenic lines produced less biomass than the control by the final season, and the two lines with moderate expression levels also exhibited altered disease susceptibilities (Baxter et al. 2018). A field experiment

Table 7 Interaction effects of DBH and sites and comprehensive evaluation of test lines. E1: Cuohai; E2: Daqing; E3: Lanxi

Test line	Mean of DBH (cm)	Values among different tested sites			Line value	Adaptation region	Comprehensive evaluation
		Cuohai	Daqing	Lanxi			
WT	9.93	−0.112	0.704	−0.592	1.01	E2	Great
XL-1	9.61	0.644	0.0730	−0.717	0.694	E1	Great
XL-9	9.59	0.621	−0.270	−0.351	0.671	E1–E3	Great
XL-7	9.27	0.241	−0.565	0.324	0.354	E1–E3	Good
XL-14	9.12	−0.247	−0.895	1.14	0.204	E3	Good
XL-13	9.12	0.586	−0.620	0.0350	0.199	E1–E3	Good
XL-3	8.55	−0.910	0.855	0.0550	−0.372	E2	Normal
XL-5	8.26	−1.30	1.27	0.0320	−0.656	E2	Worse
XL-10	8.25	0.398	0.017	−0.415	−0.672	E1–E3	Worse
XL-4	8.23	0.311	−0.119	−0.192	−0.692	E1–E3	Worse
XL-6	8.18	−0.228	−0.453	0.681	−0.740	E3	Worse

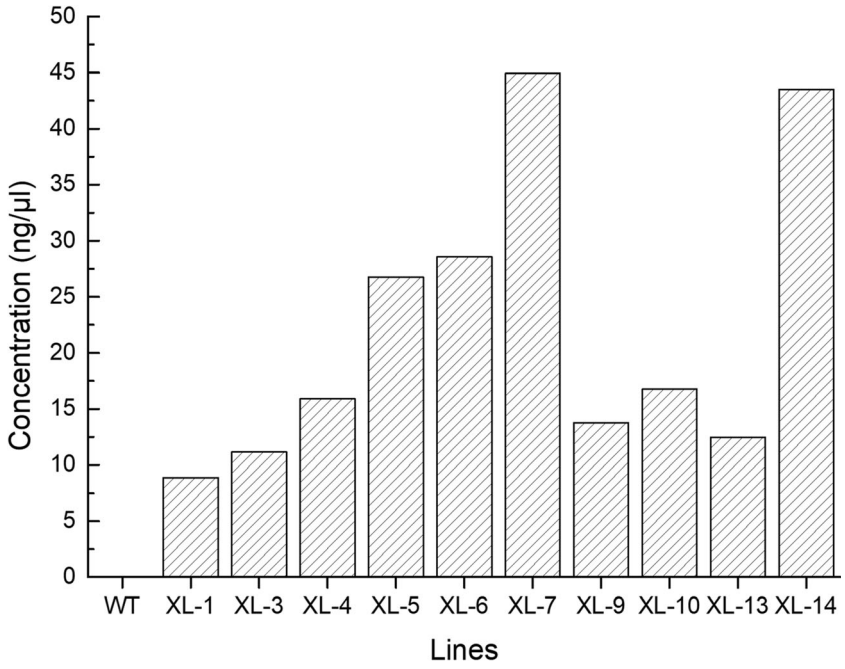

Fig. 5 Results of semi-quantitative interpretation of gene expression

was conducted on switchgrass which overexpressed the transcription factor gene of MYB4 (pvmyb4). The results showed that over two growing seasons, one transgenic event (out of eight) had important gains in both biofuel (32% more) and biomass (63% more) at the end of the second growing season relative to non-transgenic controls. These gains represent a doubling of biofuel production per hectare, which is the highest gain reported from any field-grown modified feedstock. In contrast to this transgenic event, which had relatively low ectopic overexpression of the transgene, five of the eight transgenic events planted did not survive the first field winter. The dead plants were all high-overexpressing events that performed well in the earlier greenhouse studies. Disease susceptibility was not compromised in any transgenic events over the field experiments (Baxter et al. 2015). These results highlight the importance of years of field research on transgenic plants with genes that regulate plant growth and development.

4.2 Determination of the most suitable extension area of new transgenic varieties by the regionalization test

The ultimate purpose of transgenic *P. simonii* × *nigra* is to obtain new varieties with drought resistance and salt tolerance and to then popularize them. The adaptability and stability analyses of transgenic lines were carried out by a regionalization test, which was the premise of popularizing new varieties. The Tai model is commonly used to analyze the adaptability of each line according to regression analysis. The results of the Tai model showed that XL-9 had the strongest adaptability and the best growth at the three experimental sites, followed by XL-7 and XL-13, and XL-4 and XL-10 had the worst adaptability. Therefore, the XL-9, XL-7, and XL-13 lines are

Table 8 Analysis of T-DNA insertion sites in transgenic lines

Line	Primer name	Site name	Insertion site	Designation of chromosome
XL-1	XL-1 Chr01	IS1	2,759,251 bp	Chr01
	XL-1 Chr03	IS2	11,710,668 bp	Chr03
	XL-1 Chr05	IS3	10,599,509 bp	Chr05
	XL-1 Chr09	IS4	7,653,220 bp	Chr09
	XL-1 Chr14	IS5	14,552,526 bp	Chr14
	XL-1 Chr16	IS6	9,294,838 bp	Chr16
XL-7	XL-7 Chr09	IS-XL-7	5,030,092 bp	Chr09
XL-9	XL-9 Chr05	IS-XL-9	6,685,809 bp	Chr05

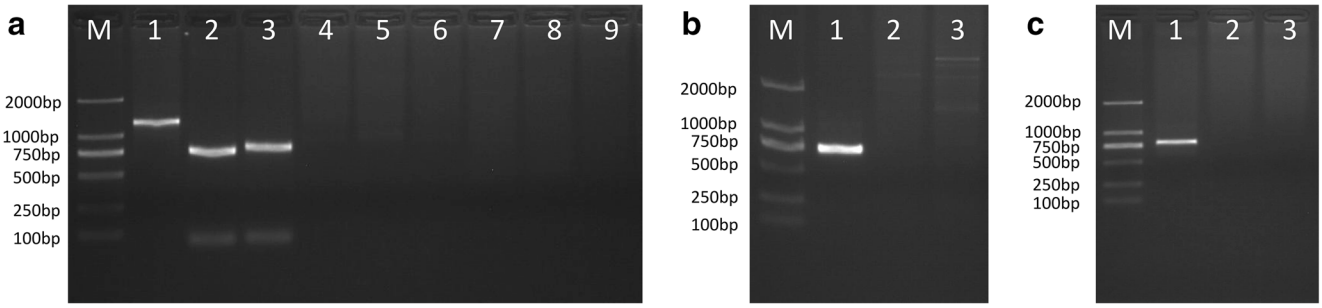

Fig. 6 Verification of T-DNA insertion sites for transgenic plants. (**a**) Line M: DNA ladder; lines 1–3: XL-1; lines 4–6: XL-7; lines 7–9: XL-9. Primer name: lines 1, 4, 7: XL-1 Chr01; lines 2, 5, 8: XL-1 Chr05; lines 3, 6, 9: XL-1 Chr09. (**b**) Line M: DNA ladder; line 1: XL-7; line 2: XL-1; line 3: XL-9. Primer name: XL-7 Chr09. (**c**) Line M: DNA ladder; line 1: XL-9; line 2: XL-1; line 3: XL-7. Primer name: XL-9 Chr05. The corresponding primer sequence can be found in Table 2 according to the primer name

the first choice for subsequent environmental release and productive trials.

4.3 The T-DNA insertion site can be used as a molecular feature of preferred transgenic lines

In the production and application of transgenic plants, the T-DNA insertion site is a label of each of the transgenic lines and an important molecular feature of the transgenic lines. The identification of T-DNA insertion sites provides technical support for the market supervision of transgenic lines after commercialization. In the past, thermal asymmetric interlaced (TAIL)-PCR, reverse PCR, exogenous junction-mediated PCR, and other techniques were often used to identify T-DNA insertion sites; however, these methods not only require complex and time-consuming processes, but also have high uncertainty, poor specificity, and a low success rate (Chen et al. 2010; Pu et al. 2016; Tsaftaris et al. 2010; Wang et al. 2011). In recent years, with the decrease in the time and cost of whole-genome resequencing, it is now being increasingly employed (Hu et al. 2016; Nordström et al. 2013). The T-DNA insertion sites of transgenic plants were identified by genomic resequencing, which has been used in *Arabidopsis thaliana*, soybean (*Glycine max*), and other transgenic plants and offers the advantages of simplicity, reliability, and high efficiency (Guo et al. 2016; Polko et al. 2012). Three transgenic poplar lines—XL-1, XL-7, and XL-9—with a potential popularization value were identified by this technique. Three T-DNA insertion sites were identified by the XL-1 transgenic lines, located on Chr01, Chr05, and Chr09, and one insertion site was identified in XL-7 and XL-9, which was located on Chr09 5,030,092 and Chr05 6,685,809, respectively. At present, the selected XL-1, XL-7, and XL-9 transgenic poplars have been approved with the administrative license for environmental release (Forest Technology Permit [2018] No. 6). The T-DNA insertion site of the selected lines can provide a reference for the subsequent productive testing of transgenic poplars.

5 Conclusions

Semi-quantitative PCR confirmed that *TaLEA* is still expressed after 9 years in the field. Additionally, three T-DNA insertion sites were identified on Chr01, Chr05, and Chr09 for XL-1 transgenic lines. One insertion site was found on Chr09 and Chr05 for XL-7 and XL-9, respectively. We confirm that preferred transgenic lines can be selected for adaptability 2 years after field experiments. These results will provide further guidance for testing of transgenic tree production.

Contributions of the co-authors Conceptualization: Jing Jiang. Methodology: Yang Wang, Jing Jiang. Software: Yang Wang, Chenrui Gu. Formal analysis: Yang Wang, Chu Wang. Investigation: Yunli Yang, Fusen Wang, Guangyu Wang, Wei Wang, Kun Chen. Data curation: Yang Wang. Writing – original draft: Yang Wang. Writing – review & editing: Qibin Yu, Jing Jiang.

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Data availability The datasets generated and/or analyzed during the current study are available in the Science Data Bank repository, https://doi.org/10.11922/sciencedb.00435

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

References

Baxter HL et al (2015) Field evaluation of transgenic switchgrass plants overexpressing *PvMYB4* for reduced biomass recalcitrance. Bioenerg Res 8:910–921. https://doi.org/10.1007/s12155-014-9570-1

Baxter HL et al (2018) Transgenic miR156 switchgrass in the field: growth, recalcitrance and rust susceptibility. Plant Biotechnol J 16: 39–49. https://doi.org/10.1111/pbi.12747

Chen L et al (2010) Isolation and heterologous transformation analysis of a pollen-specific promoter from wheat (*Triticum aestivum L.*). Mol Biol Rep 37:737–744. https://doi.org/10.1007/s11033-009-9582-7

Gang H, Li R, Zhao Y, Liu G, Chen S, Jiang J (2019a) Loss of GLK1 transcription factor function reveals new insights in chlorophyll biosynthesis and chloroplast development. J Exp Bot 70:3125–3138. https://doi.org/10.1093/jxb/erz128

Gang H, Liu G, Zhang M, Zhao Y, Jiang J, Chen S (2019b) Comprehensive characterization of T-DNA integration induced chromosomal rearrangement in a birch T-DNA mutant. BMC Genomics **20**. https://doi.org/10.1186/s12864-019-5636-y

Gao W, Bai S, Li Q, Gao C, Liu G, Li G, Tan F (2013) Overexpression of *TaLEA* gene from *Tamarix androssowii* improves salt and drought tolerance in transgenic poplar (*Populus simonii* × *P. nigra*). PLoS One 8:e67462. https://doi.org/10.1371/journal.pone.0067462

Guo B, Guo Y, Hong H, Qiu L (2016) Identification of Genomic Insertion and Flanking Sequence of G2-EPSPS and GAT Transgenes in Soybean Using Whole Genome Sequencing Method. Front Plant Sci 7 doi:https://doi.org/10.3389/fpls.2016.01009

He W, Zeng F, Chen X (2008) Stability of inheritance transferring in transgenic. Plant J Zhanjiang Normal College 29:57–61

Hu H et al (2016) GIPS: a software guide to sequencing-based direct gene cloning in forward genetics studies. Plant Physiol 170:1929–1934. https://doi.org/10.1104/pp.15.01327

Jouanin L et al (2000) Lignification in transgenic poplars with extremely reduced Caffeic acid O-methyltransferase Activity1. Plant Physiol 123:1363–1374

Li Z et al (2013) Variation and growth adaptability analysis of transgenic *Populus simonii* × *P. nigra* lines carrying *TaLEA* gene. J Beijing Forestry Univ 35:57–62

Li R, Chen S, Liu G, Han R, Jiang J (2017) Characterization and Identification of a woody lesion mimic mutant lmd, showing defence response and resistance to Alternaria alternate in birch Sci Rep-Uk 7 doi:https://doi.org/10.1038/s41598-017-11748-2

Liang C et al (2004) Construction of a BAC library of *Physcomitrella patens* and isolation of a *LEA* gene. Plant Sci 167:491–498. https://doi.org/10.1016/j.plantsci.2004.04.015

Liu G, Yang C, Cai Z, Cheng G, Zhang L (2006) Salt Tolerance of *betA* Transgenic *Populus simonii* × *P. nigra* and Selection for Superior Transgenic Plants Scientia Silvae Sinicae:33–36

Liu L, Liu F, Chen S, Lin L, Li H, Liu G, Jiang J (2013) *TaLEA* overexpression in *Populus simonii* × *P. nigra* dwarf mutants and their identification and analysis of insertion site flanking sequences journal of Beijing Forestry University 35:45-52

Liu P, Xu L, Ye J, Yang X (2014) Administration on the biosafety of agricultural GMOs in China. J Biosafety 23:297–300

Liu M et al (2015) Variation and genetic stability analyses of transgenic *TaLEA* poplar clones from four different sites in China. Euphytica 206:331–342. https://doi.org/10.1007/s10681-015-1471-7

Lu S, Yang N, Xiong T (2013) Advances in researches on LEA proteins and Lea genes. J Sichuan Forestry Sci Technol 34:26–28

Manfre AJ, Lanni LM, Marcotte WR (2006) The Arabidopsis group 1 LATE EMBRYOGENESIS ABUNDANT protein ATEM6 is required for Normal seed development. Plant Physiol 140:140–149. https://doi.org/10.1104/pp.105.072967

Meyer P, Heidmann I (1994) Epigenetic variants of a transgenic petunia line show hypermethylation in transgene DNA: an indication for specific recognition of foreign DNA in transgenic plants. Mol Gen Genet MGG 243:390–399. https://doi.org/10.1007/BF00280469

Mu H, Li Z, Li Y, Tang X, Jiang J, Yang C, Liu G (2009) Growth Manifestation of Transgenic *Populus simonii*×*P.nigra* with *betA* Gene on Low-grade Salinate. Fields J Northeast Forestry Univ

Nordström KJV et al (2013) Mutation identification by direct comparison of whole-genome sequencing data from mutant and wild-type individuals using k-mers. Nat Biotechnol 31:325–330. https://doi.org/10.1038/nbt.2515

Pasonen H, Vihervuori L, Seppänen S, Lyytikäinen-Saarenmaa P, Ylioja T, von Weissenberg K, Pappinen A (2008) Field performance of chitinase transgenic silver birch (*Betula pendula* Roth): growth and adaptive traits. Trees 22:413–421. https://doi.org/10.1007/s00468-007-0202-7

Pilate G et al (2002) Field and pulping performances of transgenic trees with altered lignification. Nat Biotechnol 20:607–612. https://doi.org/10.1038/nbt0602-607

Polko JK et al (2012) Illumina sequencing technology as a method of identifying T-DNA insertion loci in activation-tagged *Arabidopsis thaliana* plants. Mol Plant 5:948–950. https://doi.org/10.1093/mp/sss022

Pu Y, Guo C, Ping S (2016) Application Progress on amplified methods to clone flanking sequence. Biotechnol Bull 32:72–79

Shin YJ et al (2016) Characterization of *PyGUS* gene silencing in the red macroalga, *Pyropia yezoensis*. Plant Biotechnol Rep 10:359–367. https://doi.org/10.1007/s11816-016-0408-5

Sivamani E, Bahieldin A, Wraith JM, Al-Niemi T, Dyer WE, Ho TD, Qu R (2000) Improved biomass productivity and water use efficiency under water deficit conditions in transgenic wheat constitutively expressing the barley *HVA1* gene. Plant Sci 155:1–9. https://doi.org/10.1016/S0168-9452(99)00247-2

Srivastava V, Vasil V, Vasil IK (1996) Molecular characterization of the fate of transgenes in transformed wheat (*Triticum aestivum L.*). Theor Appl Genet 92:1031–1037. https://doi.org/10.1007/BF00224045

Sun Y (2014). The Analysis of salt and alkali Stress Resistence of Transgenic *Populus davidiana*×*P. bolleana* overexpresslng *TaLEA* gene. Master, Northeast Forestry University

Tai GCC (1971) Genotypic stability analysis and its application to potato regional trials. Crop Sci 11:184–190

Tsaftaris A, Pasentzis K, Argiriou A (2010) Rolling circle amplification of genomic templates for inverse PCR (RCA–GIP): a method for 5′- and 3′-genome walking without anchoring. Biotechnol Lett 32:157–161. https://doi.org/10.1007/s10529-009-0128-9

Wang M, Hua L (2018) LEA protein and its role in the process of crop resistance. J Northern Agricult 46:70–76

Wang N, Wang J, Zhang J, Zhang S (2004) Current Development of Transgenic Plants and the Segregation of Transgene in the Progenies of Transgenic Plants. Biotechnol Bull:13–17

Wang Y, Jiang J, Zhao X, Liu G, Yang C, Zhan L (2006) A novel *LEA* gene from *Tamarix androssowii* confers drought tolerance in transgenic tobacco. Plant Sci 171:655–662. https://doi.org/10.1016/j.plantsci.2006.06.011

Wang Z, Ye S, Li J, Zheng B, Bao M, Ning G (2011) Fusion primer and nested integrated PCR (*FPNI-PCR*): a new high-efficiency strategy

for rapid chromosome walking or flanking sequence cloning Bmc. Biotechnol 11:109. https://doi.org/10.1186/1472-6750-11-109

Wang H, Tian Y, Bai G, Qian Y, Chu K, Liu J (2013) Advances in influencing factors of genetic stability of transgenic plants. Guizhou Agricult Sci 41:5–8

Wang Y, Zhang Z, Wu J (2015) LEA protein and its application in improvement of stress tolerance in plants. Biotechnol Bull 31:1–9

Wang Y, Yang Y, Wang F, Wang G, Wang C, Wang W, Chen K, Gu C, Yu Q, Jiang J (2021) Growth adaptability and foreign gene stability analysis datasets of 9-year-old *TaLEA* transgenic *Populus simonii* × *nigra*. [dataset]. V2. Science Data Bank. https://doi.org/10.11922/sciencedb.00435. (2021-01-18)

Xu D, Duan X, Wang B, Hong B, Ho T, Wu R (1996) Expression of a late embryogenesis abundant protein gene, *HVA1*, from barley confers tolerance to water deficit and salt stress in transgenic Rice. Plant Physiol 110:249–257

Zegzouti H, Jones B, Marty C, Lelievre JM, Latche A, Pech JC, Bouzayen M (1997) ER5, a tomato cDNA encoding an ethylene-responsive LEA-like protein: characterization and expression in response to drought. ABA Wound Plant Mol Biol 35:847–854. https://doi.org/10.1023/a:1005860302313