Front. For. China 2008, 3(2): 226–231
DOI 10.1007/s11461-008-0029-4

RESEARCH ARTICLE

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Molecular detection and drought resistance analysis of *Sac*B-transgenic poplars (*Populus alba* × *P. glandulosa*)

Abstract The propagation of drought-resistant transgenic poplars is one of the more effective ways to improve the ecological state of arid regions. The expression of *Sac*B gene in transgenic lines of *Populus alba* × *P. glandulosa* was analyzed by a semi-quantitative RT-PCR. Drought resistance of these transgenic lines was evaluated by water stress trials in a greenhouse. Results showed that the mRNA transcription of *Sac*B was found in all transgenic lines and fructan has accumulated in the leaves of these plants. The growth, biomass and leaf water content of some transgenic lines were significantly higher than those of the control plants under drought stress. Correlation analysis indicated that the said factors were significantly and positively related to the concentration of fructan in the leaves of the transgenic lines. Results revealed that the expression of *Sac*B gene in transgenic poplars enhanced its resistance to water stress.

Keywords *Sac*B gene, *Populus alba* × *P. glandulosa*, transgenic, drought resistance

1 Introduction

Poplars are an economically important tree species with short rotations for industrial purposes. They play a significant role in water and soil conservation and maintenance of the environment. However, most poplar varieties cannot develop high yield potential due to their poor drought resistance. The development of genetic engineering has become an effective way to cultivate drought-resistant transgenic poplars within a short time.

Research on drought-resistance of transgenic plants has mainly focused on crops such as *Nicotiana tabacum*, *Medicago sativa* and *Oryza sativa* (Zhang and Shan, 1999). Poplar trees such as *P. tremula* (Wang et al., 1998), *P. tomentosa* (Wen et al., 1997), *Populus alba* × *P. glandulosa* (Zhang et al., 2005) and *P. alba* (Altman et al., 1996; Nicolescu et al., 1996; Arisi et al., 1998) have been mentioned in gene transfer research, but most of this work was limited to the laboratory stage. Results from greenhouse tests were rarely reported.

The *Sac*B gene, first obtained from the soil bacterium *Bacillus subtlis*, (Steinmetz et al., 1985; Steinmetz and Aymerich, 1986) can enhance plant drought resistance. It can encode levansucrase, a key enzyme in fructan synthesis, by catalyzing the transfer of a fructose base from one sucrose molecule to another, to compose a poly-3-fructan and one glucose molecule. Fructan is a polymer composed of sucrose and one or more fructoses. It is a water-soluble and non-reductive polysaccharide. It accounts for 15% of the important carbohydrates in angiosperms (Gramineae, Liliaceae, Compositae) (Hendry, 1993). The accumulation of fructan can adjust plants to adapt to low temperatures, high CO_2 concentrations, water deficits, and oxygen deficiency condition as well as regulate sucrose designations (Cheng et al., 2002). Capacity for drought-resistance in plants can be enhanced to a certain extent through fructan accumulation in plant cells by transgenic technology (Ebskamp et al., 1994; Van der Meer et al., 1994). *Sac*B gene has been reported to be transferred into *Nicotiana tabaccum* (Pilon-Smits et al., 1995; Zhang et al., 1998; Zhang et al., 2005), *Solanum tuberosum*, *Zea mays*, *Beta vulgaris*, *Lolium multiflorum*, *Trifolium repens* (Zhang et al., 2005), *Lycopersicon esculentum*) (Wang et al., 2004) and *P. alba* × *P. glandulosa*. Expression of *Sac*B gene in transgenic lines of *P. alba* × *P. glandulosa* was analyzed by semi-quantitative RT-PCR and fructan determination. Outcome of this study shows that the transcription of *Sac*B was detected in the transgenic plant lines and fructan buildup in the

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Translated from *Journal of Beijing Forestry University*, 2007, 29(2): 1–6 [译自: 北京林业大学学报]

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leaves was also found. Results of water stress trials in the greenhouse indicated that some transgenic lines were more resistant to water deficiency than the control plants. This report lays a foundation for the cultivation of drought-resistant transgenic poplars.

2 Materials and methods

2.1 Materials

Experimental materials consisted of transgenic lines of *P. alba* × *P. glandulosa*, which genome was proven to be integrated with a foreign *Sac*B gene through a Southern blotting test. Line numbers were as follows: S13–4, S13–9, S13–13, S13–23, S13–35, S13–27 and S13–85 with non-transgenic poplars as the control (CK).

2.2 Methods

2.2.1 RT-PCR molecular test

Total RNA extracted from the transgenic lines and control plants via Trizol, were treated with RQ1 RNase-free DNase (1 U/μL, Promega, USA) at 37°C for 15 min to get rid of DNA. The determination of RNA concentration by ultraviolet spectrophotometer (DU800) and RT-PCR (AccessQuick™ RT-PCR System Promega) was carried out with the *ubiquitin* gene as an inner reference. Primers P1 (5′-tgaggcttaggggaggaact-3′) and P2 (5′-tgtagtcgcgagctgtcttg-3′) were also used for the said determination. The primers of *Sac*B gene were P1 (5′-tggtcaggttcagccacattta-3′) and P2 (5′-gcaatcagcggtttcatcactt-3′), which were synthesized by the Beijing Baosheng Biotechnology Company. The product of the *ubiquitin* gene was 620 bp with RNA from *P. alba* × *P. glandulosa* as a negative control template. Reaction conditions were as follows: pre-denaturalization at 94°C for 5 min, then at 94°C for 1 min, at 58°C for 1 min and at 72°C for 1 min. The amplification went on for 30 cycles with the end of an extension at 72°C for 10 min. The product of the *Sac* gene was 512 bp. The negative template was RNA from *P. alba* × *P. glandulosa* and de-ionized water, while the positive template was pKP plasmid with the *Sac*B gene. Reaction conditions were the same as for *ubiquitin*. Separation of the reaction product was by means of gel electrophoresis of 0.8% agar.

2.2.2 Experimental treatment

The experiment was carried out in the Chinese Academy of Forestry greenhouse. Light transmittance into the greenhouse was excellent due to its glass structure. Air circulating in the greenhouse was refreshed through the clearstories of the roof. No actions were taken to control temperature and humidity. The temperature and relative humidity were taken twice a day from June to September. Mean temperature was 26°C at 8:00 and 33°C at 13:00 and the average relative humidity was 75% at 8:00 and 55% at 13:00.

A cutting of transgenic lines was planted on each garden pot (30 cm high and 24 cm in diameter) in mid-March, 2004. Six grams of the medium on each pot were mixed with filed soil, fine sand and peat soil (10:2:1, v/v). The size and quality of the transgenic line cuttings were kept the same across all lines. These stocks were cultivated under conditions of normal irrigation. The water control experiment was started when the stocks have grown to a height of 40 cm in early June. Two kinds of treatments were done, with a replication of nine pots for each transgenic line. The first treatment was drought stress (TR) and the other was normal irrigation (CK). During the entire growing period, stocks were watered under normal conditions to keep the soil moist with a relative moisture content of 45%–55%. This soil water content was determined by a heating and weighing method. Water supply was also quantitatively controlled to keep the said water content at 25%–35%. The stocks of the same treatment were irrigated by the same amount of water. The pots were shifted from time to time to eliminate small random variations in the environment. To ensure an adequate supply of nutrients, the stocks were fertilized once every two weeks.

2.2.3 Fructan extraction and determination

Functional leaves (the 7th–10th leaf from the top) of three transgenic lines and control were collected to determine the fructan content at the end of the experiment (July 20, 2004). Method of determination was as follows: 0.5 g leaves were grounded with 5 mL of distilled water, followed by a water bath at 80°C for 10 min; the supernatant was collected after centrifugation and the residues were extracted twice under similar conditions. The supernatants were merged for condensation by heating. Then, 1 to 3 mL of water was added to determine soluble sugar content. Due to the various molecular weights of monosaccharides, disaccharides and polysaccharides such as the three sugar, four sugar and five sugar, a sugar-pak-1 gel-type cation exchange column was used. This cation exchange column was produced by the Waters Company. Using resteamed water as the mobile phase (containing 0.1 mmol/L EDTA Na_2–Ca); free sugars were eluted in succession from big molecular weights to small ones. This has a flow rate of 0.7 mL/min and a column temperature of 90°C. Liquid chromatography was Hitachi 244 with Hitachi RI: 4 × refractive index detector. The content of DP3 and DP $\geqslant$ 4 fructans were determined by this method (Jiang et al., 2000).

2.2.4 Growth and biomass determination of transgenic lines and controls during the experimental period

The growth of transgenic lines and control plants was observed during the water control period. Before and after the water control test (dated June 5 and August 10), the plants' height and diameters at the root collar were measured twice using a ruler and Vernier caliper, respectively. Growth was obtained from the difference of the respective data before and after the tests.

On the basis of height and diameter, five medium-sized transgenic lines and control plants were washed and heated in an oven at 105°C for 15 min and then at 70°C for about 60 h until a constant weight was obtained. Dry matter was gauged by an electronic balance with 1/10000 accuracy. The average value was used for the end results. The cumulative value of the biomasses (g) was calculated from the weight loss during the water control period.

One functional leaf from three different medium-sized plants of every transgenic line from the treatment and from the control was collected on July 20.Their fresh weights were recorded. These leaves were then heated in an oven at 105°C for 15 min. These were heated further at 70°C until their weights remained constant. The relative water content was calculated as follows: leaf relative water content = (fresh weight − dry weight)/ fresh weight × 100%.

2.2.5 Statistical analysis

Multiple comparisons were performed using SPSS10.0 (for Windows).

3 Results and analysis

3.1 RT-PCR molecular test

RT-PCR results of transgenic lines are shown in Fig. 1. This indicates that one characteristic band of 512 bp was amplified in transgenic lines (4 to 10) and the plasmid pKP, without any corresponding amplified bands in the de-ionized water and control. Findings from three replications were similar and proved that the *Sac*B gene did transcript mRNA and was expressed variously in different lines, with high expression in S13–23, S13–35 and S13–72 and with low manifestation in S13–85, S13–4, S13–9 and S13–13. The amplified products of the gene-specific primers of *ubiquitin* by PCR were essentially consistent in the brightness of the bands, indicating that the initial concentration of RNA used was basically the same.

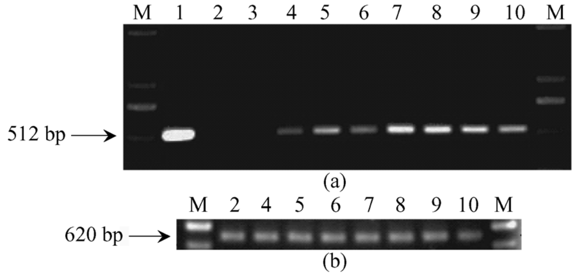

Fig. 1 RT-PCR analysis of transgenic lines
(a) *Sac*B gene; (b) *ubiquitin* internal standard gene. M: marker; 1: plasmid pKP; 2: non-transgenic plants; 3: de-ionized water; 4–10: transgenic lines S13–4, S13–9, S13–13, S13–23, S13–35, S13–72, S13–85.

3.2 Fructan content analysis

The fructan content of functional leaves from transgenic lines and the control was detected during the later part of the growing period. Results showed that fructan, the expressed product of exogenous *Sac*B in transgenic lines under the normal water supply state, had accumulated. For example, 0.228 mg of fructan had built up per gram of fresh weight of leaves of line S13–23, while fructan had not accumulated in the control plants. The fructan content was higher in transgenic lines S13–23, S13–35, S13–72 and S13–85 and lower in lines S13–9, S13–13 and S13–4 (Table 1). This result is consistent with that of a semi-quantitative RT-PCR, signifying that the *Sac*B gene has been successfully expressed in the transgenic lines.

Table 1 Comparison of fructan concentrations of transgenic plants and the non-transgenic controls (unit: $mg \cdot g^{-1}$)

treatment	S13–4	S13–9	S13–13	S13–23	S13–35	S13–72	S13–85	CK
normal irrigation	0.174	0.187	0.184	0.228	0.224	0.223	0.217	0
drought stress	0.213	0.294	0.215	0.879	0.789	0.507	0.441	0
increment	0.039	0.107	0.031	0.650	0.565	0.284	0.223	0

Compared with a normal water supply, fructan accumulation had increased in the transgenic lines under drought stress. The increase was higher in transgenic lines S13–23, S13–35, S13–72 and S13–85 and lower in lines S13–9, S13–4 and S13–13.

3.3 Growth and biomass analysis of transgenic lines during water control period

3.3.1 Growth analysis during water control period

The growth of transgenic lines and control plants was observed before and after water control (Fig. 2). All plants grew under the normal water supply setting without any significant change in external morphology. Drought stress inhibited the growth of these plants to varying degrees. The leaves of transgenic plant lines became thick and dark green. The growth of the control plants slowed

Fig. 2 Growth of transgenic plants
Three lines on the left: normal water supply; Three lines on the right: drought stress.

down significantly with the leaves turning yellow and shedding from the base (Fig. 2, right A).

The growth determination of the plants during water control presented that height and diameter of transgenic lines were higher than those of the control plants in the normal water supply condition. Findings of multiple comparisons demonstrated that there were statistically significant differences in height and diameter between S13–23, S13–35 and the control plants, but no significant difference between other transgenic lines and the control plants (Table 2).

Under the conditions of drought stress, plant growth significantly declined. The decrease in height of transgenic lines was lower than that of the control plants. Except for S13–4 and S13–9, the decrease in diameter of transgenic lines S13–13, S13–23, S13–35, S13–72 and S13–85 was lower than that of the control plants. Height and diameter of transgenic plant lines were higher than those of the control. Multiple comparison showed that height of S13–23, S13–35 and S13–72 were significantly dissimilar with the control plants. Results of the other lines were not significant. Compared with the control, the diameter of S13–23 was significantly different unlike the other lines (Table 2).

3.3.2 Biomass analysis during water control period

The stem and leaf biomasses of the plants during the water control period were also noted. Under normal water supply, the stem and leaf biomasses of transgenic lines were higher than that of the control plants. Results of multiple comparisons exhibited that stem biomasses of S13–9, S13–23, S13–35, S13–72 and S13–85 were significantly different from the control plants. The leaf biomasses of S13–23, S13–35, S13–72 and S13–85 were significantly different from that of the control (Table 3).

The accumulation of the dry matter of the stems and leaves was significantly decreased by drought stress in different ways. The dry biomasses of S13–23, S13–35,

Table 2 Growth of transgenic plants and non-transformed controls under different water treatments

lines	height/cm		diameter/cm	
	normal water supply	drought stress	normal water supply	drought stress
S13–4	133.50 ± 2.50ab	106.25 ± 3.71ab	0.54 ± 0.05ab	0.38 ± 0.01a
S13–9	136.00 ± 3.58abc	109.50 ± 2.90ab	0.56 ± 0.02ab	0.40 ± 0.01ab
S13–13	134.75 ± 4.75ab	108.00 ± 4.08ab	0.51 ± 0.02a	0.39 ± 0.01a
S13–23	148.75 ± 4.27c	126.50 ± 4.29c	0.63 ± 0.02b	0.47 ± 0.03b
S13–35	146.25 ± 4.71bc	123.25 ± 3.20c	0.62 ± 0.02b	0.45 ± 0.01ab
S13–72	140.25 ± 4.42abc	118.00 ± 5.71bc	0.58 ± 0.01ab	0.43 ± 0.03ab
S13–85	138.75 ± 4.03abc	111.25 ± 3.71ab	0.57 ± 0.04ab	0.42 ± 0.01ab
CK13	129.75 ± 4.87a	101.25 ± 3.47a	0.50 ± 0.11a	0.38 ± 0.02a

Note: *q*-tests were used for multiple comparisons with a significance level of 0.05. Same letters indicate that the difference was not significant and different letters mean that the difference was significant.

Table 3 Biomass and leaf water content of transgenic plants and the non-transgenic controls under different water treatments

lines	dry biomass/g		leaf biomass/g		leaf water content/%	
	normal irrigation	water stress	normal irrigation	water stress	normal irrigation	water stress
S13–4	15.98 ± 0.71ab	9.51 ± 0.68ab	14.65 ± 0.64a	12.90 ± 0.88b	66.28 ± 2.61ab	52.81 ± 4.49ab
S13–9	16.98 ± 0.95bc	10.06 ± 0.75abc	15.33 ± 0.91ab	13.82 ± 0.67bcd	67.14 ± 2.28ab	57.79 ± 4.58abc
S13–13	16.19 ± 0.79ab	9.73 ± 0.50abc	15.14 ± 0.91a	13.39 ± 1.18bc	67.38 ± 2.02ab	55.63 ± 3.15ab
S13–23	19.19 ± 0.12d	12.35 ± 0.51d	17.81 ± 0.59b	16.93 ± 0.78e	74.67 ± 1.81c	70.03 ± 1.53d
S13–35	18.40 ± 0.44cd	11.65 ± 0.55cd	17.72 ± 0.50b	15.75 ± 0.47de	72.07 ± 1.54bc	65.58 ± 1.22cd
S13–72	18.35 ± 0.25cd	11.37 ± 0.31bcd	17.64 ± 0.65b	15.32 ± 0.43cde	69.01 ± 1.64abc	61.42 ± 0.98bcd
S13–85	17.34 ± 0.74bcd	10.84 ± 0.59abcd	16.52 ± 0.98ab	14.59 ± 0.19bcd	68.00 ± 1.75ab	59.86 ± 1.65bc
CK13	14.68 ± 0.76a	9.06 ± 0.82a	14.11 ± 0.89a	10.60 ± 0.59a	64.86 ± 1.18a	49.31 ± 3.11a

S13–72 and S13–85 were lower than that of the control plants while the other lines' were slightly higher than that of the control. Leaf biomasses of transgenic lines were lower than that of the control. But dry biomasses of transgenic lines (still under drought stress conditions) were higher than those of the control plants. Multiple comparisons results showed that stem dry biomasses of lines S13–23, S13–35 and S13–72 were significantly different with the control. Compared with the control plants, leaf biomasses of transgenic lines were significantly different because the leaves from the base of the control turned yellow and shed (Table 3).

Leaf water content determination of the plants during water control period showed that water stress caused decline with the highest drop, 23.97% of the control, and the smallest drop, 6.21% of the control. The drop in leaf water content of transgenic lines was lower than that of the control plants. Multiple comparisons indicated that leaf water content of S13–23 and S13–35 were significantly different with that of the control under normal water supply. On the other hand, leaf water content of S13–23, S13–35, S13–72 and S13–85 were significantly different with that of the control under water stress (Table 3).

3.3.3 Correlation of growth, biomass, leaf water content and fructan content

Correlation analysis of height and diameter growth, biomass and leaf moisture content with fructan showed that growth, biomass and fructan content of the transgenic lines and control plant were positively correlated under normal water supply condition. These factors were also significantly correlated (Table 4) when the plants were subjected to drought stress. This showed that drought stress has a significant effect on plant growth, shoot dry matter accumulation and leaf water content.

Table 4 Correlation coefficients of fructan concentrations with growth, biomass and leaf water content in transgenic plants and non-transgenic plants under different water treatments

characteristics	correlation coefficient	
	normal irrigation	drought stress
height	0.7316^{*}	0.9874^{**}
diameter at root collar	0.7241^{*}	0.9895^{**}
dry biomass	0.8332^{*}	0.9759^{**}
leaf biomass	0.9341^{**}	0.9554^{**}
leaf water content	0.6502	0.9839^{**}

Note: * means significantly correlated at the 0.05 level, ** means significantly correlated at the 0.01 level. The number of samples was 4.

4 Discussion

It has been reported that fructan could be significantly amassed in *Sac*B gene-transferred tobacco and could significantly improve drought resistance in transgenic tobacco by osmotic adjustment (Ebskamp et al., 1994). Pilon-Smits et al. (1999) found that the *Sac*B gene-transferred tobacco showed no significant difference with the control under normal conditions; while under drought stress, fructan in *Sac*B gene-transferred tobacco was seven times higher than that of the control. The fresh weight was 33% higher and the dry weight 59% higher than that of the control. In particular, the root weight of transgenic tobacco increased 73% compared with the control's root weight. The gene transferred into potato leaves and tubers have resulted in a considerable accumulation of fructan, which changed the original carbon allocation pattern of potatoes (Van der Meer et al., 1994). Transgenic sugar beets grew better than the wild-type under drought stress conditions (Wang et al., 2004).

Semi-quantitative RT-PCR and fructan content determination indicated that the *Sac*B gene was expressed in the genome of *P. alba* × *P. glandulosa* at different levels. Fructan accumulated in *Sac*B transgenic poplars and transgenic lines grew normally under drought stress, while the growth of the control plants had been seriously affected through the manifestation of yellow leaves. Foliage at the base of the plants also shed due to necrosis. Followed by lines S13–85 and S13–9, all indices of S13–23, S13–35, S13–72 lines performed well, providing significant differences with the control. This was consistent with RT-PCR tests and the determination of the amount of fructan. Deterioration in growth, leaf biomass and leaf water content of transgenic plants were lower than those of the control plants, indicating higher adaptability to drought stress. In addition, growth, biomass, leaf water content of transgenic lines and control plants were significantly and positively correlated with the amount of fructan. These results showed that the exogenous gene *Sac*B increased transgenic poplar adaptability to water stress, with varying degrees of drought resistance. Differences in expression, product and target traits among various transgenic individuals are common phenomena. These are relevant to exogenous gene insertion sites, silencing and post-transcriptional regulation (Yang and Wang, 1995). Factors for the differences among the transgenic lines will be investigated in further studies.

If fructan fructose-transferase genes were transferred into fructan-accumulated plants, new types of this polymer would be generated. This would promote further accumulation and enhance plant adaptability to water stress (Sprenger et al., 1997; Vijn et al., 1997). Only a preliminary exploration of adaptability of transgenic lines to water stress was carried out in this study. Its actual capacity to drought stress needs to be tested further by field experiments. A drought trial in arid regions is now in progress.

Acknowledgements This study was supported by the Hi-Tech Research and Development Program of China ("863" Program) (No. 2006AA100109).

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