Tree Physiology 25, 1273–1281

Overexpression of *mtl*D gene in transgenic *Populus tomentosa* improves salt tolerance through accumulation of mannitol

LEI HU,[1–3] HAI LU,[1–3] QUNLU LIU,[1–4] XUEMEI CHEN[1,5] and XIANGNING JIANG[1,5]

[1] *The Tree and Ornamental Plant Breeding and Biotechnology Laboratory of State Forestry Administration, Beijing 100083, P.R. China*

[2] *College of Life Sciences and Biotechnology, Beijing Forestry University, Nr 35, Qinghua Donglu, Beijing 100083, P.R. China*

[3] *These authors contributed equally to this work*

[4] *The School of Agriculture and Biology of Science, Shanghai Jiaotong University, Shanghai 201101, P.R. China*

[5] *Corresponding authors (jiangxn@bjfu.edu.cn)*

Received April 5, 2004; accepted January 15, 2005; published online August 1, 2005

Summary The *mtl*D gene encoding mannitol-1-phosphate dehydrogenase, which catalyzes the biosynthesis of mannitol from fructose, was cloned from *Escherichia coli* and transferred to poplar (*Populus tomentosa* Carr.) through *Agrobacterium*-mediated transformation. The transgenic plants were screened and selected on Murashige and Skoog (MS) medium containing 30–50 mg l^{-1} kanamycin and verified by polymerase chain reaction (PCR) and Southern blotting. Expression of the gene led to synthesis and accumulation of mannitol in the transgenic plants. Gas chromatography and mass spectrometry (GC/MS) and capillary gas chromatography (GC) showed that transgenic plants accumulated much more mannitol in their tissues than the wild-type plants, whether cultured in vitro, or grown hydroponically or in the field.

Increased salt tolerance of transgenic plants was observed both in vitro and in hydroponic culture. The transgenic buds rooted normally on MS medium containing 50 mM NaCl, whereas wild-type buds did not. In the 40-day hydroponic experiments, transgenic poplar plants survived in a 75-mM NaCl treatment, whereas the wild-type poplar plants tolerated only 25 mM NaCl. Under the same NaCl stress, stomatal conductance, transpiration rates and photosynthetic rates were all higher in transgenic plants than in wild-type plants, whereas cellular relative conductivity was lower. We demonstrated that the *mtl*D gene was expressed in transgenic poplar plants, resulting either directly or indirectly in mannitol accumulation and improved salt tolerance. The constant mannitol concentrations in transgenic plants during the NaCl treatments indicated that mannitol accumulation caused by the *mtl*D gene was not a consequence of NaCl stress.

Height growth was reduced by about 50% in the transgenic plants compared with the wild-type plants in the absence of salt; however, relative growth rate was much less influenced by salt stress in transgenic plants than in wild-type plants. The stunted growth of the transgenic plants may in part explain their improved salt tolerance.

Keywords: GC/MS, physiological characteristics, slower growth, transformant.

Introduction

Arid and saline soils retard growth of most plants. Naturally salt- or drought-resistant plants are uncommon and usually adapted only to their natural saline or arid habitat. To remediate or afforest saline and drought-prone land, it will likely be necessary, therefore, to breed stress-tolerant plants. Plant genetic engineering and transformation provide effective means of improving stress-tolerant properties of plants (Borsani et al. 2003, Foolad 2004). Several metabolites, known as compatible solutes, play a critical role in plant stress tolerance. Typically, they are sugars, amino acids and inorganic acids (Hanson et al. 1994). Their biosynthetic pathways have been characterized and genes encoding enzymes in these pathways have been identified and used in some stress tolerance research (Zhu 2000, Cushman and Bohnert 2000), including the mannitol-1-phosphate dehydrogenase gene (*mtl*D), which results in mannitol accumulation in plant tissues.

Mannitol is a monosaccharide containing six hydroxyl groups. Initially, it was considered to be a cellular osmotic regulator (Ahdam 1979). Recently, it has been shown to have an important role in oxidation resistance. For reasons unknown, mannitol can also improve plant salt tolerance. Mannitol is a widely distributed sugar alcohol, reported in over 70 species of higher plants, including celery, olive and most rosaceous species (Rumpho et al. 1983, Loescher 1987). In most higher plants mannitol, if present, is found only in trace amounts (Zimmerman and Zeigler 1975), which are insufficient to contribute to salt or drought tolerance. The *mtl*D gene can be transferred to plants by genetic engineering to induce the accumulation of mannitol in plant tissues. The expression of a bacterial *mtl*D gene in tobacco (*Nicotiana tabacum* L.) plants results in the accumulation of mannitol (Tarczynski et al. 1992), resulting in improved salt tolerance (Tarczynski 1993). The *mtl*D gene has also been transferred to *Arabidopsis* where it enhances seed germination under high salinity conditions (Thomas et al. 1995). Recently, the *mtl*D gene has been engineered in crop plants. Eggplants (*Solanum melongena* L.) containing the *mtl*D gene survive in the presence of 200 mM NaCl

(Prabhavathi et al. 2002). Cellular accumulation of mannitol as a result of the expression of the *mtl*D gene protects transgenic wheat (*Triticum aestivum* L.) from the harmful effects of both soil waterlogging and salinity (Abebe et al. 2003). To date, the *mtl*D gene has been incorporated only in herbaceous plants. Our objective was to determine whether the *mtl*D gene can be incorporated into a species of the woody genus poplar, and if so, whether the transgenic plants have enhanced salt tolerance.

Materials and methods

*Cloning the mtl*D *gene and vector construction*

The open reading fragment (ORF) of the *mtl*D gene was cloned from *Escherichia coli* strain 13R141 by polymerase chain reaction (PCR) (Davis et al. 1988, Jiang et al. 1990). The PCRs were performed in a 50-µl reaction mixture containing 0.5 µg DNA template, 7 pmol of each primer, 5 µl 10× buffer, 1.5 mM $MgCl_2$, 100 µM of each dNTP and 5 U of DNA polymerase. The forward primer was 5′-GGTCTAGATGAAAG CATTACATTTTGG CG-3′ (the underlined sequence is the *Xba*I site). The reverse primer was 5′-CCGAGCTCCACCATT ATTGCATTGC-3′ (the underlined sequence is the *Sac*I site). The PCR mixture was incubated at 94 °C for 5 min, followed by 25 cycles of denaturation at 94 °C for 40 s, annealing at 65 °C for 40 s and extension at 72 °C for 1 min. The amplification was ended with extension at 72 °C for 10 min.

The PCR products were separated by agarose gel electrophoresis. The DNA fragment of 1.1 kb was recovered and inserted into the *Xba*I and *Sac*I restriction sites of the pUC18 vector to construct a pUM plasmid. After identification by sequencing, the ORF of *mtl*D was subcloned into the binary Ti vector pBI121 between the *Xba*I and *Sac*I sites to construct a fusion gene comprising the cauliflower mosaic virus 35S (CaMV35S) promoter, *mtl*D ORF and the *Agrobacterium tumefaciens* nopaline synthase (*nos*) gene transcriptional terminator. The resulting expression vector (pBM) was transformed into *A. tumefaciens* strain LBA4404 by the method of Höfgen and Willmitzer (1988).

Gene transformation and transgenic plants screening

The fusion gene was introduced into poplar plants by *Agrobacterium* infection. For co-cultivation, *A. tumefaciens* strain LBA4404 containing the expression vector was incubated in liquid YEB medium supplemented with 200 µM acetonesyringone at 28 °C with constant shaking (250 rpm) until the culture reached an optical density of about 0.6 at 600 nm. The *A. tumefaciens* culture was then diluted with one volume of liquid MS medium.

Leaves of *Populus tormentosa* Carr. excised from plantlets cultured in vitro were cut into discs and cultured in the dark for 2 days on co-cultivation medium (MS medium (pH 5.0) containing 2.0 mg l^{-1} 6-benzylaminopurine (6-BA), 0.05 mg l^{-1} 1-naphthaleneacetic acid (NAA), 200 µM acetonesyringone and 0.8% (w/v) agar) (Murashige and Skoog 1962). The leaf discs were then dipped in the diluted *Agrobacterium* culture for about 10 min and cultured on co-cultivation medium after the liquid on the surface of the leaf disc had been absorbed by sterilized paper. After co-cultivation in the dark for 2 days, the leaf discs were transferred to MS medium (pH 5.8) containing 2.0 mg l^{-1} 6-BA, 0.05 mg l^{-1} NAA, 300 mg l^{-1} Cefotaxime and 0.8% (w/v) agar and cultured in the light for 7 days to dispose of *Agrobacterium*. The explants were then subcultured on screening medium (MS medium (pH 5.8), 2.0 mg l^{-1} 6-BA, 0.05 mg l^{-1} NAA, 300 mg l^{-1} Cefotaxime, 30 mg l^{-1} kanamycin and 0.8% (w/v) agar) to induce adventitious buds.

The regenerated shoots were individually removed from the callus and transferred to flasks containing 40 ml of solid MS medium containing 2.0 mg l^{-1} 6-BA, 0.05 mg l^{-1} NAA, 50 mg l^{-1} kanamycin, 200 mg l^{-1} Cefotaxime and 0.8% (w/v) agar. Cultures were incubated at 25 °C, in a 16-h photoperiod at 120 µmol m^{-2} s^{-1}. Regenerated shoots were transferred to rooting medium (1/2 MS salts (pH 5.8) containing NAA 0.1 mg l^{-1}, sucrose 15 g l^{-1}, 50 mg l^{-1} kanamycin and 200 mg l^{-1} Cefotaxime). Regenerated plantlets were acclimatized in pots at 25 °C in a 16-h photoperiod and then transferred to the greenhouse.

Molecular verification

For PCR verification, the forward primer was 5′-ATCGGTCG TGGCTTTATCGG-3′ and the reverse primer was 5′-CTGCG ATCATCTTCACTGCG-3′. The DNA from wild-type plants was used as the negative control and the pUB plasmid as the positive control. The PCR was performed under the following conditions: denaturation at 94 °C for 40 s, annealing at 65 °C for 40 s, and extension at 72 °C for 1 min.

Southern blotting and Northern blotting of the target DNA fragment were carried out according to the protocol of the DIG High Primer DNA Labeling and Detection Starter Kit I (Roche Diagnostics, Mannheim, Germany).

Salt tolerance tests

Salt tolerance tests were performed in vitro and in hydroponic culture. In the tissue culture phase, wild-type plants and transformed plants were cultured in rooting medium containing NaCl (0, 25, 50, 75 or 100 mM) for 35 days. The wild-type plants served as the control. There were three replications for each treatment.

In the hydroponic experiments, the non-stressed rooted plantlets were transferred to containers filled with Hoagland solution after being cultured in the greenhouse for 3 weeks (Jones 1983, Resh 1995). After 2 weeks of acclimation to hydroponic conditions, the heights of the plants and the lengths and widths of their leaves were measured with a ruler. The plants were then subjected to NaCl stress for 40 days. Five salt treatments (0, 25, 50, 75 or 100 mM NaCl), each with three replications, were tested. The wild-type plants were used as controls.

Physiological measurements

Two weeks after the salt stress treatments in hydroponic culture, plant heights, leaf lengths and leaf widths were re-measured. Cellular relative conductivity, according to which injury in cellular membrane can be estimated, was determined with a

conductivity detector, and photosynthetic rate, transpiration or evaporation rate and stomatal conductance were measured with a CIRAS-2 Photosynthesis Instrument (PP Systems, Amesbury, MA).

Sampling

In vitro, non-stressed healthy plants were harvested to determine tissue mannitol concentrations. Plants were divided into leaves, stems and roots. Tissues were frozen in liquid nitrogen and stored at –70 °C until analyzed for mannitol.

In the hydroponic experiments, leaves at similar positions on each plant were collected every 5 days during the course of the NaCl treatments and stored at –70 °C until analyzed. At the end of the experiment, leaves, stems and roots of plants in the NaCl treatments were destructively harvested and analyzed for mannitol.

Leaves of the 1-year-old transgenic and wild-type plants growing in the field were collected to determine their soluble carbohydrate composition. The third or fourth leaf on each plant was chosen for analysis.

Extraction and pre-purification of mannitol

Between 20 and 200 mg of fresh tissue from wild-type and transgenic plants was ground in liquid nitrogen with 5 mg of internal standard (ribitol). Soluble carbohydrates, including mannitol, were extracted as described by Tarczynski (1992). The soluble carbohydrates were acetylated in the following procedures. Hydroxylamine hydrochloride was employed as the oximization reagent, acetic anhydride as the acetylation reagent, and 1-methylimidazole as the solvent and catalyst (Chen and McGinnis 1981, Hu et al. 2004). After adding 0.1 ml of hydroxylamine hydrochloride to the extraction, the reaction tube was incubated at 80 °C for 5 min. Then 0.15 ml of acetic anhydride was added, and 5 min later, 1 ml of chloroform was added to extract the carbohydrate derivatives. The aqueous phase was discarded. Water-soluble impurities in the chloroform phase were eliminated with three or more washes each with 1 ml of distilled and deionized water. The final derivative solution was dried with anhydrous sodium sulfate and stored in a closed capillary for analysis by gas chromatography/mass spectrometry (GC/MS) or capillary gas chromatography.

Separation, detection and identification of mannitol by GC/MS analysis

The acetyl-derivatized extracts were separated in a BP-15 capillary column (30 m × 0.25 mm × 0.25 µm) attached to a Finnigan TRACE GC, and detected and identified with a Finnigan VOYAGER GC/MS. The initial temperature of the column was 50 °C. After injection (1 µl; injector at 250 °C), the column was heated progressively at a rate of 20 °C min^{-1} to 200 °C, then at a rate of 10 °C min^{-1} to 280 °C. The column was kept at 280 °C for 10 min. The flow rate of the carrier gas helium was 0.8 ml min^{-1}. Temperature of the MS detector was 300 °C. Ribitol, mannitol, sorbitol, glucose, sucrose and trehalose were used as standards to determine retention times.

Capillary GC analysis

After identification by GC/MS, the derivatized samples were quantified by capillary GC analysis with a Varian CP-3800 gas chromatograph. The gas chromatograph was equipped with a glass column (capillary BP-15 column, 30 m × 0.25 mm × 0.25 µm) and a flame-ionization detector (300 °C). The initial temperature of the column was 50 °C. After injection (1 µl; injector at 250 °C), the column was heated progressively at a rate of 15 °C min^{-1} to 220 °C and kept at 220 °C for 3 min, followed by a rate of 25 °C min^{-1} to 280 °C. The column was kept at 280 °C for 10 min. Retention times of components from the GC column were determined as total ion currency (TIC) with an MS detector. Mannitol and other carbohydrates were quantified by comparison with the internal standard (ribitol). Chromatogram profiles were integrated and the data were processed with the Agilent Chromatography Star Software Version 6.0 (Agilent Technologies, Palo Alto, CA).

Results

Gene cloning and transformations

The PCR product was 1.15 kb and had a similar sequence to that of the *mtl*D gene (Davis et al. 1988, Jiang et al. 1990) deposited in GenBank (X06794, U03845). An *Xba*I site and a *Sac*I site were found at the 5′ and 3′ ends, respectively. The fu-

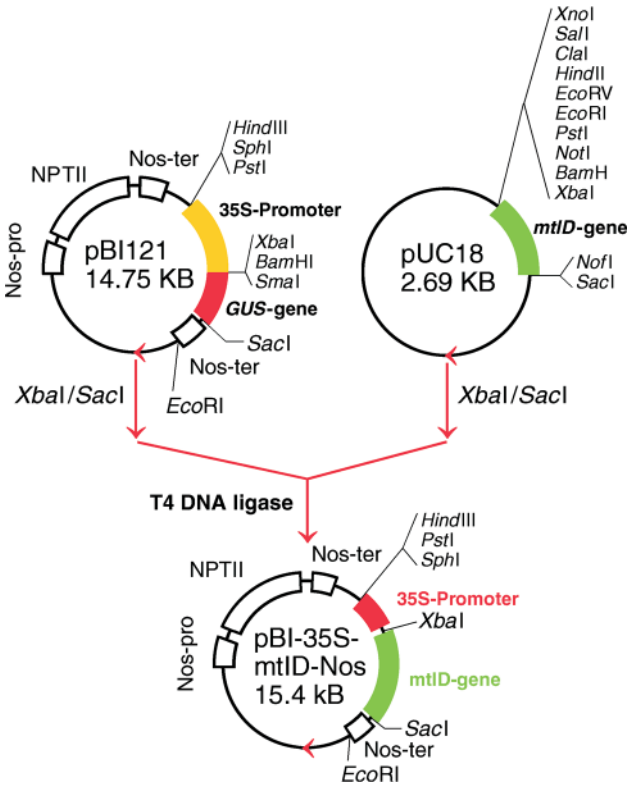

Figure 1. Procedure for constructing the expression vector (pBM) using the pBI121 binary vector as the master vector. The *GUS* gene between the *Xba*I and *Sac*I restriction sites was replaced by the *mtl*D gene.

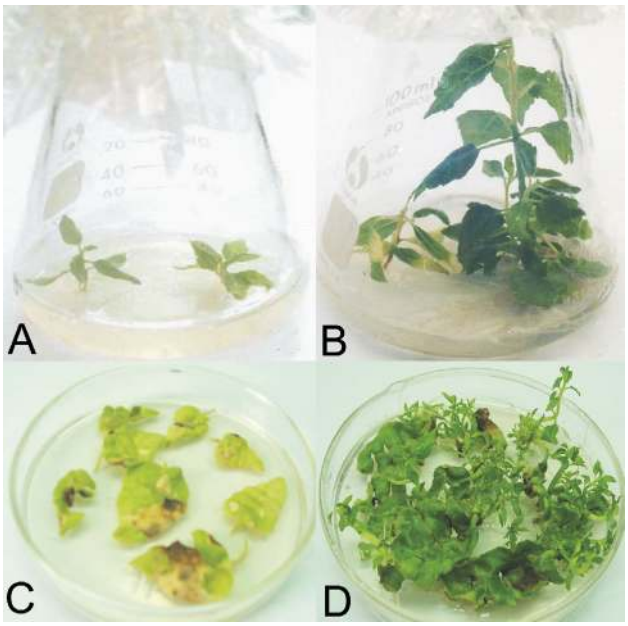

Figure 2. Wild-type (A and B left) and transgenic (A and B right) poplar shoots were cultured in the same flask containing 40 ml of rooting medium supplemented with 50 mg l^{-1} of kanamycin (A = Day 0 and B = Day 40). Leaves excised from wild-type (C) and transgenic (D) poplar plants were cultured for 30 days in the presence of 50 mg l^{-1} kanamycin on a medium inducing adventitious buds.

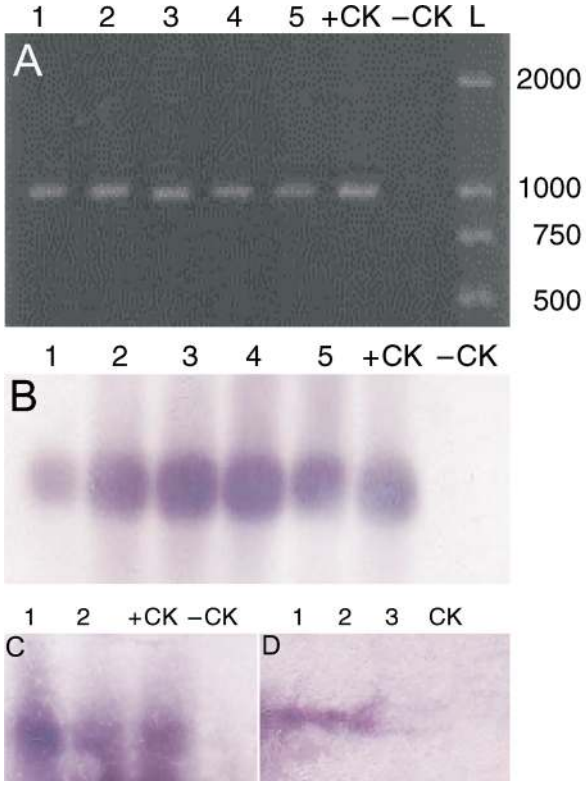

Figure 3. The transgenic poplar plantlets containing the *mtl*D gene were verified by PCR (A), PCR-Southern blotting (B), Southern blotting (C) and Northern blotting (D). In PCR, PCR-Southern blotting and Southern blotting, the pBM plasmid was the positive control (+CK) and wild-type poplar plants were the negative control (−CK), and Lanes 1–5 were the transgenic lines. In the Northern blotting, RNA extracted from wild-type poplar plants was used as the control (CK). Lane L is the kb ladder.

sion gene was constructed (Figure 1) and transferred to poplar by *Agrobacterium*-mediated transformation. The regenerated shoots were screened on MS medium containing 30–50 mg l^{-1} kanamycin. Some regenerated shoots grew normally in the screening medium, whereas the wild-type plants withered (Figures 2A and 2B) after 40 days. On the screening medium, leaves excised from the kanamycin-resistant shoots regenerated many adventitious buds, whereas leaves from wild-type plants died (Figures 2C and 2D). The results of PCR, PCR-Southern blotting, Southern blotting and Northern blotting (Figure 3) indicated that the kanamycin-resistant fusion gene was inserted into the genome of the poplar plants and overexpressed.

Five of 19 lines were found to contain the *mtl*D gene. The transgenic lines were derived from two transformation events, and more than twenty plants of the five transgenic lines were obtained. The transgenic plants were cultured in vitro and two independent lines were used in subsequent experiments.

Salt resistance of the transgenic plants

In vitro, the transgenic shoots rooted and grew normally in the rooting medium containing 50 mM NaCl, whereas the wild-type shoots did not root and wilted after 35 days (Figure 4). The transgenic buds also survived exposure to 75 and 100 mM NaCl, even though rooting was strongly inhibited in the 75 mM NaCl treatment and totally inhibited in the 100 mM NaCl treatment. Unexpectedly, in the absence of NaCl, transgenic plants rooted earlier in vitro than wild-type plants.

Transgenic plants also exhibited greater salt tolerance under hydroponic conditions than wild-type plants. The wild-type plants wilted after 15 days of exposure to 75 mM NaCl (Figure 5B) or 3 days in 100 mM NaCl, whereas the transgenic plants survived for the duration of the 40-day experiment in all NaCl treatments except for the 100 mM NaCl treatment.

Growth and physiological properties of transgenic plants

In hydroponic culture in the absence of NaCl, the height of transgenic plants was reduced by 50% compared with the wild-type plants. The length and width of leaves of transgenic plants were only half of the length and width of leaves of wild-type plants (Figure 5A, Table 1). These morphological differences were also observed during in vitro culture in the absence of NaCl but were less distinct.

Height growth of the wild-type poplar plants was severely retarded by NaCl. The plants stopped growing in the 50 mM NaCl treatment and growth was retarded by 50% in the 25 mM NaCl treatment. In contrast, the influence of NaCl on transgenic plants was not significant in either the 25 or the 50 mM NaCl treatments. However, height growth rate and leaf expan-

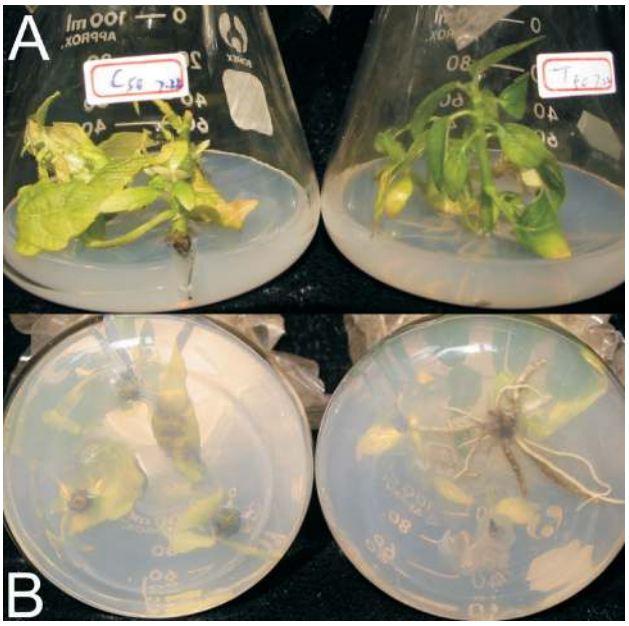

Figure 4. Transgenic and wild-type poplar shoots were cultured in vitro on rooting medium containing 50 mM NaCl for 35 days. The transgenic shoots grew well (A, right) and rooted normally (B, right), whereas the wild-type plants wilted (A, left) and did not form roots (B, left).

sion of the transgenic plants were retarded by nearly 60% in the 75 mM NaCl treatment (Table 2).

Salt stress decreased cellular relative conductivity by 1 to 2.5 times in transgenic plants compared with wild-type plants (Figure 6A). Compared with wild-type plants, stomatal conductance of transgenic plants was 2 to 3 times higher (Figure 6B); transpiration rate of the transgenic plants was 1 to 2.5 times higher (Figure 6C); and net photosynthetic rate was 1 to 2 times higher (Figure 6D). The higher the NaCl concentration, the greater the difference between the wild-type and transgenic plants in these physiological characteristics.

Analysis of mannitol

Mannitol was identified by GC/MS in both transgenic and wild-type plants (Figures 7A and 7B). Only a trace of mannitol was detected in leaf samples of wild-type plants cultured in vitro (Figure 7C), whereas substantial concentrations of mannitol were found in leaves of the transgenic plants, and glucose and sucrose concentrations were greatly reduced (Figure 7D). Capillary GC analyses showed that more mannitol was present in roots than in leaves and stems (Table 3).

Tissue mannitol concentrations were higher in hydroponi-

Table 1. Plant height, leaf length and leaf width of wild-type and transgenic poplar plants. Wild-type and transgenic buds of the same size were grown in vitro in rooting medium. Forty days later, the plantlets were acclimatized in a greenhouse for 3 weeks in a hydroponic system containing MS solution. The acclimatized plantlets were grown in Hoagland solution for 2 weeks before measurement of plant height, leaf length and leaf width. Mean values ± SE were calculated from six independent samples.

Plant	Height (mm)	Leaf length (mm)	Leaf width (mm)
Wild-type	216.2 ± 6.3	52.5 ± 6.3	47.1 ± 5.7
Transgenic	111.8 ± 12.0	27.1 ± 1.6	22.8 ± 3.1

Table 2. Plant height, leaf length and leaf width of control and salt-stressed wild-type and transgenic poplar plants. Wild-type and transgenic buds of the same size were grown in vitro in rooting medium. Forty days later, the plantlets were acclimatized in a greenhouse for 3 weeks in a hydroponic system. The acclimatized plantlets were grown in Hoagland solution and 0, 25, 50 or 75 mM NaCl for 2 weeks before measurement of plant height, leaf length and leaf width. Most wild-type plants died several days after being subjected to the 75-mM NaCl treatment. Mean values ± SE were calculated from three or more independent samples.

NaCl (mM)	Plant	Height (mm)	Leaf length (mm)	Leaf width (mm)
0	Wild-type	246.0 ± 5.3	75.0 ± 3.0	64.0 ± 3.6
	Transgenic	126.6 ± 10.4	36.3 ± 3.5	32.3 ± 2.8
25	Wild-type	231.0 ± 7.5	62.7 ± 8.0	53.0 ± 5.0
	Transgenic	123.7 ± 6.1	34.3 ± 3.5	29.7 ± 3.0
50	Wild-type	216.3 ± 5.7	53.7 ± 2.5	44.3 ± 1.5
	Transgenic	122.6 ± 4.0	33.7 ± 3.8	28.7 ± 3.8
75	Transgenic	115.3 ± 7.5	30.7 ± 2.5	25.7 ± 2.1

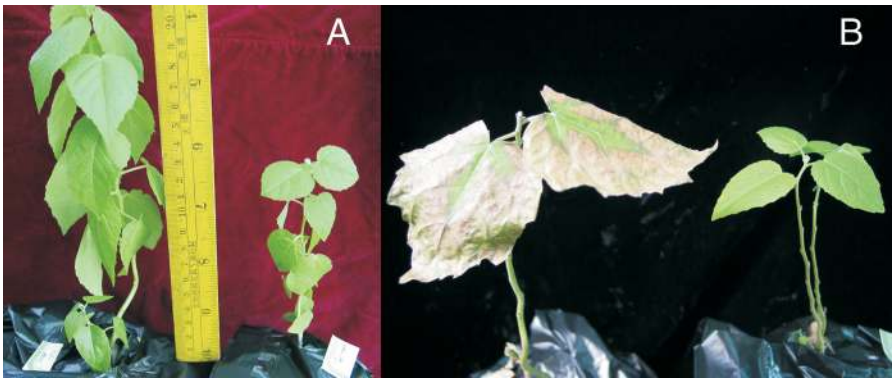

Figure 5. Transgenic and wild-type poplar plants were cultured hydroponically in Hoagland solution (A) and in Hoagland solution containing NaCl (B) for 6 weeks. (A) The growth rate of transgenic poplar plants (right) was retarded compared with that of wild-type plants (left). (B) In the 75-mM NaCl treatment, the transgenic plants survived (right), whereas the wild-type plants wilted after 15 days (left). The leaves on the lower parts of plants were sampled for mannitol analysis.

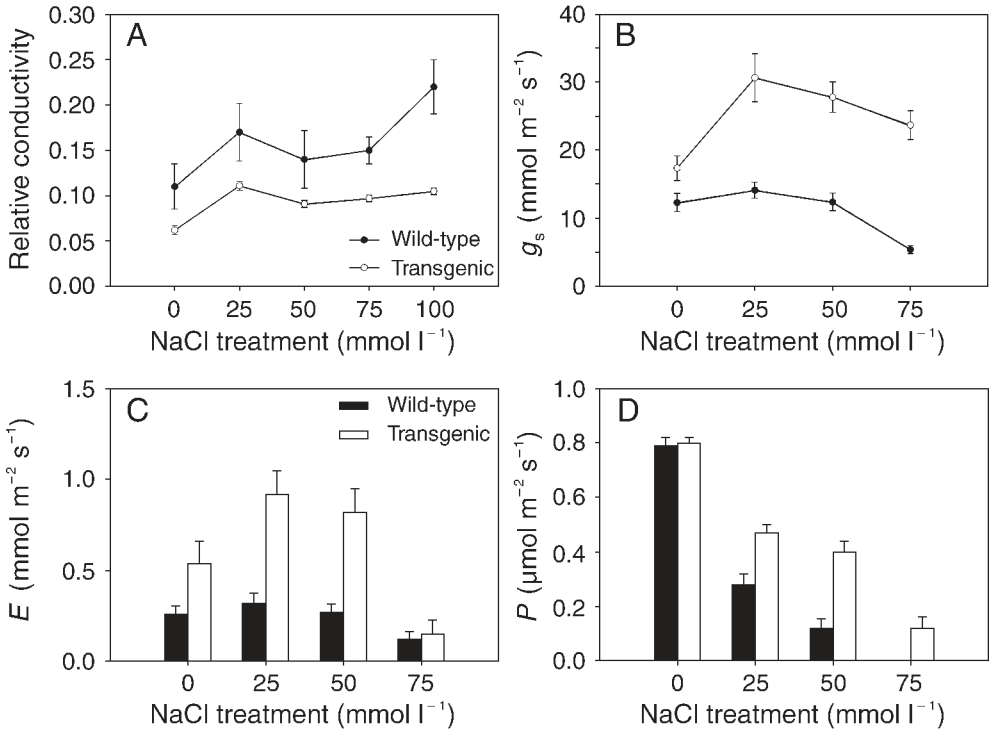

Figure 6. Effects of salt stress on the physiological traits of transgenic and wild-type poplar plants. Relative conductivity (A), stomatal conductance (g_s; B), transpiration rate (E; C) and photosynthetic rate (P; D) of transgenic and wild-type poplar plants were determined 3 weeks after subjecting the plants to five NaCl treatments.

cally cultured transgenic plants than in tissue-cultured transgenic plants. However, there was no change in mannitol concentration in the transgenic plants in response to NaCl treatments (Table 4).

Field-grown transgenic plants accumulated greater concentrations of mannitol in leaves than either tissue-cultured or hydroponically-grown transgenic plants. Foliar mannitol concentration of field-grown transgenic plants was 0.26–0.42 mg g^{-1} of fresh mass, 10× higher than in tissue-cultured transgenic plants, but the high mannitol concentration was not associated with reductions in glucose and sucrose concentrations. As observed in tissue-cultured wild-type plants, mannitol concentrations of field-grown wild-type plants were negligible (data not shown).

Discussion

Based on our molecular verification tests, salt tolerance tests and carbohydrate analysis, we conclude that expression of the fused *mtl*D gene improves the salt tolerance of transgenic

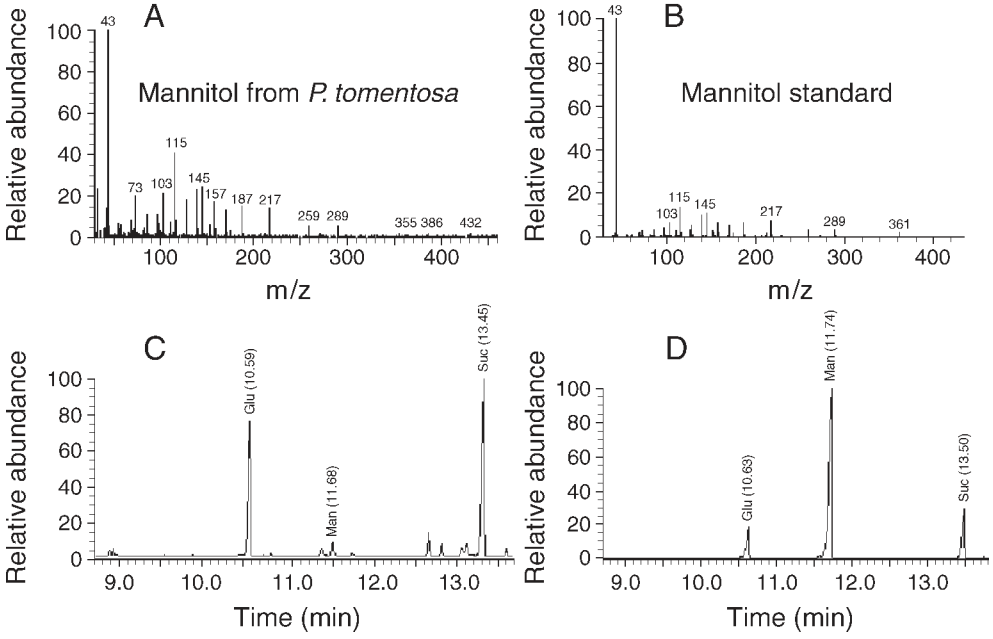

Figure 7. Mass spectra of mannitol extracts from poplar plants (A) and the mannitol standard (B) obtained by gas chromatography and mass spectrometry. The total ion currency from 9 to 13.8 min of derivatives of soluble carbohydrates in wild-type (C) and transgenic (D) poplar plants. For the two plant types, the mass of fresh tissue (0.2 g), the extraction and derivation method and injection volume (1 µl) were identical.

Table 3. Mannitol, glucose and sucrose concentrations (mg g^{-1} fresh mass) in leaves, stem and roots of transgenic and wild-type poplar plants cultured in vitro and in a hydroponic system containing Hoagland solution. Mannitol, glucose and sucrose concentrations were determined by capillary gas chromatography. Mean values ± SE were calculated from three or more independent samples.

Culture conditions	Plant	Mannitol in leaf	Mannitol in stem	Mannitol in root	Glucose in root	Sucrose in root
In vitro	Wild-type	0.0008 ± 0.0005	0.0014 ± 0.0008	0.0071 ± 0.0031	0.664 ± 0.266	0.497 ± 0.187
	Transgenic	0.003 ± 0.001	0.036 ± 0.014	0.216 ± 0.031	0.124 ± 0.073	0.038 ± 0.003
Hydroponic	Wild-type	0.004 ± 0.003	0.022 ± 0.009	0.097 ± 0.048	0.467 ± 0.066	0.591 ± 0.093
	Transgenic	0.035 ± 0.010	0.055 ± 0.029	0.273 ± 0.118	0.261 ± 0.016	0.346 ± 0.055

poplar plants through the elevation of mannitol concentrations in plant tissues. Similar results have been obtained in research with herbaceous plants (see our Introduction and Karakas et al. 1997). However, the NaCl concentration that the transgenic poplar plants tolerated was far lower than that tolerated by transgenic tobacco and eggplant. Partly, the difference may reflect differences in method of salt treatment. Adding NaCl progressively (Karakas et al. 1997) results in improved salt tolerance. In addition, differences in developmental stages may partly account for the discrepancy. At each stage of development, salt tolerance is largely controlled by a few quantitative trait loci (QTLs) with major effects and several QTLs with smaller effects (Foolad 2004), leading to different responses to stress at different developmental stages (Thomas et al. 1992). Thus the effect of the *mtl*D gene on salt tolerance of transgenic poplar may differ with plant developmental stage. Our finding that mannitol concentrations in transgenic poplar differ according to culture conditions indicates that the expression of *mtl*D gene is influenced by environmental factors or stage of plant development. However, the expression of the gene was unaffected by NaCl stress because mannitol concentrations of the transgenic plants were similar regardless of the concentration of NaCl in the growth medium.

The mechanism underlying the increase in salt tolerance of our transgenic poplar plants was not identified. It is not known if the introduction of the *mtl*D gene contributes directly or indirectly to the increased salt tolerance through an accumulation of mannitol. However, the large difference in plant tissue mannitol concentrations between transgenic and wild-type plants suggests that mannitol plays a major role in increasing salt tolerance. Mannitol functions as an osmolyte in some plant cells; however, it made no significant contribution to cellular osmotic adjustment in transgenic tobacco (Karakas et al. 1997). Mannitol also plays a role in oxidative stress protection. A bacterial mannitol-1-phosphate dehydrogenase gene was targeted to tobacco chloroplasts and the resistance to oxidative stress in transgenic tobacco was improved, evidently as a result of mannitol accumulation (Shen et al. 1997*a*). Further studies showed that mannitol might function as a free radical scavenger, helping plants resist oxidative stress by preventing hydroxyl radicals from inactivating specific enzymes (Shen et al. 1997*b*). In our poplar plants, less electrolyte was released from transgenic plant tissues than from wild-type plants under the same NaCl stress (Figure 4A), indicating that the transgenic plants were better able than wild-type plants to maintain cell membrane integrity under salt stress, which supports the hypothesis that mannitol serves a protective function.

The mannitol molecule contains many water-like groups (Loescher 1987), which enable mannitol to participate in water-enforced hydrophobic interactions or to combine with high-molecular-weight compounds in biological membranes and thus maintain their biological activity. The cell membrane is critical to the viability of plant cells and its integrity is essential for normal cell metabolism. The higher photosynthetic rates, transpiration rates and stomatal conductances of transgenic poplars compared with wild-type plants may reflect enhanced membrane integrity as a result of oxidative stress protection by mannitol.

A specific and unexpected characteristic of the transgenic poplar plants was their slower growth compared with the wild-type poplar plants. Transformation with the *mtl*D gene reduced the growth of non-stressed plants by about 50% in the hydroponic experiments and it also reduced growth of the tissue-cultured transgenic plants. This result is consistent with the response of tobacco to transformation with the *mtl*D gene (Karakas et al. 1997). Plants with high stress resistance gener-

Table 4. Mannitol concentrations (mg g^{-1} fresh mass) in leaves of transgenic poplar plants during hydroponic culture for 40 days in Hoagland solution containing 0, 25, 50 or 75 mM NaCl. Leaves were collected from each plant during the course of the NaCl treatments. Mean values ± SE were calculated from three or more independent samples.

NaCl treatment (mM)	Before treatment (Day 0)	Day 10	Day 20	Day 30	Day 40
0	0.039 ± 0.002	0.039 ± 0.002	0.038 ± 0.002	0.039 ± 0.002	0.039 ± 0.003
25	0.036 ± 0.008	0.038 ± 0.004	0.037 ± 0.004	0.036 ± 0.008	0.037 ± 0.004
50	0.037 ± 0.009	0.037 ± 0.007	0.037 ± 0.005	0.036 ± 0.004	0.037 ± 0.006
75	0.039 ± 0.003	0.038 ± 0.004	0.039 ± 0.003	0.039 ± 0.000	0.039 ± 0.001

ally have a dwarf form (Xiong and Zhu 2002), which can reduce water and energy consumption and facilitate energy redistribution. Thus, the inherently slower growth of the transgenic plants might alone be the cause of the improved salt tolerance. This suggestion is supported by the finding that plants sprayed with the growth retardant paclobutrazol show enhanced salt tolerance (Karakas et al. 1997 and references therein).

We do not know why our transgenic poplar plants were dwarfed, but there are several possible explanations. The fast-growing and large roots of the transgenic plants (data not shown) indicate that the *mtl*D gene induced an increase in the root:shoot ratio, which may account for the dwarfism of the transgenic plants. Alternatively, the *mtl*D gene may have disturbed carbohydrate metabolism as occurs in transgenic tobacco containing the *gut*D gene (Sheveleva et al. 1998). The low concentrations of glucose and sucrose in tissue-cultured transgenic poplar plants may result in decreased energy available for other metabolic processes, thus leading to reduced biosynthesis and growth.

Another possible reason for the slow growth of *mtl*D-transformed poplar may be the use of the selective marker gene, *NPT* II. However, this seems unlikely because the growth rate was unaffected by transformation with empty vector containing the *NPT* II marker gene (Dale and McPartlan 1992, Arnoldo et al. 1992, Tarczynski et al. 1993).

Stunted growth occurs in plants transformed with trehalose synthetase (*ost*A) gene or *TPS*1 gene when these genes are driven by the CaMV35S promoter (Goddijn et al. 1997, Romero et al. 1997), but not when other promoters are used (Holmström et al. 1996, Jaglo-Ottosen et al. 1998, Kasuga et al. 1999). These studies provide evidence therefore that the CaMV35S promoter may have been one of the causes of dwarfing in our transgenic plants (Kasuga et al. 1999).

Additional information about the mechanism underlying the lower growth of transgenic poplar compared with wild-type plants is needed before we can understand the roles of the *mtl*D gene and ultimately engineer woody plants able to withstand salt stress and meet industrial needs.

Acknowledgments

The work was jointly supported by the National Grand Basic Fundamental Research "973" Project (G1999016005) to Dr. Xiangning Jiang and National Natural Science Foundation Project (Grant No. 30271066) to Xuemei Chen. The research work was carried out at the Tree and Ornamental Plant Breeding and Biotechnology Laboratory of State Forestry Administration, P.R. China. The authors are grateful to Dr. Mosseller Alexander and Dr. Bruce Pendrel of the Canadian Forest Service for editing drafts, and thank Huajun Chen, Xiangwang Zou and Qing Li for their technical assistance.

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