Research paper

Efficient *Agrobacterium*-mediated Transformation of *Populus nigra x Populus tomentosa* Expressing the *MtlD* Gene Improves Salt Tolerance

Kuan-Hung Lin,[1] Min-Yi Lin,[2,4] Ta-Chung Wang,[3] Yan-San Wu[3]

【Summary】

Bacterial mannitol-1-phosphate dehydrogenase (*mtlD*) is known for its tolerance to salinity. Previous work on a number of transgenics having *mtlD* established the role of mannitol accumulation in alleviating salt stress. *Populus* species are extensively planted for landscaping, biomass production, and maintenance of the global environment due to their rapid growth and ease of reforestation. However, salt stress tremendously limits these trees' survivability, particularly within saline areas. The objective of this study was to use *Agrobacterium tumefaciens* harboring the *mtlD* gene to infect *Populus nigra x P. tomentosa* hybrids and produce salt-resistant transgenic plants using a callus organogenesis system. Our results show that all transformants presented an expected fragment of the *mtlD* transgene of 1149 bp. Significantly higher rates of callus induction, shooting, and rooting in poplar explants were observed in an MS-cultured medium containing 1 mgL^{-1} thidiazuron and 0.1 mgL^{-1} naphthaleneacetic acid compared to other of combinations plant growth regulators. The optimal infection time for *A. tumefaciens* to infect explants was 8 min, and 300 ppm of cefotaxime was the best concentration for eliminating *A. tumefaciens*. Survival and rooting rates in transgenic plants under 50, 85, and 120 mM NaCl were both significantly higher than in non-transgenic (NT) plants. In addition, transgenic poplar plants grew faster than NT plants subjected to 120 mM NaCl, as revealed by better growth, longer shoots, and greater plant heights.

Key words: *Agrobacterium tumefaciens*, mannitol-1-phosphate dehydrogenase *Populus*, salt stress.

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研究報告

歐洲黑楊X毛白楊藉農桿菌轉殖*mtl*D基因提高其耐鹽性

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摘 要

甘露醇-1-磷酸脫氫酶(*mtl*D基因)是可提高耐鹽性的基因。早期研究已對*mtl*D基因轉殖發現甘露醇積累對鹽害具緩解作用。楊樹品種廣泛，其具快速生長、造林栽植的園林綠化、生質能的生產和維護全球環境等特點。然而，鹽害影響了這些樹的生存能力，特別是在鹽鹼地區。本研究目的擬利用帶有*mtl*D基因之農桿腫瘤菌感染歐洲黑楊X毛白楊之雜交種，生產具抗鹽鹼能力的轉殖株。試驗結果顯示，所有轉殖株皆帶有1149 bp之*mtl*D基因片段。楊樹培殖體發現在含1 mg/L TDZ和0.1 mgL^{-1} NAA之MS培養基，比其它植物生長調節劑的組合，其癒傷組織、不定芽、不定根誘導率較高。較佳農桿菌感染培植體時間為8分鐘，300 ppm的抗生素是抑制農桿菌之較佳濃度。基因轉植株在50、85和120 mM之氯化鈉逆境下，其存活和發根率均明顯高於對照組植物。此外，轉基因楊樹在120 mM之氯化鈉影響下，其生長速度、芽體高度都較對照組好。

關鍵詞：農桿腫瘤菌、甘露醇-1-磷酸脫氫酶、楊樹、鹽害。

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INTRODUCTION

Abiotic stresses like soil salinity are considered to be predominant factors determining the global geographic distribution of vegetation and restrictions of crop yields in agriculture. Approximately 20% of the world's irrigated land is affected by salinity (Munns 2005). Salinity occurs due to low rainfall and high soil water evaporation rates, causing groundwater to transport soluble salts to the topsoil at levels that damage crops and adversely impact the economic welfare of farmers. Symptoms of salinity injury include the breakdown of chlorophyll, protein degradation, decreasedmembrane permeability, peroxidation, slower leaf expansion, petiole epinasty, abscisic acid accumulation, stomatal closure, declines in photosynthesis, and increased photorespiration (Hoque et al. 2007). Plants also suffer from composite stresses caused by salinity, including altered nutrient uptake, accumulation of toxic ions, disruption and water homeostasis, osmotic stress, and oxidative stress (Wang et al. 2008). Salt tolerance can be achieved by several adaptive mechanisms involving multiple processes, including physiological changes, osmotic adjustment, and avoidance of ion toxicity during salt stress. Research has proven that one of the best methods for abiotic stress tolerance is osmoregulation, especially when osmoregulatory genes are trigged in reaction to salinity (Rontein et al. 2002). Many crops do not have a sufficient ability to synthesize osmoprotectants; therefore, the metabolic engineering of osmoprotectant biosynthesis pathways is an efficient way to enhance abiotic stress tolerance (Motallebi and Rahnama 2011).

The mannitol-1-phosphate dehydrogenase (*mtlD*, EC 1.1.1.17) enzyme, encoded by the bacterial *mtlD* gene, is involved in mannitol metabolismand the biosynthesis of osmolytes, and reversibly converts fructose-6-phosphate to mannitol-1-phosphate (Davis et al. 1988). Mannitol is a 6-carbon non-cyclic sugar alcohol that is widely distributed in nature and synthesized in bacteria, fungi, algae, and lichens, and in more than 100 species of vascular plants (Iwamoto and Shiraiwa 2005). Mannitol in higher plants is commonly present at low levels that are inadequate to contribute to abiotic stress tolerance (Zimmerman and Zeigler 1975). Mannitol plays an important role in stress tolerance. Under abiotic stresses, plants can accumulate mannitol, which is regulated by inhibiting mannitol-competing pathways and reducing the consumption and catabolism of mannitol (Khare et al. 2010, Chan et al. 2011). Early studies by Tarczynski et al. (1993), Sheveleva et al. (1997), and Karakas et al. (1997) found that tobacco plants with accumulated mannitol had an increased ability to tolerate high salinity. Improved morphological and physiological characteristics associated with salinity tolerance were observed in several species expressing thebacterial *mtlD* gene (Huizhong et al. 2000, Abebe et al. 2003, Tang et al. 2005, Maheswari et al. 2010, Motallebi and Rahnama 2011, Rahnama et al. 2011, Askari and Pepoyan 2012, Sticklen et al. 2013, Bhauso et al. 2014). Stress-induced genes not only protect cells from abiotic stresses through the production of important enzymes and metabolic proteins, but also regulate signal transduction and gene expressions during stress responses (Rabbani et al. 2003). Although the roles of mannitol in alleviating salinity and osmotic-induced stresses are known for other plants, our current understanding is that the *mtlD* gene expressed in transgenic poplar plants resulted either directly or indirectly in mannitol accumulation and improved salt tolerance (Hu et al. 2005).

Populus is a genus of deciduous trees in the Salicaceae family that grow in temperate zones and is the first woody perennial to gain recognition as a model for worldwide tree-breeding programs. Poplars have several advantages as model forest trees, including rapid growth, prolific sexual reproduction, ease of cloning, relatively small genomes, facile transgenesis, and tight coupling between physiological traits and biomass productivity (Bradshaw et al. 2000, Brunner et al. 2004). To date, many nations face soil salinity and desertification problems that seriously impact agricultural and silvicultural production. The accumulation of salts in irrigated soils is a primary factor depressing forest production because most forest trees are almost universally non-halophytic **(**Volkmar et al. 1998). Trees that thrive in hypersaline environments possess specific mechanisms to adjust their internal osmotic status by accumulating low -molecular-weight organic compatible solutes such as sugars, some amino acids, and quaternary ammonium compounds, which are believed to be essential for the adaptability of plant cells to high salinity (Tang et al. 2001). *Populus* spp. are cultivated in a wide range of environments, among which *P. nigra x P. tomentosa* is one of the most widely grown, but salt stress tremendously limits its survivability, particularly within saline areas. To increase its production for economic benefits, it is necessary to identify the genetic determinants and underly-ing mechanisms controlling tolerance to soil salinity. There is great demand for salt-tolerant *Populus* species with improved properties that can take advantage of less-expensive, more- robust, and cost-effective logistical processes for potential industrial applications. Successful

attempts have recently been made in transferring genes involved in salt stress tolerance into trees, with the resulting transgenic trees exhibiting enhanced tolerance to salt stress. The functions of *mtlD* gene expression in *Populus* species under salinity are limited. Therefore, the aim of the current study was to assess the salt-stress (NaCl-mediated) tolerance of the *mtlD* gene (from *Escherichia coli*) by overexpressing *mtlD* cDNA in *P. nigra x P. tomentosa* via an *Agrobacterium*-mediated transformation and micro-propagation system. Our hypothesis was that the overexpression of *mtlD* in a transgenic poplar hybrid would induce mannitol accumulation and significantly elevate salt tolerance during seedling growth and plant development in comparison to non-transgenic (NT) plants under salinity stress. Characterization of the *mtlD* gene can provide supporting evidence for the actions of such mechanisms in the salt tolerance of transgenic trees and facilitate our understanding of the salt-response mechanism in *Populus.* To our knowledge, this is the first report of *mtlD* transgenic characterization for salt stress tolerance in *P. nigra x P. tomentosa*.

MATERIALS AND METHODS

Plant materials

Poplar plants (*P. nigra x P. tomentosa*) were obtained as gifts from the Taiwan Poplar Green Energy Co. (Taipei, Taiwan). After prelimilary tests of leaf stalks, leaf blades, ribbed leaves, and nodal segments with axillary buds, the induction rate of nodal segments with axillary buds was better, so follow-up tests used them. Explants were sterilized with 2% (v/v) mercuric chloride and 2 drops of Tween-20 for 5 min, followed by immersion in 70% ethanol for 1 min, and then rinsing with distilled-deionized H_2O.

Amplification of *mtlD* complementary (c) DNA

Total RNA was isolated from 0.1 g of leaves with a Qiagen RNeasy Plant Mini Kit (Valencia, CA, USA) and then poly $(A)^+$ messenger (m) RNA was extracted from total RNA with a Qiagen Oligotex Mini Kit according to the vendor's instructions. First-strand cDNA was synthesized from 1 μg of total RNA using RETEROscriptreverse transcription for the reverse-transcription polymerase chain reaction (RT-PCR) with an oligo (dT) primer according to the manufacturer's instructions (Ambion, city, TX, USA). Database searches for the *mtlD* gene were performed at the National Center of Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/gene/948117) with Entrez, BLAST of Gene Bank accession number NC_000913.3. Paired degenerated primers (*mtlD* 5F-ATTTCATTTGGAGAGAACACGG and *mtlD* 3R-TTATTGCATTGCTTTATAAGC) were used for amplification (http://biocyc.org/ECOLI/sequence?object=EG10616). Amplification of the salt-resistant *mtlD* gene was based on a previous study (Gaxiola et al. 2001). Briefly, a PCR was carried out in an Eppendorf Mastercycler Gradient Thermal Cycler (compary, Hamburg, Germany) with the following thermal program: initial denaturation at 92℃ for 5 min, followed by 35 cycles at 92℃ for 45 s, 50℃ for 45 s, and 72℃ for 50 s, with a final extension at 72℃ for 7 min. The products were electrophoretically separated on 1.5% agarose gels and the predicted size of 1149 bp was verified with a 1000-bp DNA marker (BM 2000 DNA Marker, Rainbow Biotechnology, city, Taiwan).

Cloning of the *mtlD* gene from poplar plants and plasmid construction

The RT-PCR of the *mtlD* gene from poplar plants displayed an expected band of 1149

bp, and products were cloned into the pUC18 vector (Genomics BioSci & Tech, Taipei, Taiwan), followed by transfer into competent DH5α cells. Blue and white screening was then used to identify transformant colonies. After plasmid DNA extraction, PCR amplification of transformed colonies was carried out, and the *mtlD* gene was confirmed by DNA sequencing (Tri-I Biotech, Taipei, Taiwan). *Escherichia coli* strain DH5α (Yi-Shan Biotech, Taipei, Taiwan) and pCAMBIA1302 (Invitrogen, Carlsbad, CA, USA) were respectively used for gene construction and transformation. *Populus mtlD* was ligated to the EcoRI/PstI site of the pCAMBIA expression vector and fused between cauliflower mosaic virus 35S RNA (CaMV35S) promoter and kanamycin coding sequences. The *mtlD* gene was driven by the CaMV35S promoter. The constructed plasmid was named pCAMBIA1302-*mtlD* (Fig. 1).

Agrobacterium-mediated transformation and callus organogenesis

A freeze-thaw method (Wise et al. 2006) was used to transform plasmids into *A. tumefaciens* strain LBA4404, and *Agrobacterium*-competent cells for transformation were prepared according to Jyothishwaran et al. (2007). PCR amplifications of the *mtlD* gene were performed following plasmid extraction. The *Agrobacterium*-containing plasmid, pCAMBIA1302-*mtlD*, was used for *Populus* transformation (Tarczynski 1992). Briefly, LBA4404 was maintained on YEP medium composed of yeast extract (10 g/L), peptone (10 gL^{-1}), NaCl (5 gL^{-1}), and agar (15 gL^{-1}). A single bacterial colony was inoculated into 20 mL of liquid YEP medium in a 125- mL culture flask. The culture was placed on a rotary shaker (170 rpm) and held at 28℃ until the cell optical density (OD_{600nm}) was between 0.6 and 0.8. The bacterial suspension was cen-

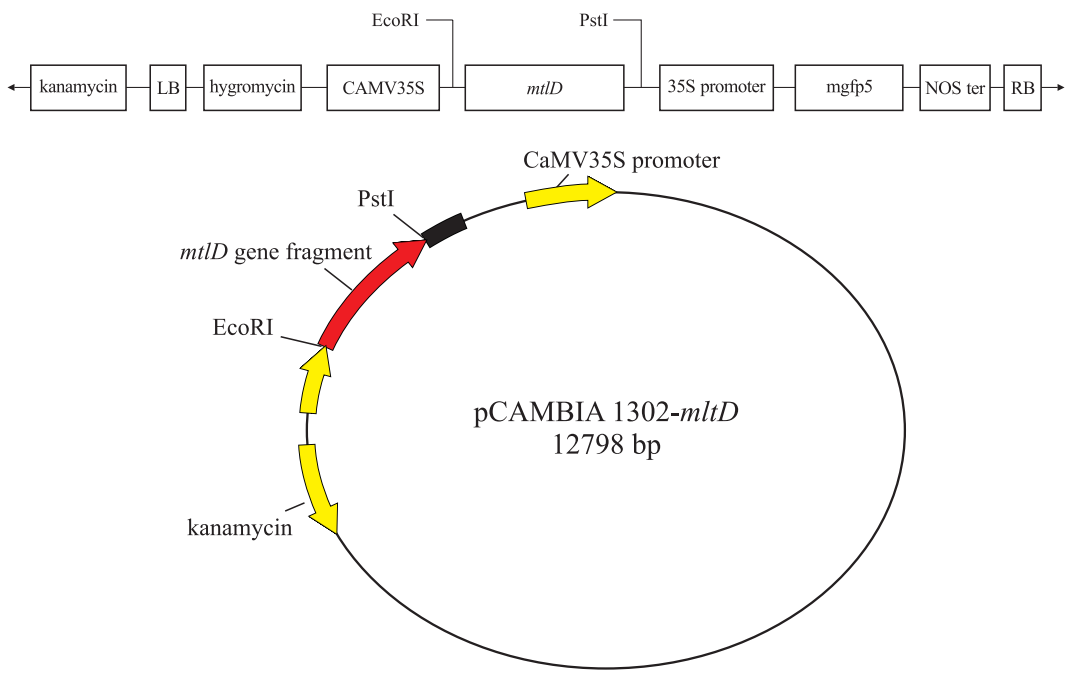

Fig. 1. Construction of the pUC18-*mtlD* expression vector (upper panel) and vector map of pCAMBIA 1302-*mtlD* (lower panel).

trifuged at 5000 rpm for 5 min. The obtained pellet was resuspended in MS (Murashige and Skoog 1962) liquid medium containing 100 μM of acetosyringone (AS). During co-cultivation, the poplar explants were placed separately in conical flasks containing *A. tumefaciens* and cultured in an MS liquid medium containing AS. Flasks were placed in the dark at 28℃ and centrifuged at 170 rpm for 5, 8, 10, 20, 30, 60, or 120 min. Explants were then removed, drained of excess bacterial broth with sterilized filter paper, and transferred to MS solid media containing AS. Explants were cultured at 28℃ in the dark for 2 d, cleansed with sterilized water, and transferred to MS solid media containing 100, 200, or 300 ppm of cefotaxime for 1 wk.

After eliminating LBA4404, explants were cultured on screening MS solid media with different combinations of plant growth regulators (PGRs) and kanamycin (30 mgL^{-1}) to induce calli, buds, and adventitious roots. Subsequently, explants were continuously subcultured every 15 d for 1 mo. Five PGR combinations were used: [Treatment 1] 1.2 mgL^{-1} 6-benzyladenine (BA) + 0.1 mg/L naphthaleneacetic acid (NAA), [Treatment 2] 1 mgL^{-1} BA + 0.1 mg/L NAA, [Treatment 3] 0.5 mg/L BA + 0.1 mgL^{-1} NAA, [Treatment 4] 1 mgL^{-1} thidiazuron (TDZ) + 0.1 mgL^{-1} NAA , and [Treatment 5] 0.5 mgL^{-1} TDZ + 0.1 mgL^{-1} NAA. Induction of calli, buds, roots, andregenerated explants was carried out according to a procedure and method altered from Tang et al. (2001) that transformed *Pinus taeda* with *A. tumefaciens* and then cultured it on differentiation medium to grow adventitious shoots directly on primary explants. All chemicals and reagents were purchased from Sigma-Aldrich (St Louis, MO, USA).

Genomic PCR analysis

Genomic DNA was extracted from 2-mo-old explants (buds) of transgenic plants and NT plants (controls). Plant samples were ground to a fine powder with a mortar and pestle in liquid nitrogen and DNA was prepared essentially as described by the manufacturer's protocols for the Qiagen DNeasy Plant Mini Kit. A PCR analysis was performed using the above-mentioned program and specific primers (*mtlD* 5F and 3R) to amplify the *mtlD* gene. PCR products were electrophoretically separated on a 1.5% agarose gel, and the predicted size of 1149 bp was verified with a 1000- bp DNA marker.

Transgenic plant survival and rooting rates, and plant growth under stress

Salt tolerance procedures were modified from Hu et al. (2005). Both transgenic and NT plantlets were subcultured in rooting media (MS + 0.3 mgL^{-1} indole butyric acid + 30 mgL^{-1} kanamycin) containing 5 different concentrations (0, 50, 85, 120, and 170 mM) of NaCl, and kept in a growth chamber under a 16- h photoperiod (100 μE m^{-2} s^{-1} white fluorescent light) at 25/20℃ (day/night) temperatures for 30 d. The number that survived and continued to grow was then counted. In addition, rooting rates of transgenic and NT plantlets were also measured. Each experiment was performed with 3 replicates. All surviving and rooted transgenic plantlets were transferred from 50- ml Erlenmeyer flasks into pots containing a perlite/vermiculite/peat moss (1:1:1 v/v/v) soil mixture, and kept in a growth chamber under the above-described conditions for recovery. Plants were watered 3 times a week and acclimatized under greenhouse conditions for 30 d. Transgenic and NT plants were then subjected to 120 mM NaCl solution for 20 d in the optimal environment of a greenhouse. Plant growth was observed, and photographs were taken at the indicated times.

Statistical analysis

Callus induction, shooting, rooting, and survival rates are presented as the mean values of 3 independent sets of experiments with a minimum of 30 explants per treatment. Generalized linear model (GLM) factor-analysis patterns were determined using SPSS software (SPSS, Chicago, IL, USA). An analysis of variance (ANOVA) was used to evaluate differences among transgenic lines for the treatments tested. Means were compared by Duncan's multiple range test at the level of $p \leq 0.05$. In addition, 10 seedlings of each NT and transgenic plant under salt stress were assessed for survival and rooting rates. Data are presented as the mean values of 2 independent sets of experiments with similar results. Statistical significance was calculated by a paired, two-tailed Student's *t*-test. A value of $p \leq 0.05$ indicated statistical significance.

RESULTS

Effects of infection time and cefotaxime tests of *A. tumefaciens* on the survival rate of explants

Leaf explants were infected with *A. tumefaciens* during cultivation at 5~120 min, followed by using 100~300 mgL^{-1} of cefotaxime for 7 d to eliminate *A. tumefaciens*. Explants were then cultured in MS medium containing 1 mgL^{-1} BA, 0.1 mgL^{-1} NAA, and 30 mgL^{-1} kanamycin for 30 d. Survival rates of tissue culture-induced explants at 5, 8, 10, 20, 30, 60, and 120 min were 15, 31.1, 24.4, 8.2, 4.3, 2.2, and 0%, respectively (Fig. 2A). This suggests that the best cultivation time for explants infected with bacteria was 8 min. Furthermore, the highest survival rate in the cefotaxime test for eliminating bacteria after co-cultivation for 8 min was 31.1% at 300 ppm of cefotaxime, followed by 27.0 and 15.0% at 200 and 100 ppm of cefotaxime, respectively (Fig. 2B). Notably, no significant differences in survival rates were observed in explants from leaf stalks, leaf blades, ribbed leaves, and stems with buds for either infection time or cefotaxime concentration (data not shown). Therefore, for the subsequent micropropagation experiments using 5 different PRGs in MS media, *A. tumefaciens* was used to infect explants with costa or stalks for 8 min, and 300 ppm of cefotaxime was used to eliminate *A. tumefaciens*.

Effects of various PGRs on explant induction

Plantlets were subcultured on MS medium with PGRs and kanamycin for 60 d and explant inductions are listed in Table 1. Induction rates of calli in treatments 1~5 were 70.63, 46.67, 37.62, 78.89, and 27.30%, respectively (Fig. 3A). The highest (38.73%) and lowest (8.41%) induction rates in shoots and buds were observed in treatments 4 and 3, respectively (Fig. 3B). Moreover, treatment 4 also displayed a significantly higher adventitious rooting rate (34.13%) compared to other treatments, ranging 31.59~16.19% (Fig. 3C). These results demonstrate that treatment 4 (1 mgL^{-1} TDZ and 0.1 mgL^{-1} NAA) was the best combination for inducing calli, shoots, and roots.

Identification of transgenic plants

LBA4404 the pUC18 containing binary vector carrying the *mtlD* gene was transferred to plantlets. To examine the presence of *mtlD* transgenes, 3 healthy, robust, independent transgenic poplar explants cultured on MS medium with TDZ and NAA (treatment 4) were selected and then detected by a DNA-PCR amplification and electrophoretic analysis. The DNA from NT plants was also isolated and subjected to a PCR analysis, followed by electrophoresis. All of the tested transformants displayed the expected band of

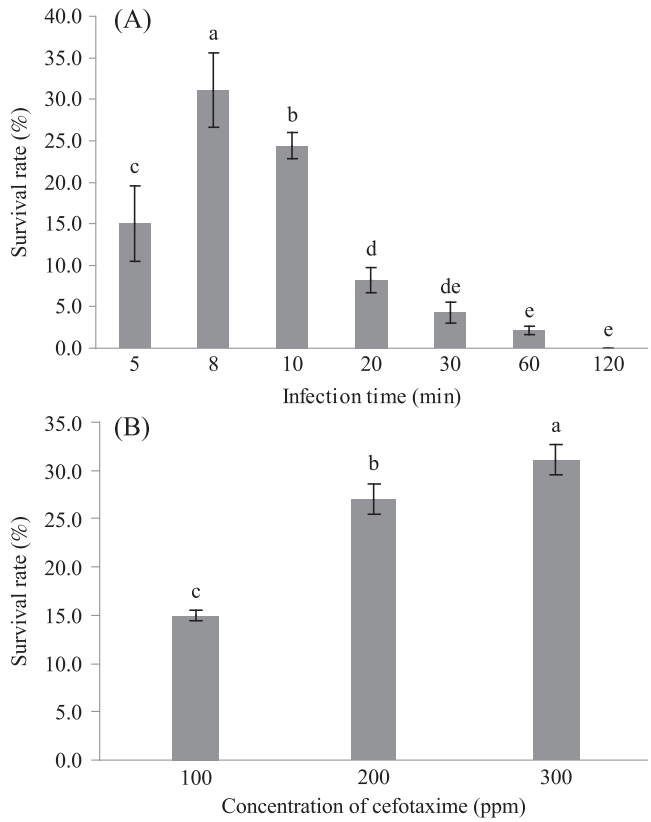

Fig. 2. Survival rates based on the infection time (A) and cefotaxime level (B) of *Agrobacterium tumefaciens* in tested explants. Values are the means of 3 replicates with the corresponding standard error (error bars). Means with the same letters do not significantly differ by Duncan's test at $p \leq 0.05$.

Table 1. Treatment of plant growth regulator combinations on induction rates of poplar explants

Treatment	Plant growth regulators	Callus induction rate (%)	Shooting rate (%)	Rooting rate (%)
1	BA 1.2 mgL^{-1} NAA 0.1 mgL^{-1}	70.63[a]	24.29[b]	31.59[b]
2	BA 1 mgL^{-1} NAA 0.1 mgL^{-1}	46.67[b]	18.10[bc]	22.22[c]
3	BA 0.5 mgL^{-1} NAA 0.1 mgL^{-1}	37.62[c]	8.41[d]	13.17[cd]
4	TDZ 1 mgL^{-1} NAA 0.1 mgL^{-1}	78.89[a]	38.73[a]	34.13[a]
5	TDZ 0.5 mgL^{-1} NAA 0.1 mgL^{-1}	27.30[c]	14.29[bc]	16.19[c]

*Among treatments, means with the same lowercase letters do not significantly differ by Duncan's test at $p \leq 0.05$.

BA, 6-benezyladenine; NAA, naphthaleneacetic acid; TDZ, thidiazuron.

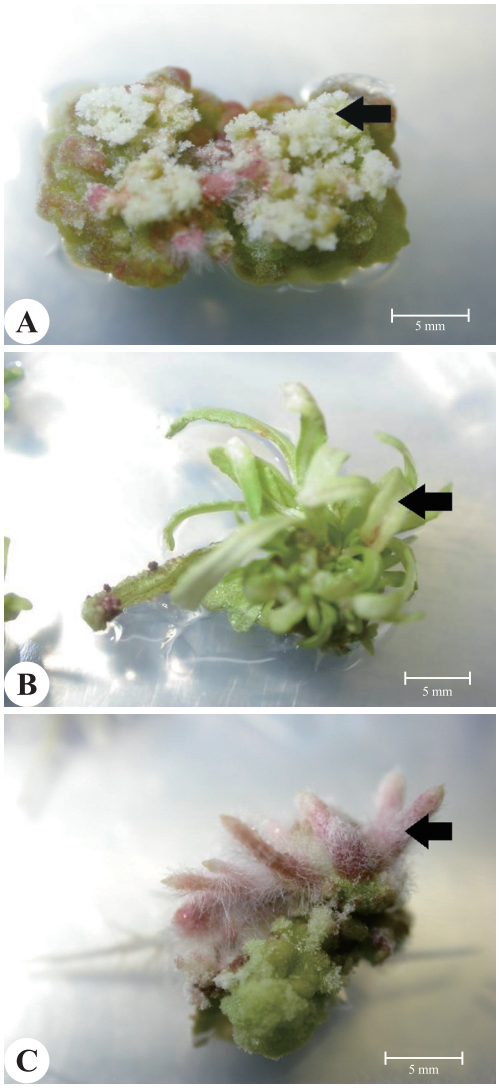

Fig. 3. Induction of calli (A), buds (B), and adventitious roots (C) in poplar explants cultured on MS medium with 1 mgL^{-1} thidiazuron and 0.1 mgL^{-1} naphthaleneacetic acid for 60 d. Calli, buds, and roots are indicated by arrowheads.

the *mtlD* gene (Fig. 4, lanes 3~5). The expected band size of 1149 bp was also observed in the positive control (plasmid carrying *mtlD* gene, lane 1) as expected, but no band was observed in NT plants (lane 2).

Salt tolerance test and phenotypes of transgenic plants grown under salty conditions

When buds cultured in treatment 4 were 1.5~2.0 cm in length, shoots were cut and transferred to rooting medium with 0, 50, 85, 120, and 170 mM of NaCl for 30 days. The effects of different NaCl concentrations on transgenic and NT plants are shown in Fig. 5. The survival rates of transgenic plantlets treated with 0, 50, 85, 120, and 170 mM of NaCl were 99.0, 91.9, 81.8, 64.3, and 0%, respectively (Fig. 5A). However, the survival rates of NT plantlets under identical treatments were 99.0, 80.0, 53.8, 15.4, and 0%, respectively. Fig. 5B shows that rooting rates in transgenic and NT plantlets under 0, 50, 85, 120, and 170 mM of NaCl were 96.2, 87.4, 40.9, 13.7, and 0%, and 97.8, 74.2, 22.5, 0, and 0%, respectively. Obviously, survival and rooting rates in transgenic plants under 50, 85, and 120 mM of NaCl were both significantly higher than in NT plants. Transgenic poplar plants survived at least 30 days and still grew roots in rooting medium containing 120 mM of NaCl solution (Fig. 6A), but NT plants were wilted and no root growth occurred in media containing 120 mM NaCl after 30 days (Fig. 6B).

The effects of salinity on plant growth after 50 d are depicted in Fig. 7. All plants were grown in pots for 30 d and exposed to 120 mM of NaCl for 20 d in a greenhouse. Before high-salinity treatment, no discernible differences in plant growth or morphology were observed between NT and transgenic plants. However, after salt treatment for 20 d, NT plant growth was stunted and impaired relative to transgenic plants. Most NT leaves visually exhibited epinasty, rolling, retardation, wrinkled stunting, and a small size (Fig. 7, right panel). Conversely, the salted transformants putatively expressing the transgene performed well in the test, and most leaves

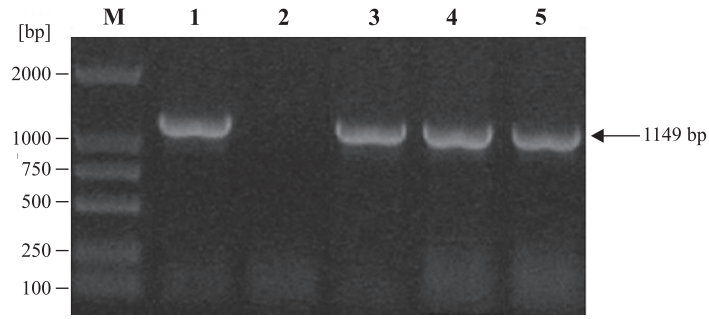

Fig. 4. Analysis of transgenic *Populus* by genomic PCR for the *mtlD* gene. The expected size of the *mtlD* gene fragment (indicated by arrowhead) was 1149 bp. M, 1000-bp DNA marker; lane 1, positive control (plasmid carrying the *mtlD* gene); lane 2, NT, non-transgenic plant; lanes 3~5, transgenic plants.

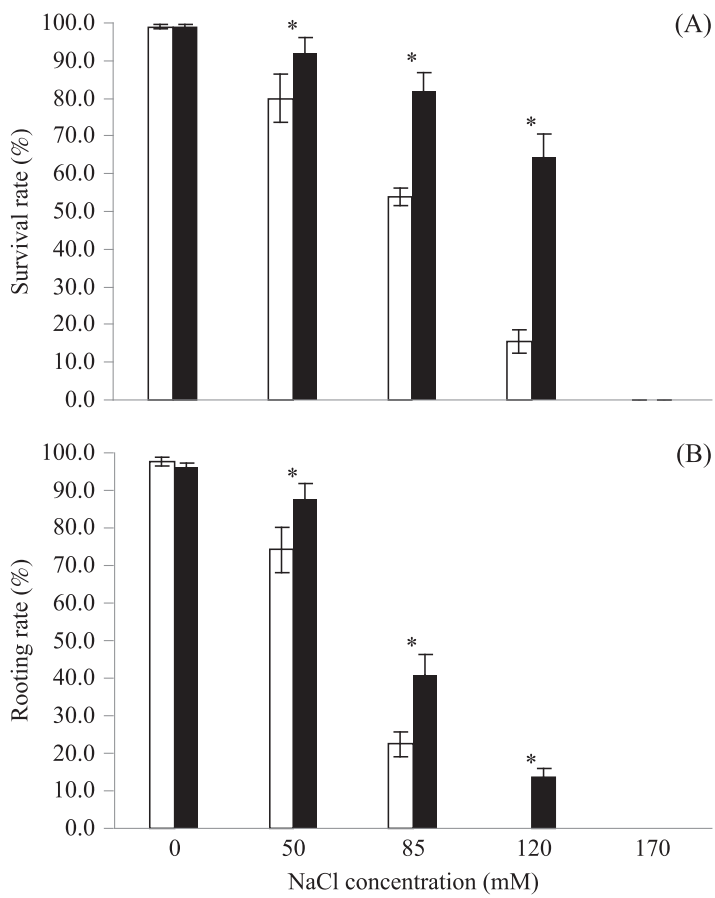

Fig. 5. Effects of different salt concentrations on transgenic andnon-transgenic (NT) plants. Survival rates (A) and rooting rates (B) of NT (white bars) and transgenic (black bars) plantlets treated with rooting medium with 0, 50, 85, 120, and 170 mM NaCl for 30 d. Values represent the means of 5 independent plants with the corresponding standard error. * Means significantly differ at the 5% level by a *t*-test.

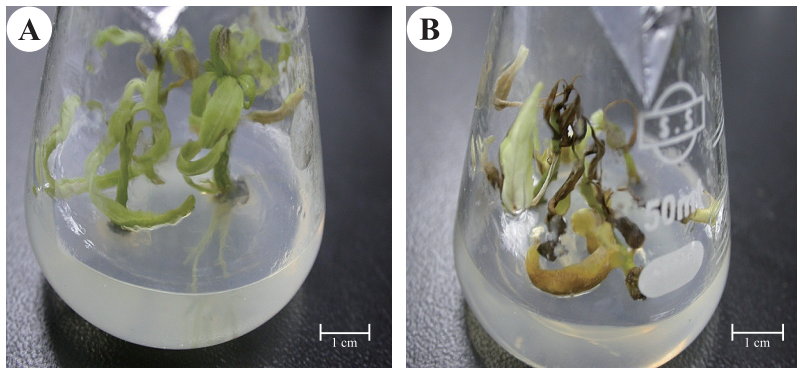

Fig. 6. *In vitro* culture of transgenic plants (A) and non-transgenic plants (B) on rooting MS medium containing 120 mM NaCl for 30 d.

Fig. 7. Salt stress tolerance in transgenic (left panel) and non-transgenic plants (right panel). All plants (30-d-old) grown in pots were subjected to 120 mM NaCl for 20 d.

looked green, healthy, and large (Fig. 7, left panel). All 50-d transgenic plants which we observed grew better than 50-d NT plants under salt stress, and there were increases in shoot length and plant height growth in transgenic plants, whereas NT plants were more retarded under salinity stress.

DISCUSSION

Populus was the first woody plant to be transformed (Parsons et al. 1986), and some *Populus* genotypes can be successfully transformed and express highly stable transgenes (Confalonieri et al. 2003, Brunner et al. 2007). In *Agrobacterium*-mediated transformation systems, co-cultivation is crucial to the processes of transformation. The optimum *Agrobacterium* concentration, precise timing, the specific medium, and lighting conditions can affect the success of transfer (t) DNA transfer during co-cultivation (De Buck et al. 2000). Excessive prolongation of this period results in the browning death of co-cultivated

explants. Cheng et al. (1997) studied wheat transformation mediated by *A. tumefaciens* and found that increasing the duration of infection and co-cultivation was beneficial to the transfer of DNA but resulted in cell injury. Tepfer (1995) pointed out that the shorter the time that explants and bacterium were co-cultured, the less damage there was to plant tissues. Different infection time periods occur in *Agrobacterium*-mediated transformations (Wu et al. 2003, Tao and Li 2006, Ding et al. 2009), suggesting that the infection time is much more important than the inoculation stage. Furthermore, antibiotics can also inhibit the growth of *A. tumefaciens* and affect explants grown in different concentrations or types (Teixeira and Fukai 2001). The optimal concentration of cefotaxime for sugarcane somatic embryogenesis from callus culture to maximize somatic embryogenesis is 500 ppm (Mittal et al. 2009). Moreover, higher cefotaxime concentrations accelerate wheat callus browning and inhibit subsequent regeneration (Yu and Wei 2008). Those results suggest that selection of an appropriate cefotaxime concentration can affect growth and explant survivability. In our study, we showed that the optimal infection time of 8 min enhanced the success of explant transformation, but after 8 min, co-cultivated bacteria underwent excessive proliferation that resulted in explant death. In addition, the cefotaxime concentration did not affect poplar shoots, but as the cefotaxime concentration decreased, the survival rate of explants decreased due to increasing bacterial proliferation.

Most protocols in poplar transformation use direct or indirect *in vitro* shoot organogenesis, followed by rooting. One of the main determining factors affecting the transformation efficiency is the origin and physiological condition of the explants. Although explants from leaves, petioles, and stems have been successfully employed in transformation tests, the success rates with different types of explants are highly variable and species dependent. Additionally, the variability of explants may be because frequent metabolic activity in vigorously dividing cells facilitates DNA insertion, and tissues, and cells undergoing vigorous division possess more cell wall -binding sites for adherence of *Agrobacterium*, thereby enhancing the transformation efficiency (De Velde et al. 2003). Yevtushenko and Misra (2010) studied the efficiency of *Agrobacterium*-mediated transformation in a hybrid poplar using various types of explants and found that petioles and leaves had higher transformation frequencies than stem segments. In our study, a successful *Agrobacterium*-mediated transformation was also observed using leaves as explants.

Callus induction usually requires the presence of auxin (e.g., NAA or IBA) and is performed in the dark or under very- low- intensity light. The propensity of different types of explants (e.g., leaf, stem, or petiole) to produce calli is highly genotype-specific. Direct or indirect shoot organogenesis requires high levels of cytokinins, such as BA and TDZ. Rooting is accomplished in auxin-dominant media and is generally a non-limiting step in poplar transformation and regeneration (Busov et al. 2005, 2010). In our study, using MS media with 1 mgL^{-1} TDZ + 0.1 mgL^{-1} NAA (treatment 4) to induce poplar buds was more effective, while using BA (treatments 1~3) resulted in lower induction bud rates in compared to TDZ (treatments 4 and 5). TDZ has been among the most active cytokinin-like substances for woody plant tissue culture and facilitates efficient micropropagation of many recalcitrant woody species (Huetteman and Preece 1993). Howe et al. (1994) studied the *Agrobacterium*-mediated transformation

of hybrid poplar suspension cultures, and found that BA had a negative effect on shoot regeneration. In *Agrobacterium*-mediated transformation with *Populus*, Wang et al. (2011) reported that the addition of TDZ improved regeneration and transformation efficiencies. Our study also found TDZ to be more effective than BA for induction rates of poplar explants.

Prabhavathi et al. (2002) showed that after the *mtlD* gene was introduced into eggplant (*Solanum melongena* L.) by *Agrobacterium*-mediated transformation, transgenic seeds germinated, and seedlings grew well on 200 mM salt-MS basal medium, whereas the wild-type control seeds did not germinate at the same NaCl level. Canola (*Brassica napus* L.) transgenic seeds expressing the *mtlD* gene germinated, and their seedlings survived up to 24 d under *in vitro* conditions on MS medium containing 350 mM of NaCl, whereas their wild-type control seeds failed to germinate at the same NaCl concentration (Motallebi and Rahnama 2011). The expression of the *mtlD* gene in transgenic potato plants resulted in increased salt tolerance under both *in vitro* and hydroponic stress conditions with 100 mM NaCl compared to control NT plants (Rahnama et al. 2011). Moreover, NT Indica rice plants withered at 200 mM NaCl, whereas transgenic rice plants withstood such a stress (Punji et al. 2007). *mtlD* transgenic Chinese white poplar (*P. tomentosa*) survived up to 40 d in a 75 mM NaCl hydroponic culture, while their NT counterparts only survived a concentration of 25 mM NaCl (Hu et al. 2005). In our study, after 30 d of salt-stress, survival and rooting rates of transgenic and NT *Populus* plants were affected as salt concentrations increased. Survival and rooting rates were both significantly higher in transgenic plants over NT plants at different levels of salt stress except in the no-stress and lethal-stress (170 mM NaCl) conditions. In addition, 120 mM NaCl was the tolerance threshold for transgenic survivability and rooting (Fig. 5). The deleterious effects of NaCl on plant survivability and rooting might be attributed to a decrease in the osmotic potential of the growing medium, ion toxicity, and nutrient ion deficiencies (Motallebi and Rahnama 2011). Therefore, different plant species with the *mtlD* transgene show various levels of salt tolerance, and expression of the *mtlD* gene is also affected by salt concentrations.

Plant growth and plant height in transgenic and NT plants were not visually different under the no-salt condition. However, *Populus* transgenics grew well under 120 mM NaCl compared to NT plants. A apparent reductions in shoot elongation, length, and plant height were observed in NT over transgenic plants (Fig. 7). Salt stress had a harmful effect on the height of NT plants, but transgenic plants exhibited stronger resistance to salt tolerance and showed less salt injury. Transgenic plants exhibited unique abilities and specificities through *mtlD* in response to salt stress. Although different lines of transgenic *Populus* displayed variations in phenotype and differential *mtlD* gene expressions in various transgenic lines were associated with the salinity stress response, all transgenic *Populus* plants showed greater resistance to salinity than NT plants because of increased *mtlD* gene expression, which led to more osmoprotectant agent production in transgenic plants. This clearly indicates that the *mtlD* transgene was induced and expressed, resulting in enhanced tolerance for plant growth under salt stressing. Salinity stressing is one of the most serious factors limiting the distribution and productivity of crops and forest trees; therefore, we hope that the research presented here will take us a step closer to developing a *P. nigra*

x P. tomentosa hybrid that can tolerate salinity in those parts of the world that suffer from losses to this abiotic stress factor. The results obtained in this study also suggest the feasibility of utilizing the salt-tolerant *mtlD* gene in transgenic poplar trees for tree-processing industries. This investigation could also be useful for future studies on genetic engineering of other forest trees, including other of the genera Salicaceae.

CONCLUSIONS

An efficient *A. tumefaciens*-mediated transformation protocol for the stable integration of the bacterial *mtlD* gene into *Populus* was developed, and the overexpression of the *mtlD* gene in a transgenic *Populus* hybrid improved its salt-stress tolerance. It is possible that the synthesis and accumulation of mannitol in *mtlD* transgenic plant tissues might induce a series of signal transduction pathways, and *mtlD* might play a major role in defense against salinity and have a stress-signaling function. This in turn may activate various tolerance responses against salinity, which ultimately increase the stress tolerance of transgenics. Further analysis of these transgenics needs to be done in order to determine how the synthesis of mannitol changes expressions of *mltD* and other genes.

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