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Overexpression of the chloride channel gene (*GmCLC1*) from soybean increases salt tolerance in transgenic *Populus deltoides* × *P. euramericana* 'Nanlin895'

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Abstract

The *GmCLC1* encodes a vacuolar Cl^- transporter protein that sequesters ions from the cytoplasm to the vacuole, thereby reducing the toxic effects of salt stress. We constructed a *GmCLC1* overexpression vector, pGWB402Ω, and introduced it into poplar hybrid, *Populus deltoides* × *P. euramericana* 'Nanlin895', by *Agrobacterium tumefaciens*-mediated transformation. PCR and Southern blot analyses showed that *GmCLC1* was integrated into the genome of the hybrid poplar line. When transgenic plants overexpressing *GmCLC1* were subjected to salt stress, the contents of soluble protein and photosynthetic pigments and the activity of antioxidant enzymes (POD and SOD) were increased, and the MDA concentrations were significantly decreased in transgenic lines compared with the control lines. Also, the defoliation rates of the transgenic plants were significantly lower than that of the control plants. These results suggest that overexpression of *GmCLC1* plays a significant role in improving salt tolerance of poplars, reducing damage to membrane structures, and enhancing osmotic adjustment and antioxidative enzyme regulation during salt stress.

Keywords: *GmCLC1*; *Populus*; overexpression; salt tolerance; transgenic.
Abbreviations: TDZ_thidiazuron; 6-BA_6-benzylaminopurine; CK_control; SOD_superoxide dismutase; POD_peroxidase; MDA_malondialdehyde; MS_Murashige and Skoog (1962); ROS_reactive oxygen species; H_2O_2_hydrogen peroxide; TBA_ thiobarbituric acid.

Introduction

Populus is not only an important forest genus worldwide, but is also a major renewable resource that is highly valued by the pulp and paper industry for their fast growth and high-quality fiber (Bradshaw et al., 2000). Additionally, *Populus* has many features that make the species a suitable model for forest biotechnology, such as fast growth, a relatively small genome, ease of vegetative propagation, high frequency of *in vitro* regeneration, facile transgenesis, high-quality genetic maps, and the first completed woody plant genome database (Taylor, 2002; Sterky et al., 2004; Tuskan et al., 2006). *Populus deltoides* × *P. euramericana* 'Nanlin895' is an elite poplar hybrid clone which is extensively used for plantation across large portions of east-south China. However, salinity stress is a serious factor limiting the development of the poplars due to large area around long coastline in the east-south China. Photosynthesis and growth are impaired in plants subjected to salt stress mainly because of the accumulation of toxic ions, particularly Na^+ and Cl^-, in plant tissues (Hasegawa et al., 2000a; Abogadallah, 2010). Cytosolic and organelle processes are equally sensitive to Na^+ and Cl^- in both glycophytic and halophytic species (Bohnert and Jensen, 1996). Some crops, such as cotton (*Gossypium* spp.), wheat (*Triticum aestivum*), and rice (*Oryza sativa*), are more sensitive to Na^+ than to Cl^-, whereas many plant species, including tobacco (*Nicotiana tabacum*), tomato (*Lycopersicon esculentum var. esculentum*), barley (*Hordeum vulgare* subsp. *Vulgare*), grape (*Vitis vinifera*), sugar maple (*Acer saccharum*), citrus (*Citrus aurantium L.*), and soybean (*Glycine max L.*), are more sensitive to Cl^- than to Na^+ (Luo et al., 2003; Li et al., 2006; Munns and Tester, 2008; Zhang, 2011). In these species, Cl^- homeostasis is an important mechanism for NaCl tolerance. In concert with K^+ channels and the Na/K pump, Cl^- channels participate in the transmembrane movement of salt (Rubio et al., 1995). Cl^- is an energetically efficient osmolyte for osmotic adjustment and is compartmentalized into the vacuole to minimize cytotoxicity (Niu et al., 1995; Blumwald et al., 2000). The Cl^- chemical gradient is a major contributor to the maintenance of steady state levels of Na^+, K^+, and Ca^{2+} at both the cellular and whole plant levels. Maintenance of a favorable K^+/Na^+ ratio can play an essential role in salt tolerance (Hasegawa et al., 2000b; Teakle and Tyerman, 2010). The bulk of intercellular Cl^- movement and movement from the cytoplasm to the vacuole is mediated by ion transport channels located in the plasma membrane and tonoplast. Cl^- channels allow the passive diffusion of negatively charged ions along electrochemical gradients or the transport of positively charged ions. These channels can also conduct other anions such as HCO_3^-, I^-, SCN^-, and NO_3^-. Hydrophobic analysis suggests that there are different Cl^- channel structures (Suzuki et al., 2006). Membrane-localized chloride channels may function to expel cytoplasmic Cl^- and thereby lower the concentration of toxic ions in the cytoplasm. Alternatively, Cl^- channels may sequester cytoplasmic Cl^- into vacuoles to create an osmotic gradient, as an adaptation to NaCl- or drought-induced physiological stress (Jain and Selvaraj, 1997). The *GmCLC1* gene encodes a vacuolar Cl^- transport protein that moves ions from the cytoplasm into the vacuole to reduce the toxic effects of salt (Li et al., 2006). CLC proteins are involved in Cl^- movement through intracellular compartments. The first *CLC* genes in plants were cloned independently from tobacco (Lurin

et al., 1996) and *Arabidopsis* (Hechenberger et al., 1996). *GmCLC1* was discovered in soybean (*G. max L.*) and *GmCLC1* located in the tonoplast was found in the *GmCLC1-YFP* (yellow fluorescent protein) transgenic tobacco (Li et al., 2006). Moreover, the protein conferred salinity tolerance to transgenic cells by sequestering Cl^- from the cytosol into the vacuole. To date, information concerning the role of CLC proteins in tolerance to salt stress in a forest tree species is lacking. The objective of this study was to analyze the role of *GmCLC1* in tolerance to salt stress in poplars. We inserted the *GmCLC1* gene from soybean into the genome of the poplar hybrid clone *Populus deltoides* × *P. euramericana* 'Nanlin895' by *Agrobacterium tumefaciens*-mediated transformation and determined the physiological and biochemical effects of *GmCLC1* overexpression as well as its effects on antioxidant defense and osmotic protection against salt stress.

Results

Confirmation of transgenic plants by PCR and Southern blot analysis

Thirty kanamycin-resistant plants were produced by *A. tumefaciens*-mediated transformation, and nine putative transgenic lines (C4, C5, C8, C11, C15, C18, C22, C24, and C25) were selected for molecular analysis. *GmCLC1* gene amplification by PCR gave the expected 609-bp fragment in tested transgenic lines, confirming that the *GmCLC1* gene had inserted into the poplar genome (Fig. 1). Southern blot analysis showed that most transgenic plants had 1-2 copies of the transgene. Only the C15 line lacked the diagnostic hybridization band. The differences in the hybridization pattern among the transgenic lines may be attributable to unequal exchange of homologous chromosomes, resulting in gene rearrangement, or to a base mutation at an *Xho*I restriction enzyme site. *GmCLC1* transcripts were detected in most of the PCR-positive lines.

Increased salt tolerance in GmCLC1 transgenic plants

Salt sensitivity was evidenced by partial defoliation in plants after 5 days of exposure to NaCl. Small differences were apparent between transgenic and CK plants grown for 5 days on MS medium (without agar) supplemented with 50 mM or 100 mM NaCl, but the differences were much larger with 150, 200, and 300 mM NaCl (Fig. 2a). The rate of leaf abscission differed significantly between the transgenic and CK plants with increasing culture time at 100-300 mM NaCl (Fig. 2a, b). The defoliation rates of the transgenic lines C8, C11, and C24 were lower than that of CK plants, especially at 150, 200, and 300 mM NaCl (Fig. 2). Even though the defoliation rate was similar between the CK and C8 lines through day 15, the transgenic C8 line showed less sensitivity to NaCl (Fig. 2). Growth at 300 mM NaCl caused complete defoliation of the CK plants, which then died (Fig. 2e). The C8 transgenic line was selected to compare differences in growth between transgenic plants and the control line (Fig. 3). Under mild salt stress (100 and 150 mM NaCl), no obvious growth reduction was observed in either the transgenic or control plants, while the leaves of the control poplars began to fall and the transgenic turned slightly brown (Fig. 3a,f). However, with the higher (200 and 300 mM) NaCl concentration treatments, defoliation of plants increased in both the transgenic and control plants (Fig. 3b, c, h, i). However, the transgenic plants had higher NaCl tolerance than the control plants. Indeed, all the control plants died, while the transgenic plants were still alive under the higher (200 and 300 mM) NaCl concentration treatments

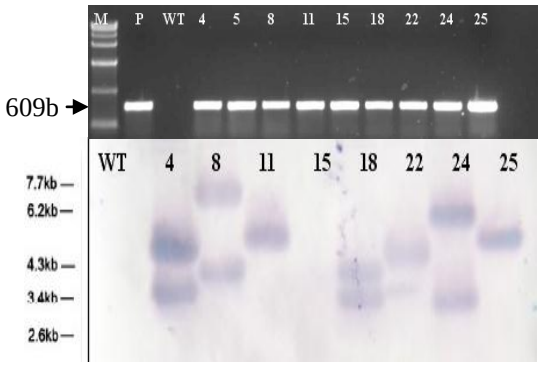

Fig 1. PCR and Southern blot analysis of transgenic plants. A 609-bp PCR product was detected in selected transgenic lines. M, 250-bp NDA ladder (Takara); P, PCR product with *GmCLC1* plasmid DNA as template; WT, PCR product with control plant genomic DNA as template; lanes 4-12 (4, 5, 8, 11, 15, 18, 22, 24, 25), PCR products with genomic DNA from Kan-resistance transgenic lines as template. For Southern blot analysis of transgenic plants, genomic DNA was digested with XhoI, electrophoresed, and probed with a DIG-labeled 609-bp PCR product of the coding region, as above. The number of bands reflects the number of transgene insertions. Molecular weight DNA markers are shown on the left. WT, the control plant; lanes 2-9 (4, 8, 11, 15, 18, 22, 24, 25), Kan-resistance transgenic lines, lane 5(15) are for the non-transformed plant.

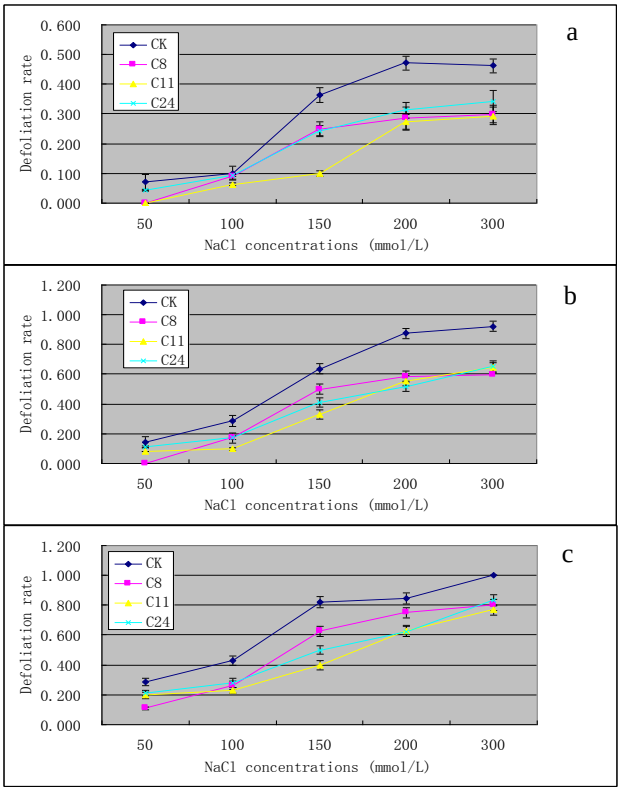

Fig 2. Effect of salt (NaCl) stress on leaf abscission of CK and transgenic 'Nanlin895' plants. The plants were grown in MS medium (without agar), supplemented with different concentrations of NaCl for 5 days (a), 10 days (b), and 15 days (c). Defoliation rate of CK plants was significantly higher than that of transgenic plants. CK, control plants; C8, C11, C24, transgenic plants.

Fig 3. Growth of CK and transgenic 'Nanlin895' plants in MS medium (without agar) supplemented with different concentrations of NaCl (50, 100, 150, 200 or 300 mM NaCl). All the plants were under NaCl stress for 15 days. The C8 line was taken as the template of transgenic plants. a-e, the control plants; f-k, transgenic plants (line C8).

(Fig. 3d, e, j, k). The enhanced growth of the transgenic plants relative to CK plants under salt-stress conditions suggests that the overexpression of *GmCLC1* increased the salt tolerance of 'Nanlin895' poplar plants.

Analysis of physiological parameters with NaCl treatment

After 2 weeks of growth at different NaCl concentrations, CK plants and *GmCLC1* transgenic plants were analyzed for differences in several physiological parameters. At higher salt concentrations, the chlorophyll content was decreased in both the control and transgenic plants, but the decrease was significantly less in the transgenic lines. Between 50 and 300 mM NaCl, the relative chlorophyll content was reduced in CK, C8, C11, and C24 plants by 86.22%, 67.57%, 51.17%, and 55.39%, respectively. At 300 mM NaCl, the CK plants had 39.87%, 27.68%, and 28.80% of the relative chlorophyll content of the C8, C11, and C24 transgenic plants, respectively (Fig. 4a). Soluble protein is an osmotic regulator in plants, and the accumulation of soluble protein can contribute to salt stress tolerance by reducing the osmotic pressure in the cell. In CK and transgenic plants, the soluble protein content was slightly increased at 100 mM NaCl, followed by a decline at higher NaCl concentrations (Fig. 4b). Overall, the decrease in soluble protein content was significantly greater in CK plants than in the three transgenic lines ($P < 0.05$). Thus, low-level salt stress appears to induce the synthesis of new proteins, and higher NaCl concentrations may inhibit this protein synthesis. In contrast to the decrease in relative chlorophyll content, the MDA content increased with increasing NaCl concentration in both CK and transgenic plants. In CK plants, the MDA levels were 22.40, 27.03, and 32.71 μmol/g at 150, 200, and 300 mM NaCl, respectively. By comparison, the MDA levels at 150, 200, and 300 mM NaCl were significantly lower in the transgenic lines: 15.61, 18.99, and 28.09 μmol/g, respectively, for C8; 14.84, 19.12, and 25.17 μmol/g, respectively, for C11; and 16.33, 20.16, and 27.34 μmol/g, respectively, for C24. At 150 mM NaCl, the MDA levels of the C8, C11, and C24 lines were 69.69%, 66.25%, and 72.90%, respectively, of the MDA content of CK plants (Fig. 4c). SOD protects against reactive oxygen species (ROS) during periods of environmental stress, and thus the increased tolerance to salt stress in the transgenic plants might have been due to changes in SOD activity. At 50 mM NaCl, the SOD activity was similar between transgenic and CK plants. At higher (300 mM) NaCl concentrations, the SOD activity in CK plants decreased by as much as 81.56%, which was significantly greater than the average reduction of 55.66% in the *GmCLC1* transgenic plants (Fig. 4d). Thus, despite the decreases at high NaCl concentrations, SOD activity remained significantly higher in transgenic plants compared with CK plants. SOD consumes superoxide O_2^- thereby enhancing tolerance to oxidative stress, but it also produces H_2O_2. POD catalyzes the decomposition of toxic H_2O_2 to oxygen and water (Farhoudi et al., 2012). POD activity, which increased at 100 mM NaCl and then declined at higher NaCl concentrations, differed significantly between transgenic and CK plants at NaCl concentrations of 100 and 300 mM ($P < 0.05$; Fig. 4e). The effects of NaCl on MDA content, relative chlorophyll content, and SOD activity were smaller in transgenic plants than in CK plants, and the effects differed significantly between transgenic and CK plants at 150 and 200 mM NaCl ($P < 0.05$) (Fig. 4a-c). These results indicate that salt tolerance was improved in *GmCLC1* transgenic plants relative to wild-type control plants.

Discussion

In plants, the maintenance of ion homeostasis is vital for surviving salt stress. Salt stress accelerates the degradation of plant chlorophyll and reduces the stability of cell membranes, resulting in decreased light absorption by chloroplasts and decreased photosynthesis (Munns, 2002; Kumar et al., 2005; Yu et al., 2006). In contrast to the extensive studies regarding the role of Na^+ related stress, few studies have examined the effects of Cl^-. Previous physiological studies have suggested that Cl^- homeostasis is an important mechanism of NaCl tolerance in some plants (Abel, 1969; Zhang et al., 2011). Tonoplasts in higher plants are permeable to Cl^-, and Cl^- and NO_3^- are the major anions that enter the vacuole (Kastner and Sze, 1987). Cl^- transport into tonoplasts has been demonstrated in several plant species (Plant, 1994; Pei et al., 1996; Wissing and Smith, 2000), and the majority of cellular Cl^- may be located in vacuoles (Trebacz et al., 1997). The expression of *GmCLC1* helps to prevent the toxic damage to cytoplasmic and organelle membranes caused by excess Cl^- (Li et al., 2006). In the present study, the expression of *GmCLC1* improved the salt tolerance of *Populus*.Because leaves are the site of photosynthesis, the number of leaves reflects the growth status of a plant. Toxicity normally results when certain ions are taken up with the water and accumulate in the leaves during water transpiration to an extent those results in damage to the plant. Under stress conditions, leaves fall from the plants probably due to the accumulation of toxic Cl^- ions. Defoliation rates reflect the capacity of a plant to defend against salt stress (Brugnoli et al., 1992). Two transgenic lines (C8 and C11) did not defoliate under the treatment with 50 mM NaCl during the first 5 days，and even after 15 days, few of the C8 or C11 plants defoliated. This showed that under mild salt stress, transgenic plants could maintain normal growth while that of the control plants decreased (Fig. 3). When treated with high salt stress (200 and 300 mM), the control plants could not survive but all the transgenic plants remained unaffected until day 16. Some of the transgenic lines stopped growing (Fig. 3). Chlorophyll content is one of the few physiological parameters that exhibit a close correlation with salt tolerance. Under salt-stress conditions, the accumulation of Cl^- in cells affects photosynthesis. A decline in leaf chlorophyll content was observed in all plants in response to salt stress in the present study. The accumulation of toxic ions also induces quantitative and qualitative changes in the protein content of plant cells. An increase in the amount of soluble proteins may reflect the synthesis of osmotic or structural proteins, particularly proteins involved in cell wall modification. A higher content of soluble

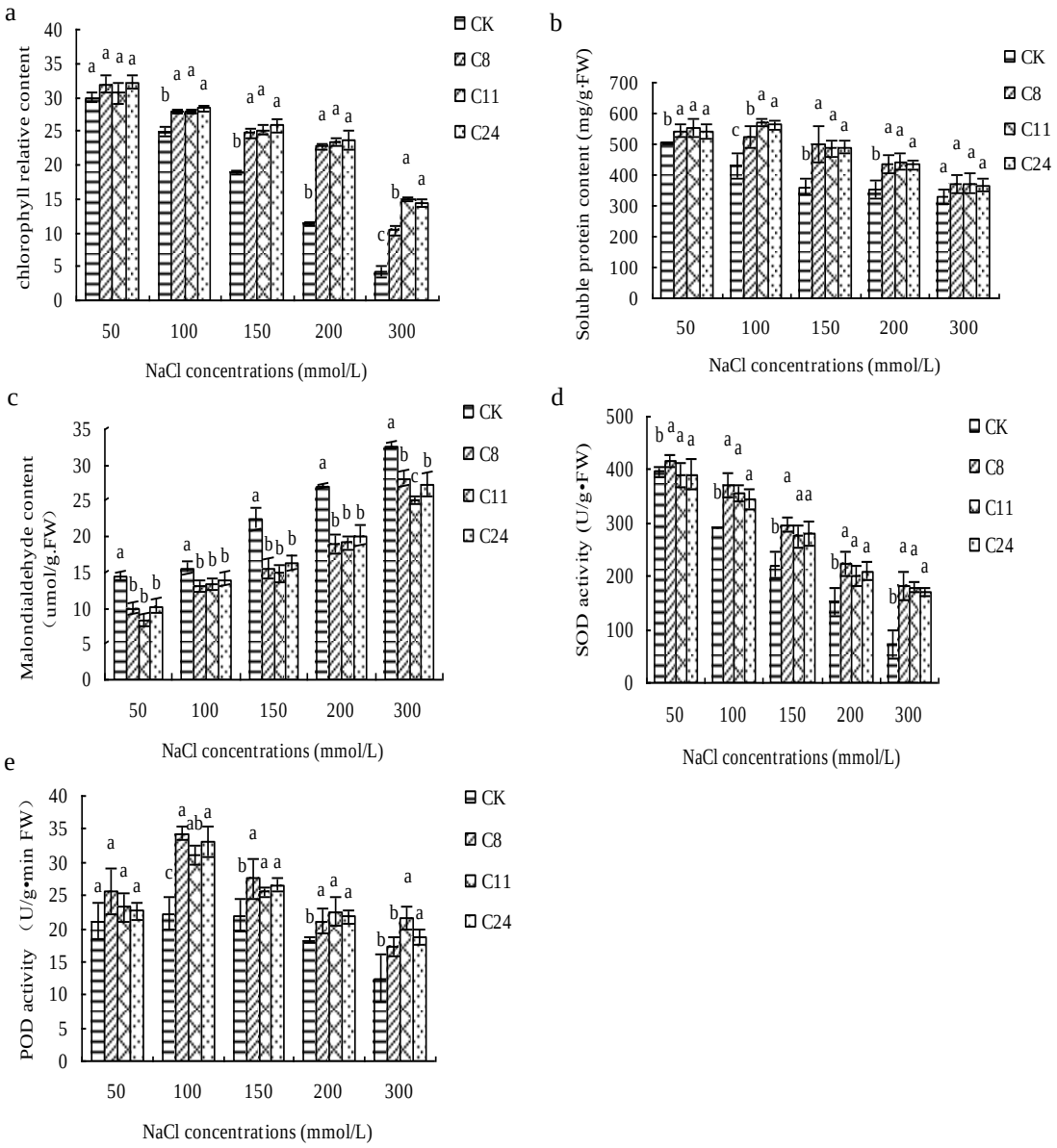

Fig 4. Effects of different NaCl concentrations on the biological characteristics. Plants were grown in MS medium (without agar), supplemented with different concentrations of NaCl for 15 days. a, chlorophyll relative content; b, soluble protein content; c, MDA content; d, SOD activity; e, POD activity; CK, control plants; C8, C11, C24, transgenic plants. Different letters above each column indicate significant differences at $P < 0.05$.

proteins has been reported in salt-tolerant cultivars of rice under salt-stress conditions (Lutts et al., 1996). High salt concentrations also affected protein synthesis in the present study. The effects of salt stress on chlorophyll content and soluble protein content were less in transgenic plants overexpressing *GmCLC1* than in CK plants (Fig. 4a, b). Severe salt stress can cause oxidative damage, and the nornal dynamic balance of the ROS system would be broken. MDA is a secondary end product of polyunsaturated fatty acid oxidation, which is widely used to measure the extent of lipid peroxidation, as indicator of oxidative stress (Lin and Kao, 2000). MDA concentration increases progressively with the progression of salinity, indicating that the degree of stress and the level of lipid peroxidation are aggravated with stress time. In our study，it increased in all plants after 15 days of salt stress. However, transgenic lines and control lines responded differently to leaf MDA contents under salt stress. A large increase in MDA content in the leaf due to salinity was observed in the control plants than in the transgenic plants (Fig. 4c). The difference between the control and transgenic plants may have been caused by the expression of *GmCLC1*. Furthermore, a negative correlation was observed in leaf MDA contents under salt stress, indicating that to a certain extent, high lipid peroxidation resulted in decreased relative content chlorophyll. Such accumulation of MDA content, coupled with reduced plant growth, under salt stress is consistent with the studies of Li (2010) and Koca et al. (2007) in tomato and sesame, respectively. Limiting of MDA content appears to be

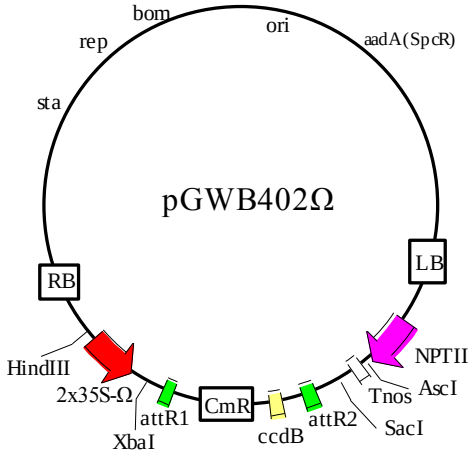

Fig 5. Schematic illustration of the binary vector pGWB402Ω. sta, region for stability in *Agrobacterium*; rep, broad host-range replication origin; bom, *cis*-acting element for conjugational transfer; ori, ColE1 origin; RB, right border; LB, left border; addA, spectinomycin-resistant (SpcR) marker used for selection in the bacteria; ccdB, negative selection marker used in the bacteria; CmR, chloramphenicol resistance marker; P35S Ω, a modified CaMV35S promoter in which the enhancer regions are duplicated, along with the Ω translational enhancer from tobacco mosaic virus (2× 35S Ω).

beneficial to plant photosynthesis. Thus, *GmCLC1* is helpful for plant growth under salt stress. Oxidative stress induced by salt stress in plants increases lipid peroxidation and hydrogen peroxide (H_2O_2) levels. These changes were associated with decreased SOD and POD activities (Mittova et al., 2004). Production of ROS under salt stress is potentially harmful to plants. However, plants possess several antioxidant systems that protect them from these potential cytotoxic effects. SOD is a major scavenger of superoxide O_2^- and its enzymatic action results in the formation of H_2O_2 and O_2. Its role in providing protection to plants against oxidative damage is well established. Increased total SOD activity coincides with the anoxic phase in the resistant varieties but not in the sensitive ones (Monk et al., 1987). The increase in SOD activity was vital in protecting plants against the oxidant stress. When SOD activity was high, ROS, (especially the O_2^- radical), scavenging was appropriately high and thus damage to membranes and oxidative stress decreased, leading to an increase in tolerance to oxidative stress. Salt stress increased O_2^- level in cells; if this is not scavenged by SOD, it can react with vital biomolecules (Mittler, 2002). With respect to SOD activity decreased in all plants in our research (Fig. 4d), indicating that the certificates control plants were sensitive to salt stress. It is also consistent with the results after NaCl treatment, even in the *GmCLC1* transgenic plants, SOD activity decreased. The osmotic and/or ionic effects of the Na+ and Cl- might have directly affected the SOD protein integrity directly and caused subsequent reductions in the SOD activity, as demonstrated by Hernandez et al. (1994), working with cowpea leaf protoplasm subjected to different NaCl concentrations. Our results are also similar to the findings of Quartacci and Navaro (1992), who found decreased SOD activity in sunflower seedlings under drought stress. Moreover, these result indicated a negative relationship between SOD activity and MDA content. This relationship could explain the variation trend in POD. In our experiment, stress induced enhanced POD activity, while the activity of SOD declined, as observed in rice roots exposed to salt stress (Khan et al., 2002), indicating that POD had a higher capacity for the decomposition of H_2O_2 generated by SOD. At the beginning of the NaCl treatment, a great amount of H_2O_2 accumulated and MDA content increased, which caused enhanced POD activity (Fig.4e). Given that total SOD activity suffered a salt-induced decrease following the NaCl treatment, lower H_2O_2 production would have been expected. Accompanied by damage from the ions accumulation, POD activity decreased. However, the decreases in SOD and POD activities with increasing NaCl concentrations were also significantly less in *GmCLC1* transgenic plants than in control plants, indicating that SOD and POD activities in *GmCLC1* transgenic plants were less sensitive to NaCl than those in control plants. Salt stress also induces oxidative stress in plants and increases lipid peroxidation and H_2O_2 levels. These changes were associated with decreased SOD and POD activities (Mittova et al., 2004; Azooz et al., 2009). As a product of lipid peroxidation, MDA is a prominent indicator of membrane impairment in plants. Compared with control plants, *GmCLC1* transgenic plants showed smaller increases in MDA content over a range of NaCl concentrations in the present study. The decreases in SOD and POD activities with increasing NaCl concentrations were also significantly less in *GmCLC1* transgenic plants than in control plants, indicating that SOD and POD activities in *GmCLC1* transgenic plants were less sensitive to NaCl compared with the activities in wild-type 'Nanlin895' plants. Under salt stress, the transgenic plants overexpressing *GmCLC1* displayed higher relative chlorophyll content, higher soluble protein content, enhanced antioxidant enzyme activities, and lower MDA levels compared with these values in control plants. These results suggest that overexpression of *GmCLC1* played a significant role in improving the salt tolerance of poplars, reducing damage to membrane structures, and enhancing osmotic regulation and antioxidant enzyme activities during salt stress.

Materials and Methods

Plant material and overexpression vector

Poplar hybrid female clones (*Populus deltoides* × *P. euramericana* 'Nanlin895') were used for genetic transformation. The binary vector pGWB402Ω, shown schematically in Fig.5, carries a modified CaMV35S promoter (2 × 35S-Ω) with duplicated enhancer regions and the Ω translation enhancer from tobacco mosaic virus (Nakagawa et al., 2007).

Plant transformation

'Nanlin895' poplar leaves were excised (discs of 1-2 cm^2) and pre-cultured for 3 days on MS1 medium (MS plus 0.5 mg/L 6-BA and 0.002 mg/L TDZ). *Agrobacterium tumefaciens* strain EHA105 carrying the overexpression vector was used to infect plants. After cocultivation on MS1 in the dark for 4 days, the discs were washed with sterile water containing 100 mg/L cefotaxime and transferred to MS2 medium (MS plus 0.2 mg/L 6-BA, 0.001 mg/L TDZ, 100 mg/L cefotaxime, and 20 mg/L kanamycin). Regenerated shoots were cultured with 20 mg/L kanamycin in 0.5 × MS. Kanamycin-resistant plants were selected for propagation.

PCR and Southern blot analyses

PCR and Southern blot analyses were performed with the 1st generation transgenic (T_0) plants to confirm the presence of the

transgene. Genomic DNA was isolated from leaves (0.5 g) of transgenic and control (CK) plants as described previously (Kang et al., 2010). To ensure that the samples were free from *Agrobacterium* contamination, the samples were taken from shoots grown for 4 weeks on rooting medium with 23 mg/L kanamycin. Primers were designed to amplify a 609-bp fragment of the chloride channel gene *GmCLC1* using the Primer 3 software: GmCLC1-F: 5'-ATGGATGCAAACCCTG-AGC-3', GmCLC1-R: 5'-CTTCCTCTTTGATTTTGCCAG-3'. For Southern blot analysis, 4 μg of genomic DNA isolated from the leaves of transformed and control poplar plants was digested with *Xho*I, electrophoresed in 0.8% agarose gels, and transferred onto Hybond N^+ nylon membranes using a vacuum blotting system. Southern blot analysis was performed according to standard procedures using the 609-bp coding region fragment as a hybridization probe. The probe was labeled using a DIG DNA labeling and detection kit (Roche, Mannheim, Germany) following the manufacturer's instructions.

NaCl treatment and measurement of physiological parameters

The transgenic plants and CK plants were grown in the culture room with 16 h cool white fluorescent light (~200 μmol m^{-2} s^{-1}), and temperature was kept at 20-24°C. When the plants grew to around 8-10 cm tall, they were transferred from the culture room to the greenhouse. The greenhouse was kept in the following growth conditions: 20–24°C in the daytime and 16-18°C overnight, 40-60% relative air humidity, and 16 h cool white fluorescent light (~300 μmol m^{-2} s^{-1}). All plants were cultured in MS medium (without agar) containing a range of NaCl concentrations (50, 100, 150, 200, or 300 mM) for 2 weeks, after which physiological measurements were recorded. To measure the activities of superoxide dismutase (SOD, E.C. 1.15.1.1) and peroxidase (POD, E.C. 1.11.1.7), leaf samples were collected from greenhouse-grown plants at the mid-vegetative stage of development (Kalu and Fick, 1983). Whole leaves were ground in liquid nitrogen in a mortar, and 0.4 mL of homogenizing buffer (50 mM KH_2PO_4, pH 7.8) was added to extract the soluble proteins. The samples were clarified by centrifugation (15,000 × *g*, 20 min, 4°C), and the supernatant was removed to a clean tube. The pellet was suspended in 0.8 mL of the same buffer, the suspension was centrifuged (15000 × *g*, 15 min), and the supernatant was removed. The two supernatants were combined and stored on ice until used for antioxidant enzyme activity measurements. The concentration of soluble proteins was determined using the Bradford protein assay (Bradford, 1976). SOD activity was estimated by recording the decrease in absorbance of the superoxide nitro blue tetrazolium (NBT) complex due to the enzyme (Sen et al., 1993). About 3 mL of reaction mixture, containing 0.2 mL of 100 mM methionine, 0.2 mL of 1mg/mL NBT, 0.2 mL of 3 mM EDTA, 2.15 mLof 100 mM potassium phosphate buffer, and 0.05 mL of enzyme extract were mixed in test tubes. The reaction was started by adding 0.1 mL riboflavin (60 μM) and placing the tubes below a light source of two 25 W florescent lamps for 15 min. The reaction was stopped by switching off the light and covering the tubes with black cloth. Blanks and controls were run in the same manner but without illumination or enzyme, respectively. Absorbance was recorded at 560 nm and one unit of enzyme activity was taken as the quantity of enzyme that reduced the absorbance reading of samples to 50% in comparison with tubes lacking enzymes. POD activity was assayed using a guaiacol reaction. Supernatants were treated with 2 mL of 100 mM phosphate buffer (pH 7) containing 1% guaiacol and 40 mM H_2O_2. Production of the reaction product was monitored spectrophotometrically at 470 nm. Leaf chlorophyll content was determined using a chlorophyll meter (SPAD-502; Minolta, Tokyo, Japan) according to the equipment instructions. The measurements were carried out at 11:00 am in each experiment. The chlorophyll contents of flag leaves from CK and transgenic plants were measured under salt-stress conditions, and six pieces from leaves about each plant were used for the measurements, with five repeats for each leaf. The average value was taken as the relative chlorophyll content of the leaf blade. MDA was measured by a colorimetric method (Stewart and Bewley, 1980). Briefly, 0.5 g of leaf samples was homogenized in 5mL of distilled water. An equal volume of 0.5% thiobarbituric acid (TBA) in 20% trichloroacetic acid solution was added and the sample incubated at 95°C for 30 min. The reaction stopped by putting the reaction tubes in an ice bath. The samples were centrifuged (10000 × *g*, 20 min). After centrifugation, the absorbance of the supernatant was read at 532 and 600 nm. The contents of MDA present were calculated from the extinction coefficient of 155 mM^{-1} cm^{-1}. using the following formula: MDA content (μmol g^{-1} Fw) = (A 532 nm-A 600 nm) $V_1V_2/(156 \bullet V_3 \bullet W)$, where V_1 is the volume of the reaction solution, V_2 is the volume of the extract medium, V_3 is the volume of the testing supernatant, and W is the fresh weight of the leaf.

Statistical analysis

Differences in physiological parameters between transgenic plants and control plants at five NaCl concentrations were determined using ANOVA, with mean separation by Duncan's test using the SPSS 15 software (SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant when the confidence intervals showed no overlap for the mean values with an error of 0.05.

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