



Full Length Article

Overexpression of the *Sorghum bicolor* K⁺/Na⁺ Transporter Gene, *SbSKC1*, Enhances Salt Tolerance in Poplar (*Populus tomentosa*)

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Abstract

The *SbSKC1* gene is instrumental in plant salt stress response. In the current study, the *SbSKC1* gene from *Sorghum bicolor* was overexpressed in poplar, using *Agrobacterium*-mediated genetic transformation, to study the effects of *SbSKC1* on poplar salt tolerance. Differences in salt tolerance between wild type and transgenic poplar were compared by determining phenotypic and physiological indexes for the two strains. Under normal growth conditions, wild type and transgenic plants exhibited normal growth with no significant differences between the two strains. However, upon exposure to 150 mM salt stress, transgenic poplar grew significantly better than wild type, and wild type plants experienced more severe leaf blight than the transgenic plants. At the same time, the chlorophyll content of the transgene changed significantly more than the wild type. Root elongation is obvious, but biomass is not significant. In addition, transgenic plants maintained a high K⁺/Na⁺ ratio. Protective enzyme activities were enhanced in transgenic plants after salt stress. Alterations in malondialdehyde (MDA) levels indicated that transgenic seedlings developed less membrane injury during salt stress. When expression levels of genes related to salt-tolerance were compared in transgenic versus wild-type poplar plants, the results showed that transgenic plants increased expression levels for *ERF76*, *SOD1*, *LEA1*, *P450*, and *Laccase1* genes. We conclude that *SbSKC1* confers salt stress tolerance by maintaining an elevated K⁺/Na⁺ ratio, suppressing membrane damage through enhanced activity of the antioxidant system, and increasing expression of genes related to salt tolerance. © 2020 Friends Science Publishers

Keywords: Poplar; *SbSKC1* gene; Plant biomass; K⁺/Na⁺ ratio; Antioxidant enzyme; RT-PCR

Abbreviation: SOD (superoxide dismutase); POD (peroxidase); CAT (catalase); MDA (malondialdehyde); GUS (β-glucuronidase); *SbSKC1* (*Sorghum bicolor SKC1* gene); 6-BA (6-Benzylaminopurine); NAA (1-Naphthaleneacetic acid)

Introduction

Soil salinization is a universal problem all over the world (Li *et al.* 2012), resulting in growth limitations in most plant species and substantially decreased crop yields (Ruiz-Lozano *et al.* 2012). Crucial metabolic processes, including photosynthesis, growth, energy, lipid metabolism and protein synthesis, are damaged by salinization, causing great harm to the sustainable development of agricultural and forestry (Boscaiu *et al.* 2008; Reis *et al.* 2012). About 1.5 billion hm² of land is salinized in China, and this area is increasing (Yang 2008). Thus, the development of new saline-alkali tolerant tree varieties is urgently needed. In traditional mutation breeding, trait predictability of the target species is relatively poor, and production of new saline-alkali tolerant germplasm is very difficult and inefficient. Transgenic technology can speed up breeding and improve its efficiency by

manipulating and transferring specific genes. For example, transgenic lines of poplar, which grow normally when subjected to 200 mmol/L sodium chloride were generated using *OsNHX1* gene transfer (Wang *et al.* 2005). Salt tolerance was also improved after transformation of *GmNHX1*, *AtNHX1* and *HbNHX1* genes into poplars (Xu *et al.* 2007; Sun *et al.* 2009).

Plants have developed mechanisms to survive the deleterious effects of salt stress, including adaptations in water/osmotic homeostasis and ion balance, damage deterrence (Munns 1993; Chen and Polle 2010; Lata *et al.* 2011), transport and/or compartmentation of Na⁺ and K⁺, and enhancement of the antioxidant system. Ion separation has been the main focus of salt tolerance research and much attention has been paid to maintaining ion homeostasis (Ren *et al.* 2005; Zhang *et al.* 2019), but little research has focused on ion transporters. The *SKC1* gene, which encodes

for a sodium transporter of the HKT family, was originally cloned as a salt tolerant gene in rice. The sodium transport proteins of the HKT family are specific for Na⁺ transport and do not participate in the transport of K⁺, Li⁺, and other cations (Ren *et al.* 2005). Stress resistance in plants is improved by the up-regulation of the *SKC1* gene, triggered by environment stress, such as salt, drought, or low temperature (Yu *et al.* 2014; Yao *et al.* 2017).

To study the effects of *SKC1* on salt tolerance in poplar, the *SbSKC1* gene was cloned from sorghum and transformed into poplar. Salt stress resistance was improved in transgenic plants. Protective enzyme activities and expression of salt-resistant genes were determined to reveal the regulatory mechanism of *SbSKC1* gene in the development of salt tolerance in poplar. The results of this study provide the basis for further development of new varieties of poplar with strong salt tolerance.

Materials and Methods

Plant materials

Poplar sterile seedlings were provided by the Institute of Agricultural Bioengineering of Guizhou University. Transgenic poplar (*Populus tomentosa* Carrière) seedlings were grown at 25°C, in 10–20% humidity, with a 16 h light photoperiod, and in a mixture of nutrient soil and perlite (3:1, v/v) (transplanted one per pot, placed in a greenhouse). Sodium chloride treatment was applied after 6 weeks of transplanting. Plants were treated with 150 mM NaCl solution when the plants when the plant grows 45 d, and subsequent experiments were carried out at the same plant height.

Total RNA isolation

Total RNA was extracted from 0.1 g leaf samples (wild type and transgenic poplar) with a High efficiency Plant RNA Kit (Omega, USA). The cDNA was produced from the RNA template (1.5 µg) with M-MLV reverse transcriptase kit (TaKaRa, Dalian, China) according to the manufacturer's directions.

Cloning of *SbSKC1* and construction of prokaryotic and plant expression vectors

A cDNA fragment of *Sorghum bicolor SKC1* containing an open reading frame was used as a template, obtained by reverse transcription using AP (F:5'-AAGTCCATCTCCGTCCCTAGGCC-3'; R:5'TTAGCCTAGCTTCCATGCCTGACC3') sequence as primers. The PCR product was inserted into T vector (TaKaRa, Dalian, China) for sequencing. The pSH737 vector was cut with *Bam*III and *Xba*I, and the *SbSKC1* sequence was cut with restriction sites. The vector pSH737-*SbSKC1* was produced using T4 DNA ligase. The vector

contains the *SbSKC1* expression element, and the 35S promoter driving the *GUS::NPTII* fusion gene as a screening marker and reporter (Fig. S1).

Plant transformation and transgenic plant detection

SbSKC1 was transformed into poplar using *Agrobacterium*-mediated leaf discs. *Agrobacterium* containing expression vector was suspended in MS liquid medium, and *agrobacterium* concentration OD₆₀₀ was adjusted to 0.6. The poplar leaves were resuspended for 6–8 min and dried the bacteria on the leaves with sterile paper before they were placed on the co-cultivation medium (MS+0.1 mg L⁻¹ NAA) for 2 d at 25±2°C under no light. Then, the co-cultivation leaves were transferred onto the callus induction medium (MS +2.0 mg L⁻¹ ZT+1.0 mg L⁻¹ NAA+200 mg L⁻¹ Timentin+50 mg L⁻¹ Kanamycin pH 5.80), value-added medium (MS +1.0 mg L⁻¹ 6-BA+ 200 mg L⁻¹ Timentin +50 mg L⁻¹ Kanamycin+30 g·L⁻¹ Sugar + 8 g·L⁻¹ agar powder pH 5.80), bud induction medium (MS +2.0 mg L⁻¹ ZT+1.0 mg L⁻¹ NAA+200 mg L⁻¹ Timentin+50 mg L⁻¹ Kanamycin+30 g·L⁻¹ sugar + 8 g·L⁻¹ agar powder pH 5.80), rooting medium (MS + 0.1 mg L⁻¹ NAA+200 mg L⁻¹ Timentin+50 mg L⁻¹ Kanamycin +30 g·L⁻¹ Sugar + 6.5 g·L⁻¹ agar powder pH 5.80). When the stem were 3–5 cm, the resistant shoots were isolated from callus and transferred them to rooting medium, rooted shoots were transplanted into pots. From the bottom to the top take the second and three leaves from each plant were analyzed using PCR (Yao *et al.* 2016) to determine if *SbSKC1* integrated into the plant.

Determination of growth of transgenic plants

Determination of agronomic traits based on this experimental design, the measurement of agronomic traits in three WT plants and three transgenic plants. The plant root length (cm) was measured with a meter ruler. The fresh weight of the transgenic plants after the 10 d stress was measured, the roots were washed with water before weighing, and then the plants were placed in a constant temperature drying oven at 80°C to dry 48~72h, the dry weight was measured when the weight of each tissue was constant.

Measurement of Na⁺, K⁺ and chlorophyll content

Poplar leaves (three wild-type sampling and three transgenic sampling) with consistent growth were washed with deionized water and dried for 15 min at 105°C, then baked at 70°C until there were no changes in weight. The dried material (0.5 g) was placed in a muffle furnace at 500°C until each sample was reduced to white ash. After cooling to room temperature, each sample was dissolved in nitric acid solution and a flame photometer (Flame Photometer 6400, Wincom Co. Ltd., Shanghai, China) was used to determine Na⁺ and K⁺. Measurements for each sample were repeated three times (Wei *et al.* 2012). Each transgenic and WT line

was tested in triplicate and the experiment was repeated three times. Specific chlorophyll concentration was determined using WT and transgenic leaves obtained from each treatment plate of the previously described chlorophyll concentration study. Leaves were blotted dry and 100 mg of tissue from each sample was placed in a 1.5 mL microcentrifuge tube. The samples were re-suspended in 80% acetone, ground with a disposable pestle, and incubated in darkness for 30 min. Total chlorophyll (mg g⁻¹ FW) was determined using absorbance at 645 and 663 nm according to the equation: 20.2 A₆₄₅ + 8.02 A₆₆₃ (Chory *et al.* 2014).

Measurement of MDA content and SOD, POD and CAT activities

Potassium phosphate buffer solution (1 mL, pH 7.8) was combined with 0.1g of plant sample (three wild-type and three transgenic mixed sampling TP1, TP2, TP3). Liquid nitrogen was used to freeze samples before they were ground into a homogenate. Homogenized samples were centrifuged (8000×g, 10 min, 4°C) and the supernatant was put on ice. SOD, POD and CAT activities, as well MDA content (Keming Biotechnology Co., Ltd. Suzhou China) content, were determined according to kit instructions (Zhang *et al.* 2009).

Quantitative RT-PCR analyses

RNA was extracted from Wild type (three wild-type sampling) and transgenic poplar leaf samples (three transgenic sampling TP1, TP2, TP3) (0.1 g) were harvested from similar areas of the plants using RNAiso Plus (TaKaRa, Tokyo, Japan). Total RNA was treated with RQ1RNase-Free DNase (Promega, USA) to clear genomic DNA, after which first-strand cDNA was synthesized (Fermentas, Burlington, ON, Canada). RT-PCR was performed in a CFX100 Real-Time PCR instrument (Bio-Rad Biotechnology Co., Ltd., Hubei China) using SYBR Premix Ex Taq (TaKaRa) according to the manufacturer's instructions. Relative levels of transcripts were determined by normalizing expression against *EF1* gene transcript levels. SYBR Green RT-qPCR assays were utilized for gene expression analysis. Gene expression levels were calculated with the 2^{-ΔΔCT} method. PCR was performed using CFX100 with a standard RT-qPCR reaction mix. The amplification cycling conditions were as follows: 3 min at 98°C, 15 s at 95°C, 1 min at 60°C, for 39 cycles. Each sample was analyzed three times, with three replicates each. Experiments were performed in at least three times. Primer sequences for *SbSKC1* and the salt tolerance-related genes are described (Supplement Table 1S).

Statistical analysis

In the absence of any difference between wild-type and

transgenic plants (NaCl treatment), design of the experiments was completely randomized with three replications. Excel 2003 and SPSS22 were utilized for statistical analyses. For multiple variable comparisons, one-way ANOVA followed by Duncan's multiple range post hoc analysis was utilized, *P* < 0.05 was considered statistically significant.

Results

Characterization of *SbSKC1* and molecular detection of transgenic poplar

The ORF of *SbSKC1* was obtained from *S. bicolor* (Accession #: XM_002457691.1). Based on the sequence information, the *SbSKC1* gene was 1497 bp and encode 498 aa. The cloned *SbSKC1* sequence was consistent with the database coding sequence. In order to better reveal the genetic relationship of *SbSKC1* in various plant species, we constructed a phylogenetic tree using the amino acid sequences obtained from the GenBank database. Blast analysis showed that *SbSKC1* shared a high degree of sequence similarity with *Brachypodium distachyon* (XP_003564102.3) during evolution (Fig. 1). To elucidate the function of *SbSKC1* in poplar; we cloned *SKC1* from *S. bicolor*, transformed into poplar plants, and used PCR amplification to identify *SbSKC1* expression in the transgenic poplar strains. Transgenic poplar strains produced a band at 165 bp, while wild type poplar plants did not (Supplement Fig. S2).

SbSKC1 enhances root elongation, biomass production and chlorophyll concentration

SbSKC1 is crucial to plant responses to various stresses. The practice of transforming foreign genes into plants is a proven method for improving resistance to various abiotic stresses. Therefore, we investigated whether *SbSKC1* improves salt tolerance in poplar. Under normal conditions, wild type and transgenic poplar plants both displayed normal growth. Wild type plants gradually withered, whereas transgenic poplar plants continued to grow normally when exposed to salt stress for 10 d (Fig. 2a). The results showed that after 10 days of salt treatment, transgenic poplar roots (Fig. 2b) increased by 39.4, 81.3 and 32.3%, respectively, and the transgenic poplar biomass increased wild-type poplar but not significant (Fig. 2c). The results showed that the chlorophyll content in TP1 and TP3 increased by 39.25 and 52.88%, respectively, after salt stress (Fig. 2c). These results demonstrated that transforming *SbSKC1* into poplar plants improves their salt tolerance.

Effects of *SbSKC1* expression on Na⁺ and K⁺ concentrations on plants under salt stress

SbSKC1 encodes a sodium ion transporter belonging to the

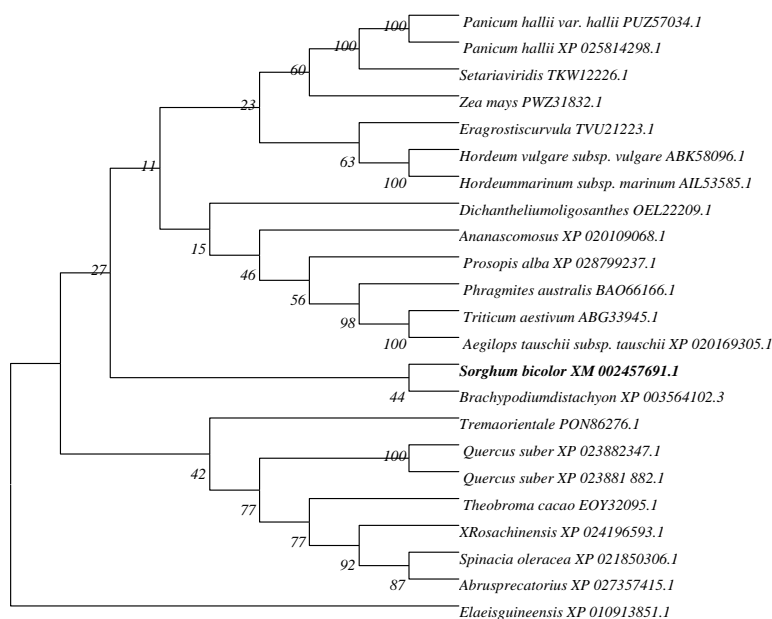


Fig. 1: Phylogenetic tree analysis of *SbSCK1* in poplar and other species. Sequence alignment was performed using Clustal X software, and the phylogenetic tree was created and visualized using MEGA5

Protein sequences used for alignment were as follow: *Zea mays* (PWZ31832.1); *Setaria viridis* (TKW12226.1); *Dichanthelium oligosanthes* (OEL22209.1); *Phragmites australis* (BAO66166.1); *Eragrostis curvula* (TVU21223.1); *Panicum hallii* var. *hallii* (PUZ57034.1); *Triticum aestivum* (ABG33945.1); *P. hallii* (XP_025814298.1); *Aegilops tauschii* subsp. *Tauschii* (XP_020169305.1); *Brachypodium distachyon* (XP_003564102.3); *Hordeum vulgare* subsp. *Vulgare* (ABK58096.1); *H. marinum* subsp. *Marinum* (AIL53585.1); *Ananas comosus* (XP_020109068.1); *Prosopis alba* (XP_028799237.1); *Theobroma cacao* (EOY32095.1); *Spinacia oleracea* (XP_021850306.1); *Abrus precatorius* (XP_027357415.1); *Quercus suber* (XP_023882347.1); *Trema orientale* (PON86276.1); *Q. suber* (XP_023881882.1); *Rosa chinensis* (XP_024196593.1); *Elaeis guineensis* (XP_010913851.1)

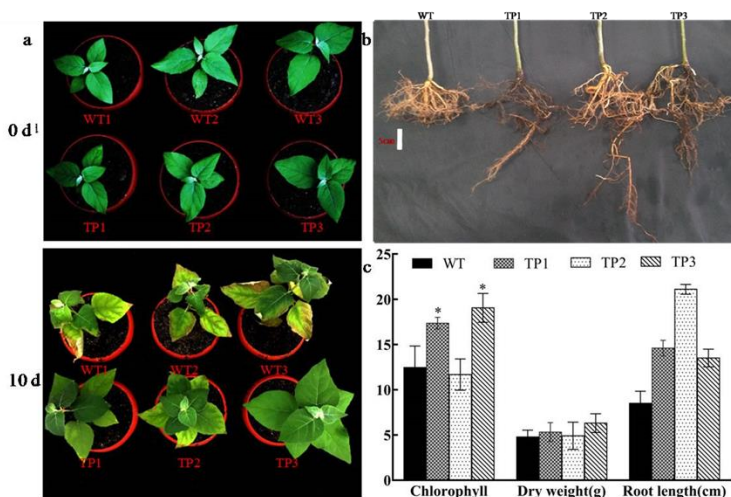


Fig. 2: Analysis of wild type and transgenic poplar plant phenotypes following exposure to salt stress. (a) Plants were relocated to pots with soil and vermiculite (3:1) when they were 10 cm high. After 3 weeks of growth, plants were subjected to 150 mM NaCl for 10 days. After treatment, wild type plants displayed clear signs of salt stress, while transgenic plants displayed little to no damage. WT1-3: wild type poplar, TP1-3: transgenic poplar. (b) Transgenic and wild-type poplar root growth after 10 days of treatment and (c) Chlorophyll content ($\text{mg g}^{-1}/\text{FW}$), root dry weight (g) and root length (cm)

HKT transporter family. To confirm that *SbSCK1* transports Na^+ and/or K^+ in transgenic plants, we examined Na^+ and K^+ levels after subjecting plants to normal and salt stress conditions. Under normal conditions, Na^+ and K^+ levels were similar in wild type and transgenic plants (Fig. 3).

After exposure to salt stress, Na^+ and K^+ levels were increased in transgenic plants compared to levels prior to NaCl exposure. However, compared to wild type plants, Na^+ was decreased and K^+ levels rose in transgenic plants after salt stress (Fig. 3a–b). Thus, *SbSCK1* regulates cation

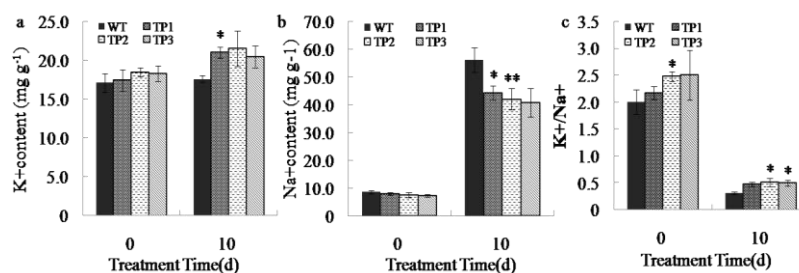


Fig. 3: Ion concentration in wild type (WT) and transgenic (TP1, TP2, TP3) poplar plants before and after 10 d of 150 mM NaCl exposure. WT and TP samples were mixed samples from WT and TP1-3, respectively. Data are shown as means \pm SD. (n=3; **P<0.01, WT vs TP)

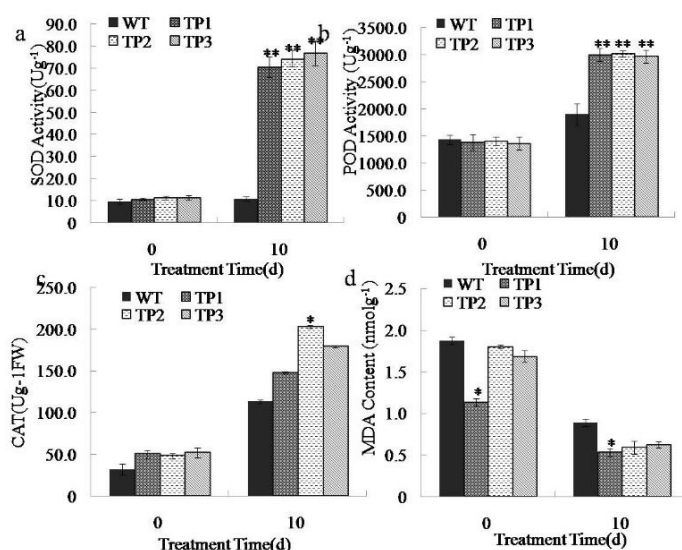


Fig. 4: SOD, POD, CAT activities and MDA levels (a-d, respectively) in wild type (WT) and transgenic (TP1, TP2, TP3) poplar in normal conditions and after 10 d exposure to 150 mM NaCl. Data are shown as means \pm SD from four replicates. (*P<0.05; **P<0.01, WT vs. TP)

content. Furthermore, salt stress induced an elevated K^+/Na^+ ratio in the leaves of transgenic lines compared to wild type plants, as shown in Fig. 3c.

***SbSCK1* expression increases antioxidant enzyme activity after salt stress**

SOD, POD and CAT activities in whole seedlings under normal and salt stress conditions are shown in Fig. 4. No significant differences in SOD, POD and CAT activities were detected when plants were grown under normal conditions. When subjected to salt stress for 10 d, SOD, POD and CAT activities were significantly elevated in transgenic plants compared to wild type plants, indicating that *SbSCK1* improves plant response to salt stress by elevating antioxidant enzyme activities.

***SbSCK1* expression suppresses MDA after salt stress**

Antioxidant enzymes scavenge reactive oxygen species

(ROS) and, thereby, lower membrane lipid peroxidation (Gechev *et al.* 2006). MDA is produced by ROS-mediated lipid peroxidation and is used as a biomarker for ROS-mediated injuries in plants. Under normal growth conditions, no differences in MDA levels were detected (Fig. 4d). In contrast, transgenic lines contained significantly lower levels of MDA compared to wild type plants after 10 d of salt stress (Fig. 4d). These data indicate that transgenic plants developed less membrane damage compared to wild type, and suggest that overexpression of *SbSCK1* protects plants from salt stress-induced membrane lipid peroxidation.

***SbSCK1* influences the expression of salt-tolerant genes**

SbSCK1 transgenic poplar were more tolerant to salt stress than wild type plants, however, the molecular mechanism of this improved tolerance is unknown. We, therefore, investigated the expression of salt-tolerant genes, including *ERF76*, *SOD1*, *LEA1*, *P450* and *Laccase1*, as well as the expression of *SbSCK1* in wild type and transgenic poplar

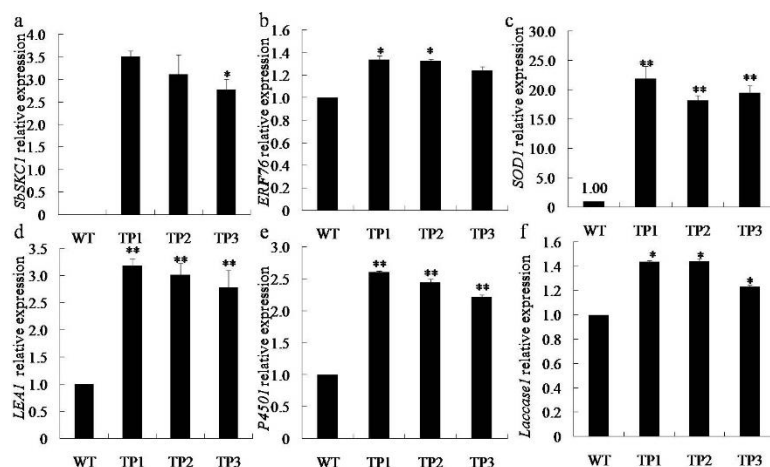


Fig. 5: Salt tolerance-related genes and *SKC1* gene expression in wild type and transgenic plants subsequent to 150 mM NaCl exposure for 10 d. *SbSKC1* overexpression in poplar induced stress related gene expression. Expression levels of *SbSKC1* (a), *ERF76* (b), *SOD1* (c), *LEA1* (d), *P450* (e) and *LacI* (f) in intact leaves were determined using RT-qPCR. Means \pm SD are shown. (n=3; * p<0.05; **P<0.01; WT vs. transgenic)

after 10 d exposure to salt stress. *ERF76* gene expression was 1.34-fold higher (Fig. 5b), *SOD1* was 16.7–21.91 folds higher (Fig. 5c), *LEA1* was 1.72–3.18 folds higher (Fig. 5d), *P450* was 2.23–2.61 folds higher (Fig. 5e), and *Laccase1* was 1.36–321.43 folds higher in transgenic plants compared to wild type (Fig. 5f).

Discussion

When soil contains elevated sodium salts, plants take up more Na^+ and less K^+ . The Na^+ uptake and dissemination throughout the plant govern salt sensitivity (Ruiz-Lozano *et al.* 2012). Na^+ is toxic to cell metabolism, thus, plants have evolved a number of biochemical and molecular mechanisms to manage the deleterious influences of elevated soil salinity. Genes, which are crucial for ionic homeostasis, regulate Na^+ and/or K^+ transport and compartmentation. Ion transporters include the *SOS1* Na^+/H^+ antiporter, the *HKT* transporter family and the *NHX* tonoplast Na^+/H^+ antiporter family (Blumwald and Poole 1986; Munns 2005; Ruiz-Lozano *et al.* 2012). In this paper, salt tolerance was investigated after overexpression of *S. bicolor SKC1* in poplar. Results demonstrate that, compared to wild type plants, salt stress induces a less damaged phenotype in *SbSKC1* transgenic poplar. Transgenic plants displayed only slight leaf wilting, suggesting that *SbSKC1* improves the salt tolerance of poplar plants.

High salt conditions can cause plants to dramatically increase their Na^+ content, resulting in toxicity. The absorption of Na^+ and K^+ by plants is antagonistic (Shen 2010), such that when one cation increases, the other decreases (Wu *et al.* 2010). Plants need to sustain a high ratio of K^+ to Na^+ for proper growth. In poplar expressing *SbSKC1*, we found that K^+ levels average increased by 16.4%, Na^+ average decreased by 27.25%, and K^+/Na^+

average increased by 58.73%, relative to wild type plants. These results indicate that *SbSKC1* overexpression regulates cation content. When comparing antioxidant enzymes in transgenic and wild type plants, no significant differences were observed when plants were grown under normal conditions. In contrast, the activity of SOD was average increased by 616.67% in transgenic plants, the activity of CAT was average increased by 56.8% in transgenic plants, while POD activity average increased by 56.32% compared to wild type plants when both were subjected to salt stress. Furthermore, MDA content average decreased by 30.45% in transgenic plants versus wild type, indicating less membrane lipid peroxidation and the maintenance of proper membrane function under NaCl stress. Thus, *SKC1* transgenic plants display an improved ability to remove free radicals, which facilitates superior growth in salt alkali environments.

The expression of *SKC1* gene affects the expression of salt-tolerant genes. Ethylene response factor (ERF) transcription factors respond to drought and salinity stress (Yan *et al.* 2013), and overexpression of these genes enhances plant resistance to abiotic stress (Hu and Liu 2011; Nakano *et al.* 2006). For example, overexpression of *ERF76* increases salt tolerance in plants (Yao *et al.* 2016). Meanwhile, *SOD1* improves plant disease resistance and late embryogenesis abundant (LEA) proteins inhibit protein aggregation caused by water stress (Goyal *et al.* 2005). Plant salt tolerance is improved by salt stress-induced P450 gene expression (Mao *et al.* 2013). *Laccase* gene expression boosts drought tolerance in transgenic *Arabidopsis* strains, SOD, POD and CAT activities are elevated and MDA levels are decreased in transgenic *laccase* strains compared to wild type plants (Chen *et al.* 2004; Tao *et al.* 2005; Zhang *et al.* 2012). We demonstrate, in this study, that *SbSKC1* gene

overexpression in poplar increases the expression of *ERF76*, *SOD1*, *LEA1*, *P450* and *laccase1* compared to wild type plants following salt stress. These data suggest that *SbSKC1* may be involved in the process of ion osmotic during salt stress, and might function to improve plant salt tolerance.

Conclusion

The overexpression of *SbSKC1* in transgenic poplar increases cation content, antioxidant enzymes, and salt tolerance-related genes to improve salt tolerance. Poplar is somewhat inferior to herbaceous plant models, such as *Arabidopsis thaliana* and tobacco. Nonetheless, transformation of exotic genes in poplar is important for studying gene function and physiology of tree species.

Acknowledgments

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