Transgenic Res (2014) 23:817–826
DOI 10.1007/s11248-014-9808-x

BRIEF COMMUNICATION

# Overexpression of *At*STO1 leads to improved salt tolerance in *Populus tremula* × *P. alba*

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Received: 8 January 2014 / Accepted: 30 May 2014 / Published online: 15 June 2014


**Abstract** One of the major abiotic stress conditions limiting healthy growth of trees is salinity stress. The use of gene manipulation for increased tolerance to abiotic stress has been successful in many plant species. Overexpression of the *Arabidopsis SALT TOLERANT1* (STO1) gene leads to increased concentrations of 9-*cis*-epoxycarotenoid dioxygenase3, a vital enzyme in *Arabidopsis* abscisic acid biosynthesis. In the present work, the *Arabidopsis* STO1 gene (*At*STO1) was overexpressed in poplar to determine if the transgene would confer enhanced salt tolerance to the generated transgenics. The results of multiple greenhouse trials indicated that the transgenic poplar lines had greater levels of resistance to NaCl than wild-type plants. Analysis using RT-PCR indicated a variation in the relative abundance of the STO1 transcript in the transgenics that coincided with tolerance to salt. Several physiological and morphological changes such as greater overall biomass, greater root biomass, improved photosynthesis, and greater pith size were observed in the transgenics when compared to controls undergoing salt stress. These results indicated overexpression of *At*STO1 improved salt tolerance in poplar.

**Keywords** Salt tolerance · Poplar · *Arabidopsis* · STO1 · Pith

## Introduction

Exposure to saline conditions is a detriment faced by many plants regardless of distance from large saltwater sources. According to the USDA nearly 30 % of irrigated lands are of limited use because of salt intrusion, natural weathering or natural rainfall-based accumulation. Irrigation of plants or agricultural crops is the main cause of salt buildup in arid regions and areas where drainage in inadequate to remove excess salt (Khan and Duke 2001). Some of the earliest research data on plant growth in marginal soils indicated that temperature and soil moisture capacity directly affected growth, productivity, and salt tolerance (Ahi and Powers 1938; Hayward and Bernstein 1958). Plants respond to the stress in a number of different ways before visual symptoms become apparent. Increased salt levels disrupt soil osmotic potentials and interfere with the careful ion balances needed for many woody plant species (Atia et al. 2011). In

**Electronic supplementary material** The online version of this article (doi:10.1007/s11248-014-9808-x) contains supplementary material, which is available to authorized users.

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addition to ABA accumulation, salt stress often leads to increased levels of reactive oxygen species (ROS) within the cell (Zhang et al. 2001). These ROS, hydroxyl radicals ($OH^-$), hydrogen peroxide ($H_2O_2$) and superoxide ($O_2^-$) interfere with normal cell functions such as homeostasis. This oxidative stress is responsible for extensive cell injury (Zheng et al. 2009; Krasensky and Jonak 2012). The most common outward appearances of plants suffering from salt stress is often stunted growth however a plethora of responses could be seen based on the plant species or salt concentration (Munns and Tester 2008). In woody plants premature leaf abscission, necrosis, leaf burn, and chlorosis also result from excess salt accumulation.

Poplars, trees from the genus *Populus*, are deciduous broadleaf trees found in North and Central America as well as Europe and Asia. Black cottonwood (*Populus trichocarpa*) was the first tree species to be sequenced (Tuskan et al. 2006) and is now one of the standard model tree species used in research. *Populus tremula* × *P. alba* (717-1B4) is a poplar hybrid that has been used extensively in transgenic research studies worldwide because of its fast growth the ease with which it can be transformed.

In plants, a plethora of research is available concerning genes identified to improve plant tolerances to salt. Expression analysis experiments in *Arabidopsis* have shown that one such gene, ABA DEFICIENT 3 (ABA3), may serve a critical role in regulation of stress-responsive genes and data has suggested that salt, drought, and cold tolerances may be improved by overexpression of ABA3 (Xiong et al. 2001). Studies in maize, oilseed, and rice produced similar results (Tan et al. 1997; Flowers 2004; Yang et al. 2009). Overexpression of a functionally similar $Na^+/H^+$ transport gene (NHX1) in tomato yielded a similar result as transgenic plants growing in 200 mM NaCl for 11 weeks were indistinguishable from non-transgenic plants growing in water alone. Wild-type tomato plants growing in 200 mM NaCl exhibit extreme chlorosis, a wilty phenotype, and extensive cell death (Zhang and Blumwald 2001). The *Arabidopsis* salt overly sensitive (SOS) pathway is essential for conferring salt tolerance in *Arabidopsis*. Overexpression of the SOS1, SOS2 and SOS3 genes also led to improvements in salt tolerance (Zhu 2003; Quan et al. 2007; Yang et al. 2009). When salt stressed, SOS3 (calcium sensor) activates SOS2 (kinase). SOS2 activation favorably regulates SOS1 (sodium/proton antiporter). Thus, these members of the SOS pathway represent a regulatory mechanism for controlling salt tolerance (Shi et al. 2000; Quan et al. 2007). Many genes have roles in salt and drought tolerance. Some of these genes work best in conjunction with others, such as the SOS1, -2, and -3 genes (Zhu 2003; Yang et al. 2009). These SOS genes have been overexpressed in a number of crops including cotton, wheat and tomato. The observed tolerances have been compared to studies with *Arabidopsis* and other crop cultivars (Katiyar-Agarwal et al. 2006; Chinnusammy et al. 2007; Fujibe et al. 2006; Martínez-Atienza et al. 2007). Wang et al. (2010) overexpressed a MnSOD gene (*Ta*MnSOD) from salt cedar (*Tamarix androssowii*) in the poplar hybrid *P. davidiana* × *P. bolleana* to demonstrate increased in salt tolerance in the hybrid. Jiang et al. (2012) overexpressed an *Arabidopsis* vacuolar $Na^+/H^+$ antiporter (*At*NHX1) to demonstrate the conferred improvement in salt tolerance in a *P.* × *euramericana* 'Neva' hybrid poplar. Both of these studies also produced plants able to grow in 200 mM NaCl solutions. Most recently, Tang and Page (2013) overexpressed the late embryogenesis abundant (LEA) gene *AtEm6* which is required for normal seed development in three plant species rice (*Oryza sativa* L.), cotton (*Gossypium hirsutum* L.), and white pine (*Pinus strobes* L.). The authors observed increased expression of $Ca^{2+}$- dependent protein kinases and tolerance to 300 mM NaCl in transgenic cell lines.

STO1 levels have been shown to be decreased in *Arabidopsis* mutants with improved salt stress tolerances (Ruggiero et al. 2004) however recent overexpression studies of STO1 in species other than *Arabidopsis* are lacking in the literature. Earlier studies of *Arabidopsis* STO1 mutants showed increased biomass accumulation during salt stress conditions (Tan et al. 1997; Iuchi et al. 2001). Interestingly, this increase in biomass was not accompanied by an accumulation of ABA, as there normally would be under abiotic stress. Overexpression of the STO1 gene in *Arabidopsis* protected root development and increased salt tolerance in *Arabidopsis* plants exposed to up to 200 mM NaCl (Helaly 2004). STO1 overexpression was also shown to allow transgenic *Arabidopsis* plants to better absorb water due to their more extensive root system (Nagaoka and Takano 2003). At least ten isoforms of proteins with 44–68 % identity to *At*STO1 have been identified in other plant

species (Helaly 2004). This study seeks to examine whether overexpression of the *At*STO1 gene in poplar would lead to an increased tolerance to saline conditions and similar morphological responses to those seen in *Arabidopsis*.

## Materials and methods

### Plant transformation and regeneration

Poplar 717-1B4 (*P. tremula* × *P. alba*) transformations were based primarily on the protocol of Ma et al. (2004), however specific changes were introduced. The *Agrobacterium* strain used here was EHA105 (Cseke et al. 2007) rather than C58. The binary vector backbone used was pBI121 rather than pART27 to carry the complete cDNA sequence of the *Arabidopsis* STO1 gene for this work. Kanamycin concentrations used for selection were 50 mg $L^{-1}$ rather than 25 mg $L^{-1}$ (Cseke et al. 2007). Use of timentin was restricted to 200 mg $L^{-1}$ as opposed to 400 mg $L^{-1}$ during the pre-selection process. Pre-culture on callus induction medium for both 717-1B4 and NM6 genotypes was done for 12 days rather than 14 days based on data from Noël et al. (2002). In addition, SOC media was used instead of LB media to improve transformation efficiencies. To control *Agrobacterium* contamination 1.6 mM Timentin (300 mg $L^{-1}$) was added to callus and shoot induction media. Poplar shoot cultures were grown under cool-white light (275 μmol $m^{-2}s^{-1}$) at 22–24 °C (16:8) on shelves in a growth room. Transformations were verified via PCR and the sequencing of several randomly selected PCR products to confirm target gene amplification. This project produced 15 transgenic lines for use in salinity tolerance studies after PCR characterization.

### PCR and RT-PCR analysis

Genomic DNA from *Arabidopsis* leaves was isolated using TRIZOL reagent (Invitrogen) and the resultant concentrations determined using a Nanodrop 8000 (Thermo Scientific). Total RNA was extracted from leaves of putative transgenic and wild-type poplar plants for subsequent RT-PCR using the RNeasy plant mini kit (Qiagen). The resultant RNA concentration was determined using a Nanodrop 8000 (Thermo Scientific). Five micrograms of DNA were reverse-transcribed using iScript cDNA Synthesis kit (Bio-Rad), and 1 μL of cDNA was used as a PCR template. PCR primers were designed based on sequences provided by TAIR (http://www.arabidopsis.org) and GenBank (NCBI; http://www.ncbi.nlm.nih.gov/). The *At*STO1 gene was amplified using F1 (5′-GAAAAAT GGCTTCTTTCACGG-3′) and R1 (5′-GCAGAGCAT CCCACTGGTAA-3′). Additional data to confirm that the amplicon was the *At*STO1 gene were obtained by cloning the PCR product into a T-easy cloning vector (Promega) and sequencing the product.

### Salt tolerance assays

For verification of salinity tolerance, three transgenic lines were selected from the eighteen lines generated. These lines were selected based on performance in preliminary salinity tolerance experiments in vitro. Preliminary salt concentrations were 0, 50, 75, 100, 125, 150, 175, 200, 250 and 300 mM. No significant differences were displayed between 0 and 50 mM (data not shown) and 100 and 125 mM exhibited very similar responses (data not shown) after performing a factorial analysis. Concentrations that induced mild, moderate, and severe responses in WT plants during in vitro analysis were selected for further experimentation. Concentrations above 200 mM resulted in chlorosis and leaf burns. Transgenic and WT poplar plants were transferred from the lab to the greenhouse 1 week after acclimatization. Poplar plants were grown under in a combination of natural and cool-white light (275–400 μmol $m^{-2}s^{-1}$). Temperature was maintained between 22–24 °C (16:8) within the greenhouse zone.

Each of the plants were transferred to round (25.4 cm × 30.5 cm) pots filled with a 4:1 soil mixture of Sun Gro Redi Earth plug and seedling mix (Sun Gro Horticulture). All plants were fertilized upon initial placement in the greenhouse and every 3 weeks thereafter with acidified water supplemented with a combination of two water-soluble fertilizers (3:1 mixture of 21 N–2.2P–16.6 K and 15 N–2.2P–12.5 K, respectively; The Scotts Co., Marysville, OH) to provide the following (in mg $L^{-1}$): 200 N, 26 P, 163 K, 50 Ca, 20 Mg, 1.0 Fe, 0.5 Mn and Zn, 0.24 Cu and B, and 0.1 Mo. This nitrate form was 76 % of nitrogen provided. Irrigation water was supplemented with 93 % sulfuric acid (Brenntag, Reading PA) at 0.08 ml $L^{-1}$ to reduce alkalinity to 100 mg $L^{-1}$ and pH to a range of 5.8–6.2. (http://www.hort.

purdue.edu/hort/facilities/greenhouse/soilFert.shtml). The plants were placed in watering trays filled with liquid fertilizer for approximately 15 min before the trays were emptied and the plants were placed back on the greenhouse benches. Plants were watered every 2 days with water by placing individual pots onto watering trays (28 cm × 43 cm × 5 cm) that were constantly refilled over the course of 15 min. The pots were then removed and allowed to drain when replaced on the greenhouse bench. Salinity tolerance tests began 6 mo after plants were placed in the greenhouse. Solutions of 75, 150, and 200 NaCl were used in lieu of water over the course of 30 days. Salt solution treatments occurred every 2 days. There were three replicates per treatment and this study was conducted in triplicate.

### Toluidine blue staining

Staining of cross-section tissues of control and transgenic plants was performed based on the protocol by O'Brien et al. (1964). These cross-sections were used to visually enhance morphological changes to the pith in response to growth in saline conditions.

### Measurement of chlorophyll content

Chlorophyll contents were determined using the acetone extraction method (Schaper and Chacko 1991). A 1 g sample of fresh leaf tissue was homogenized in 10 ml of chilled 80 % acetone before transferring to a light-safe tube to prevent chlorophyll degradation. The extract was centrifuged at 6,000 rpm for 3 min at 4° to pellet the leaf material. Each sample was done individually to prevent bias as acetone continues to leach over time. The supernatant was removed and diluted to 100 ml with chilled 80 % acetone before determining absorbance. Chlorophyll concentration was determined using the following formula: $Chl_{ttl} = 7.15\,(A_{663}) + 18.71\,(A_{646})$ (Hasbullah et al. 2012). Samples were collected and extracted in triplicate from three individual plants. No plant was sampled more than once during the study.

### Physiological and vegetative measurements

Net photosynthetic rate ($P_{net}$), stomatal conductance (gs), and intercellular $CO_2$ ($C_i$) concentrations were measured with a steady-state gas-exchange system (for photosynthesis–transpiration) that incorporates an infrared gas analyzer (LI-6400×T, Li-Cor, Lincoln, Nebraska, USA) 24 h after the final salt treatment in the greenhouse. Physiological measurements were collected at 10 am from the third fully formed leaf from the top of the plant. Plant heights and basal stem diameters were determined at the end of the 30 days study using a soft fabric tape measure. Stem sections were collected 10 cm above the root collar for each plant. Whole plants were removed from pots, rinsed profusely with deionized water, and dried at 70 °C for 72 h before dry weights were measured. Root length data during in vitro analysis was collected by adding water to the cultures to loosen the media from the jar. The entire plantlet was removed and carefully soaked in warm water to separate the gel media from the roots. Lengths were measured after all media had been removed. These length measurements were not included in the study as many fine roots were damaged in the process. Observations were based on visual inspection and photographic analysis. Root biomass data were also collected at the end of the study. Roots were excised from the stem using a razor blade and weighed.

### Statistical analysis

All measurements were taken in triplicate from three individual plants per treatment and variable. Calculation of standard error of the raw means (±SEM) and use of one-way analysis of variance (ANOVA; Model I) techniques were used to determine significance of vegetative growth and root biomass data results. Results of $p < 0.05$ were deemed significant (Tukey's HSD). All measurements for statistical analysis were the result of $n = 3$. All analyses of statistical data were performed using SAS software (SAS Institute Inc. 2008).

## Results

### Generation and validation of transgenic poplar

Agrobacterium-mediated transformation of poplar 717-1B4 (*P. tremula* × *P. alba*) with the pBI121 overexpression vector carrying the cDNA sequence of the *Arabidopsis* STO1 gene led to the development of eighteen transgenic lines initially identified by screening for kanamycin (50 mg $L^{-1}$) resistance. Transformation and subsequent propagation efforts produced

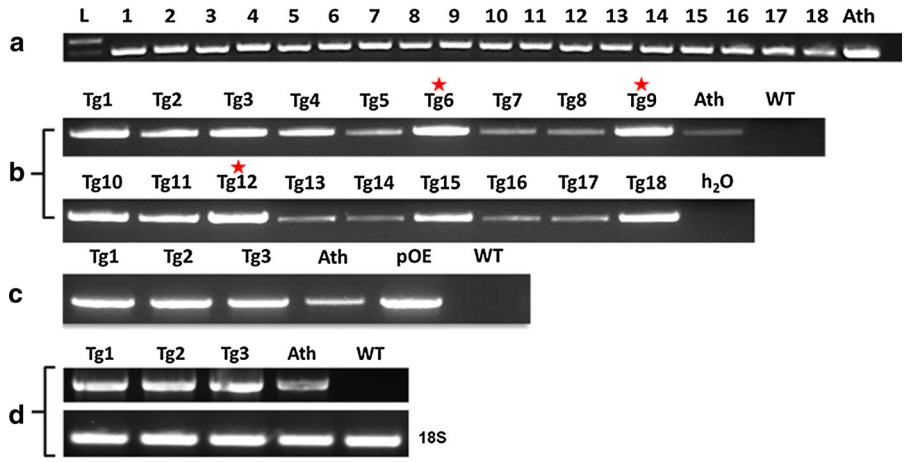


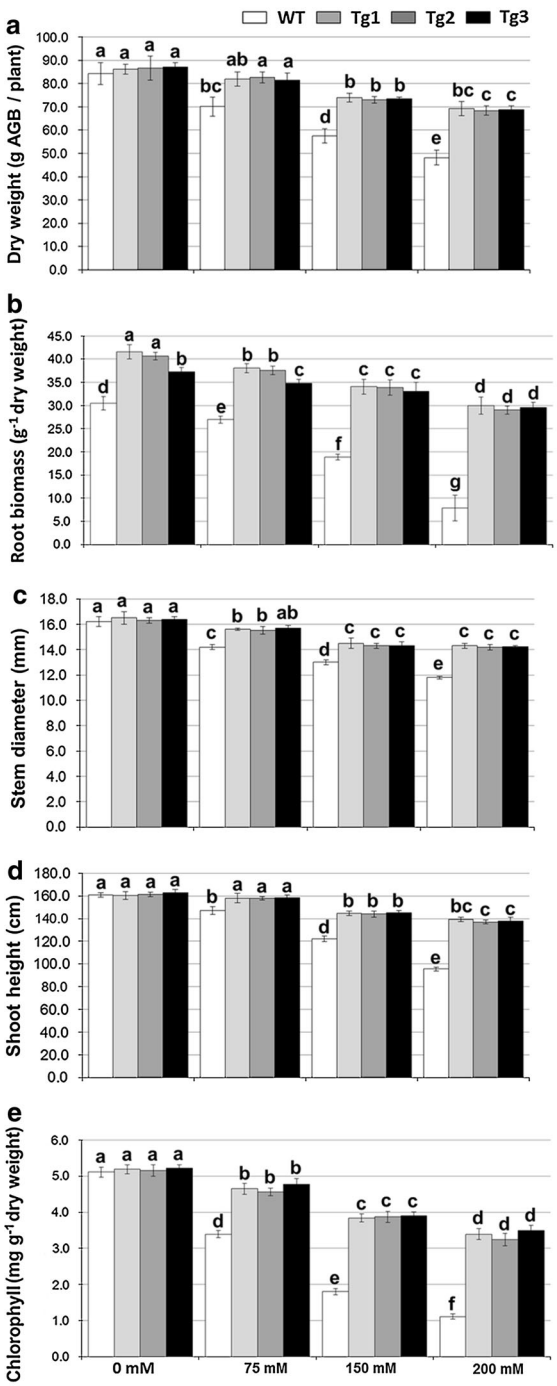

**Fig. 1** Verification of transgenic poplar. **a** PCR amplification of the *At*STO1 gene in 18 individual *poplar lines* and an *Arabidopsis* control plant. **b** RT-PCR verification of *At*STO1 expression in the 18 transgenic lines while in vitro. Those lines selected for further study were starred. **c** Test of RT-PCR expression in the three *selected lines*, an *Arabidopsis* plant, a pooled cDNA sample of other putative transgenic poplar, and a non-transgenic (WT) poplar sample after 2 weeks in the greenhouse. **d** RT-PCR of poplar transgenics 6 mo after being transferred to the greenhouse

50 ramets each of the 18 independent transgenic poplar lines carrying the *At*STO1 gene. Each plantlet was further verified with PCR amplification of the entire 1,800 bp coding sequence of the *Arabidopsis* STO1 gene once plantlets were rooted in tissue culture (Fig. 1a). All 18 transgenic lines were further validated by semi-quantitative RT-PCR while still in vitro (Fig. 1b). The three lines chosen for further study were transferred to the greenhouse and retested for the *At*STO1 gene after 2 weeks (Fig. 1c). Analysis of RT-PCR data showed that all three poplar lines continued to express the *At*STO1 gene 6 mo after being transferred to the greenhouse, an indication that the expression was stable rather than transient (Fig. 1d).

## Effect of salinity stress on poplar

Efforts to determine if the transgenics were better able to withstand salt stress were initiated 6 mo after plants were acclimated to the greenhouse from tissue culture. Comparison of dry weight of aboveground biomass (AGB) between transgenic and WT control plants in the greenhouse indicated that no differences were observed under normal watering conditions. Significant variations in AGB were only seen upon initiation of the salt study (Fig. 2a). Notable differences in root dry weight, stem diameter, and shoot height were seen when plants were exposed to salt treatments. Transgenics were able to survive without visible adverse effects when watered with several increasingly stringent concentrations of NaCl while WT plants decreased in root dry weight by 34, 46, and 74 % when compared to untreated control plants. This study also revealed that overexpression of *At*STO1 increased lateral root growth by an average of 29 % and overall root length by nearly 70 % among poplar transgenics (Supplemental Fig. 1). Stem diameter decreased by 13, 20, and 28 % while shoot height decreased by 7, 23, and 40 % respectively when compared to initial values (Fig. 2b–d). Examination of chlorophyll content indicated that WT and transgenic plants maintained the same chlorophyll content under normal conditions however both groups had decreased chlorophyll contents during the salt stress period. WT plants had a very significant decrease of 77 % while transgenic lines averaged a decrease of 36 % at the highest salt concentration (Fig. 2e).

Analysis of poplar lines overexpressing *At*STO1 also showed that these lines demonstrated greater net photosynthetic rates ($P_{net}$), stomatal conductances (gs), and intercellular $CO_2$ concentrations ($C_i$) upon initiation and throughout the course of the salt study than control plants (Fig. 3). There were morphological changes visualized between transgenics and control plants in addition to the described physiological changes. Control plants displayed larger pith sizes




◀ **Fig. 2** Effect of salinity on growth characteristics of poplar plants grown under greenhouse conditions. **a** Total plant aboveground biomass (AGB) data were collected and compared at the completion of the 30 days salt study. **b** Root biomass, **c** stem diameter, **d** shoot height, and **e** chlorophyll content data were also collected and graphed after the 1 mo salt treatment. Means with the *same letter* were not significantly different at $p < 0.05$ (Tukey's HSD). *Error bars* (±SEM)

than transgenics after salt exposure in this study (Fig. 4).

As seen in *Arabidopsis*, the overexpression of STO1 led to an increased tolerance to saline environments in the poplar transgenics generated in this study (Fig. 1, Supplemental Fig. 2, 3). Data results indicated that transgenic plants generated in this study maintained significantly greater shoot heights, stem diameters, root biomass volumes, and total AGB than control plants (Fig. 2a–d). Differences in overall root lengths were also identified. Similar results were observed in a study where the poplar gene *PeHA1* was overexpressed in *Arabidopsis* (Wang et al. 2013). Interestingly, the control plants maintained lower $P_{net}$, gs, and $C_i$ than transgenics even before being watered with saline water an indication that the transgenic plants possessed enhanced gas exchange capabilities (Fig. 3). This phenomenon has been reported in other transgenic studies involving the overexpression of salt tolerance genes (Jiang et al. 2012; Gao et al. 2013).

## Conclusion

Overexpression of *At*STO1 explained the ability of transgenics to withstand the salt treatments and to generate greater biomass than controls. Improvements in physiological traits of transgenics when compared to control plants were uncovered when data from gas exchange measurements were graphed. Deficiencies in growth and development and photosynthetic activity are often the result of salt stress (Han et al. 2013) as observed in the control plants however transgenic lines were able to assimilate greater amounts of $CO_2$ than controls before and while being challenged with increasing saline concentrations. The transgenics had a much more gradual decrease in photosynthetic activity thus providing an explanation for the generation of greater volumes of biomass. Ruggiero et al. (2004) noted that interference with proper functioning of NCED3 led to non-linear, and currently unexplained, phenotypic changes. A possible theory to explain this is that overexpression of *At*STO1 led to a decrease in ABA synthesis. Stomata could have remained open longer therefore prolonging gas exchange and biomass assimilation. As transgenic

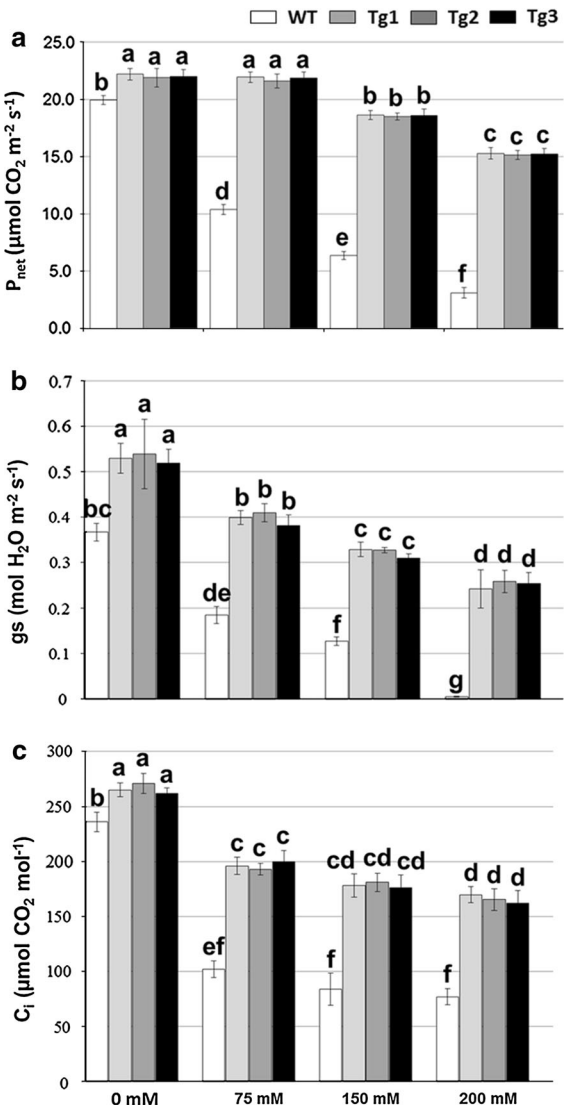



**Fig. 3** Effect of salinity on gas exchange of greenhouse-grown poplar. **a** Net photosynthetic rates ($P_{net}$), **b** stomatal conductances (gs), and **c** intercellular $CO_2$ concentrations ($C_i$) of transgenic and wild-type (WT) poplar 24 h after completion of the salinity experiment. Means with the *same letter* were not significantly different at $p < 0.05$ (Tukey's HSD). *Error bars* (±SEM)

plants showed few water stress symptoms, it is proposed that despite stomata remaining open longer; transgenics had greater water resources available and were not stressed. Biomass accumulation slows and ABA levels rise during periods of osmotic stress in *Arabidopsis STO1* mutants (Iuchi et al. 2001; Ruggiero et al. 2004). Here, control plants were significantly deficient in root biomass assimilation when compared to transgenics when watered with water only.

Plant responses to salt stress in other species have included not only changes to external morphology but internal morphology as well. Adnan et al. (2013) noted increased salt concentrations resulted in increased pith cell areas in 3 cultivars of calico plant (*Alternanthera bettzickiana* (Regel) G. Nicholson) foliage. Nargis et al. (2013) noted an increase in pith corresponded to increased levels of salinity in the desert halophyte *Aeluropus lagopoides* (Linn.) Trin. ex Thw. Studies in soybean (*Glycine max*) have revealed that salt water exposure led to increased pith sizes and decreased xylem vessel diameters (Dolatabadian et al. 2011). Previous studies have shown that plant responses to variation in gibberellin metabolism have included altered biomass accumulation, $P_{net}$, and pith sizes (Achard et al. 2006; Biemelt et al. 2004; Bonawitz and Chapple 2010; Colebrook et al. 2014). In this work, increased pith was observed in control plants when challenged with saline conditions however there was no observable increase in pith among the transgenic plants generated (Fig. 4). The increased pith size of control plants exposed to saline conditions compared to transgenics in this work hints that gibberellin metabolism may be altered when *At*STO1 is overexpressed. No significant or observable differences in the other growth characteristics were identified before salt water treatments. Numerous significant differences between controls and transgenics were observed under increasingly stringent saline concentrations.

In conclusion, these data confirmed that overexpression of *At*STO1 improved shoot and root biomass and salt resistance in transgenic poplar. It is likely that these poplar transgenics are better able to segregate $Na^+$ ions to the vacuole as has been seen with the overexpression of several other salt tolerance genes (Leidi et al. 2010; Jiang et al. 2012). Other plausible theories are that apoplastic barriers in the roots prevent $Na^+$ ion flow and improve plant tolerance and survival as described by Krishnamurthy et al. (2011) and Han et al. (2013). Overexpression of STO1 in *Arabidopsis* led to the overexpression of NCED3 thus it is reasonable to conclude that this same response occurred in poplar. Enhancement of a gene vital in ABA biosynthesis is another possible explanation for the improved biomass accumulation seen in poplar transgenics before being challenged with saline conditions. The removal of excess $Na^+$ ions to the vacuole

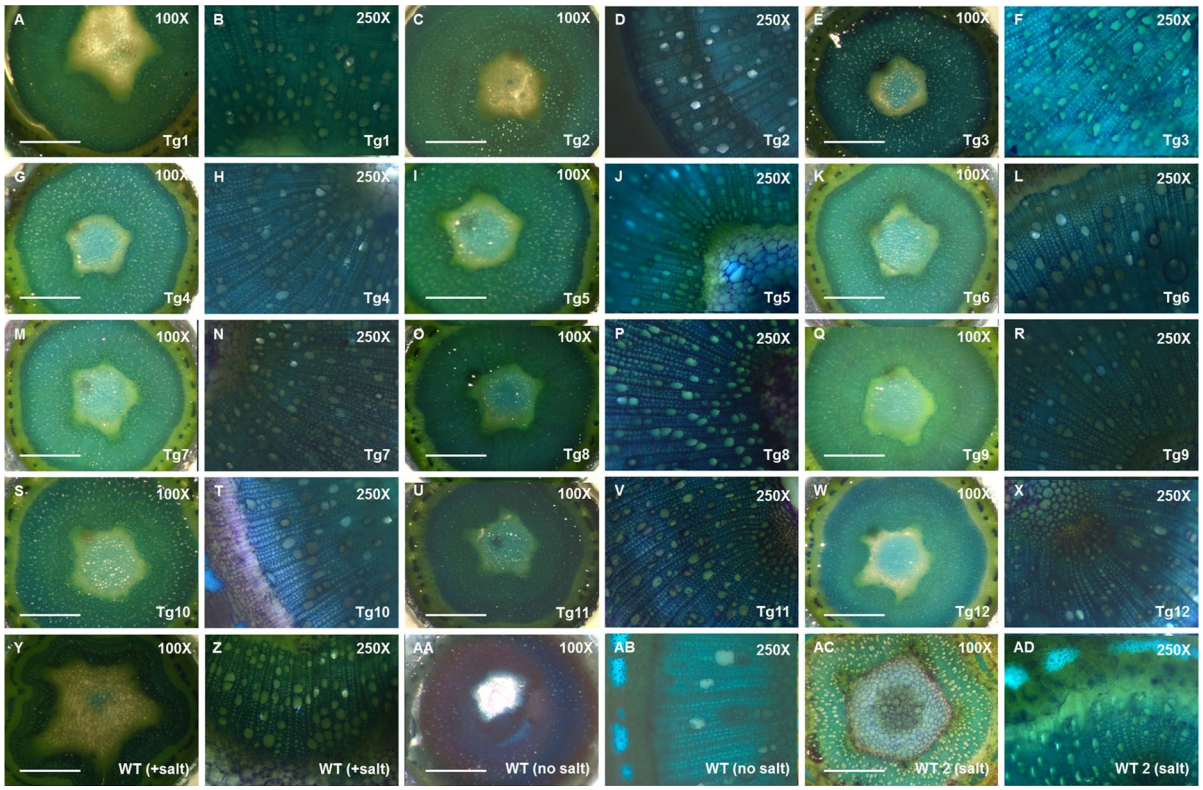



**Fig. 4** Examination of pith sizes in control and transgenic poplar seedlings. **a**–**x** Cross-section views of transgenic plant piths and xylem and **y**–**z** 717-1B4 (WT) after 30 days exposure to 200 mM NaCl. **aa**–**ab** *Cross section* of 717-1B4 (WT) without salt exposure and **ac**–**ad** a second hybrid poplar genotype *P. nigra* × *P. maximawiczii* (WT2) after 30 days with saline exposure. Pith images at ×100. Xylem images at ×250. *Bar* = 250 μm

may be activated by overexpression of *At*STO1 and potentially a poplar version of *At*NCED3. Sequestration of $Na^+$ ions to the vacuole could be the regulatory mechanism used by these poplar transgenics to survive saline conditions.

These theories are credible based on studies that show poplar genes can function in *Arabidopsis* and vice versa; therefore, it is also possible that downstream epistatic effects could be similar. Although a more thorough account of potential mechanisms of action for this gene were not available for poplar, these results demonstrate a possible method for conferring salt tolerance in poplar without intricate manipulations such as gene stacking. Additional studies will be needed to further characterize the effect of this gene on plantation grown poplar trees however, at present, *At*STO1 overexpression is a conceivable method for both engineering salt tolerance and improved biomass accumulation in poplar.

**Acknowledgments** The authors would like to thank Drs. Jaemo Yang and Zhonglian Huang for their comments and critiques of a previous version of this manuscript. Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the US Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that also may be suitable.

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