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# OVER EXPRESSION OF A VACUOLAR $H^+$-ATPase C SUBUNIT GENE MEDIATES PHYSIOLOGICAL CHANGES LEADING TO ENHANCED SALT TOLERANCE IN HYBRID POPLAR

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## Abstract

The salt stress response of *Puccinellia tenuiflora* (Grisebach) Scribner & Merrill vacuolar $H^+$-ATPase c subunit gene (*PutVHA-c*) was studied. Transgenic *Populus* plants for overexpressing *PutVHA-c* gene was generated following *Agrobacterium*-mediated genetic transformation using explants of a hybrid poplar plant. PCR, Southern and Northern blot analysis showed that the *PutVHA-c* gene was integrated and expressed in the transgenic *Populus* plants successfully. Compared to the wild-type (WT), transgenic punching leaves experienced less injury than the WT punching leaves after soaking in solutions of NaCl and $NaHCO_3$. Under the different salt stress conditions, superoxide dismutase (SOD) activities of transgenic *Populus* were enhanced significantly whereas malondialdehyde (MDA) level was lower than WT *Populus* significantly, moreover the soluble sugar content was also higher in transgenic *Populus*. Therefore, the overexpression of *PutVHA-c* gene in transgenic *Populus* cause biochemical changes that are known to correlate with improved salt tolerance.

## Introduction

Vacuolar $H^+$-ATPase c (*VHA-c*) genes have been cloned from some plant species, such as *Tamarix hispida* (Willd) (Gao *et al*. 2011), *Mesembryanthemum crystallinum* (Linn) (Tsiantis *et al*. 1996) and *Pennisetum glaucum* (Leeke) (Tyagi *et al*. 2005). *VHA-c* gene expression was affected by environmental stresses according to previous research and it is reported to be induced by the salt stress in *M. crystallinum* (Tyagi *et al*. 2005, Low *et al*. 1996). In *P. glaucum*, *VHA-c* gene's transcripts were also increased by salinity and drought stresses (Tyagi *et al*. 2005). The *VHA-c* gene from *Spartina alterniflora* (Linn.) Loisel enhanced the tolerance of salt (Baisakh *et al*. 2012). In addition, *VHA-c* gene was found to be overexpressed in plants under different stress conditions, such as low temperature stress, osmotic stress, salt and heat (Tyagi *et al*. 2005, Kluge *et al*. 2003, Lehr *et al*. 1999, Chen *et al*. 2002).

*P. tenuiflora* a monocotyledonous halophyte species is planted in Songnen plain of northeast China which is the saline-alkaline in nature. The salt content of saline-alkaline soils is high, the water and nutrition contents are low. So the poor soil conditions limit the crop growth directly. In this study, the *VHA-c* gene (*PutVHA-c*) was used to transform a hybrid poplar plant. Poplar has several exceptional qualities, such as a high capacity for vegetative propagation and a fast growth rate. *Populus davidiana* Dode × *P. bollena* Lauche is the hybrid cultivar of poplar that uses *Populus bolleana* as male parent and *Populus davidiana* Dode as female parent, which is planted on a significant scale in northeast of China due to its characteristics as graceful shape, cold resistance, fast growth, and suitability to develop transgenic progeny. A hybrid poplar *Populus davidiana* Dode × *P. bollena* Lauche was chosen for transformation. *Populus* was planted in northeast China and has a well-characterized morphology with cold resistance features.

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Although *VHA-c* has been used to transform successfully in a wide range of plants. Gramineae has received little attention for transformation of woody plant. Under these circumstances in the present study, a hybrid poplar plant was attempted to transform using a Vacuolar $H^+$-ATPase c subunit gene obtained from *P. tenuiflora*.

## Materials and Methods

The hybrid poplar *(Populus davidiana* Dode × *P. bollena* Lauche) was selected for transformation. *Populus* leaf explants of a suitable leaf age (30-days-old) were obtained from *in vitro* grown plantlets. The WT and transgenic *Populus* leaves were soaked in solution containing 60 mM $NaHCO_3$, 175 mM NaCl, and the $H_2O$ as control.

Transgenic *populus* was generated using an *Agrobacterium*-mediate protocol (Han *et al*. 2013). $OD_{600}$ = 0.8-1.0 (*Agrobacterium* concentration), and 20-30 min (infection time) were used for transformation. To obtain regenerated transgenic shoots, the MS medium containing 0.2 mg/l NAA, 0.5 mg/l BA, 200 mg/l cefotaxime and 50 mg/l kanamycin was used for leaf discs cultivation following infected with *Agrobacterium.* When the kanamycin-resistant adventitious shoots grew to about 2 cm in length, they were transferred to the MS medium with 0.5 mg/l NAA, 50 mg/L kanamycin, 200 mg/l cefotaxime for root formation.

*DNA extraction and PCR analysis:* The genomic DNA of 16 transgenic lines and wild type plants were isolated following CTAB method (Porebski *et al*. 1997). The primers 5 - ATGTCG TCGGTG TTCAGCG -3 and 5 - ATCTGCGCGGGATTGGCCG -3 were used to amplify a 498-bp fragment corresponding to the coding region of *VHA-c* gene.

RNA was extracted from WT poplar and 7 independent transgenic *Populus* leaves following the manufacturer's instructions of RNeasy Mini Kit (Qiagen) for total RNA extraction. Southern and Northern hybridization was done according to Sambrook *et al*. (1989). LAS-4000 plus image analyzer was used for the signal detection (Fuji Film, Tokyo, Japan).

*Analysis of anti-oxidation injury:* The punching leaves from the three transgenic plants lines (lines 1, 2, 3) and the WT were soaked in solutions of 175 mM NaCl and 60 mM $NaHCO_3$ for 72 hrs.

*Analysis of superoxidase, soluble sugar content and malondialdehyde content:* WT and three transgenic *Populus* lines (lines1, 2, 3) and WT were under the $NaHCO_3$ and NaCl stress testing. The 3-5 cm plants containing 4 leaves were cultivated in the medium of rooting (MS + 0.5 mg/l NAA) containing 100 mM NaCl and 3 mM $NaHCO_3$ for 7 days, respectively. The leaves were harvested from each treatment for analysis. In each sample superoxidase activity was measured by the method described by Wang *et al*. (2010). The procedure of soluble sugar contents and malondialdehyde content extraction and analysis were determined as described previously (Hodges *et al*. 1999, Leon *et al*. 2001, Klimov *et al*. 2002).

## Results and Discussion

The optimization and the transgenic plants development are presented in Fig. 1.

*The assay of PCR:* The *PutVHA-c* gene was transferred into *Populus* by *Agrobacterium*-mediate protocol. Following to kanamycin screening, 16 putatively transformed plants were obtained. All the putatively transformed *Populus* plantlets and WT were analyzed by PCR. From Fig. 2, it is apparent that 15 transforments contained pBI121-PutVHA-c plasmid segment (498 bp), whereas WT had no band. Thus the *PutVHA-c* gene was transformed into the 15 individual samples successfully.

*Southern and Northern blot analysis:* Seven independent transgenic lines genomic DNA were used for Southern blot analysis. All the lines contained the distinct bands, except the lane 1, which had non-specific DNA bands (line1, Fig. 3a). As shown in Fig. 3a, the T-DNA integrated into the poplar genome randomly.

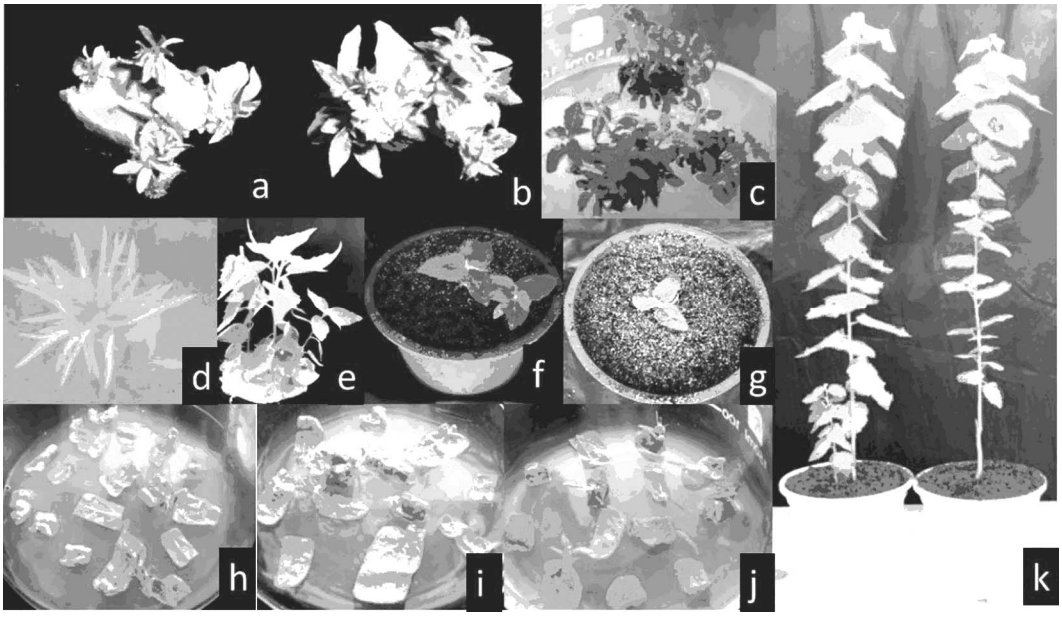


Fig. 1. High frequency plant regeneration, shoot induction, rooting, micropropagation and high frequency transformation of *Populus davidiana* Dode × *P. bollena* Lauche. a and b. Regenerated shoots from leaf explants, c. Elongation of regenerated shoots. d. Roots regenerated. e. Regenerated plantlets with medium from flask; f and g. Growth of regenerated plantlets in pots after 20 d of transfer. h i and j. Transgenic shoots differentiation. k. Growth of regenerated plantlets in pots after six months of transfer.

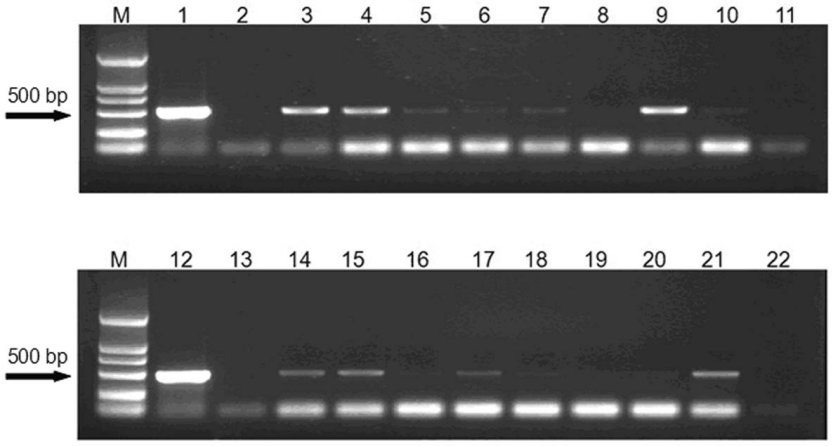


Fig. 2. PCR analysis of total genomic DNA isolated from transformed and WT plants by amplification of the *VHA-c* gene. Lane M, DL2000; Lane1 and 12, plasmid pBI121; Lane2, 13, WT plants; Lanes 3-10, Lanes 14-21, transgenic *Populus* plants; Lanes 11, 22, $H_2O$.

From the Northern blot analysis, the WT plant had no hybridization signal (Lane 1, Fig. 3b), and transgenic lines had a distinct band, which predicted *PutVHA-c* mRNA as seen from lines 2-8 and Fig. 3b. The results showed that the seven transgenic poplar lines 2-8 were integrated and expressed by *PutVHA-c* gene successfully (Fig. 3b).

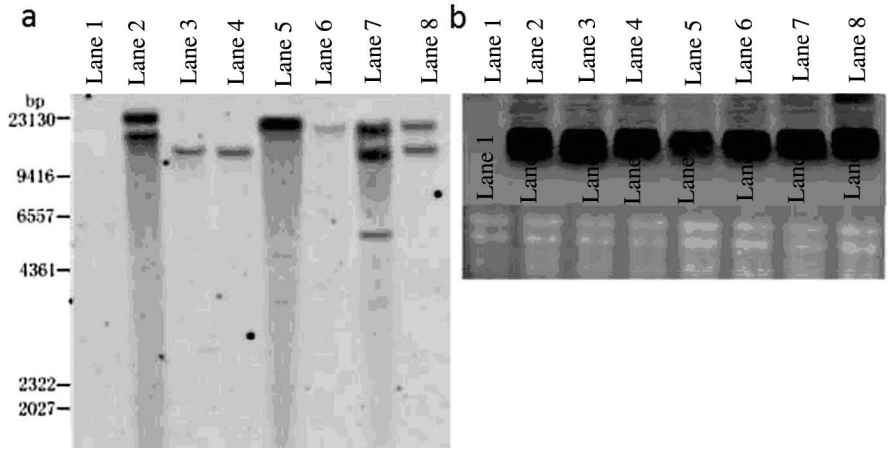


Fig. 3. (a) Transgenic poplar Southern blot analysis. Lane 1: control, WT poplar, Lanes 2-8: transgenic *populus* lines; (b) Northern blot analysis. Lane 1: control, WT poplar, Lanes 2~8: transgenic *Populus* lines.

*The leaf anti-oxidation injury analysis:* The results in Fig. 4. For 72 hrs, the transgenic leaves experienced less injury than WT leaves. Based on the results, the *PutVHA-c* gene improved the capacity of *Populus* for salt resistance.

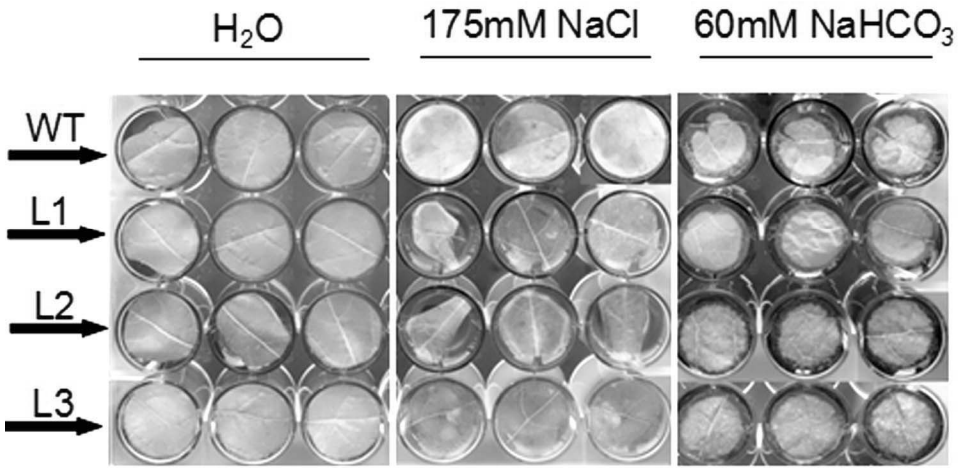


Fig. 4. Anti-oxidative injury of WT *Populus* and transgenic *Populus* leaves under salinity.

*Transgenic and WT plants SOD activity analysis:* SOD play an important role in protecting aerobic organisms, which are damaged by oxygen toxicity (Bennicelli *et al.* 1998). The SOD activity under the NaCl and $NaHCO_3$ treatments are presented in Fig. 5a,b. Transgenic plants SOD

activity was elevated under normal conditions markedly. Under the salt stress, the WT and transgenic plants SOD activity increased. The WT plants SOD activities were lower than the transgenic plants. These results suggested that *PutVHA-c gene* overexpression can increase SOD activity and improved the transgenic *Populus* for salt resistance.

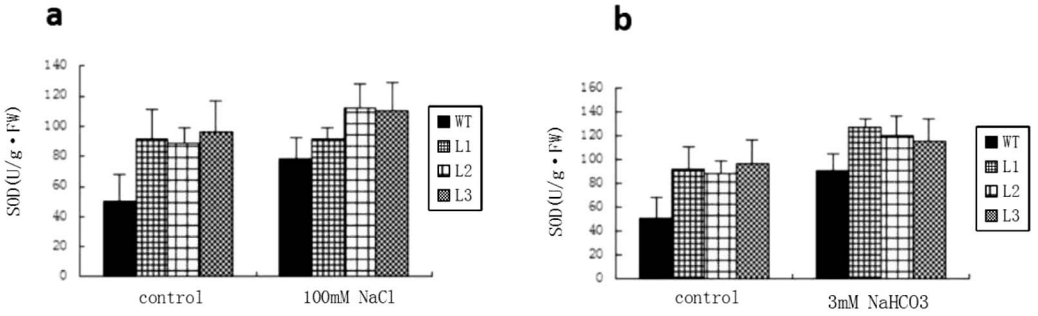


Fig. 5. SOD activity of WT *Populus* and transgenic *Populus* leaves with (a) 100 mM NaCl and (b) 3 mM $NaHCO_3$.

*Transgenic and WT plants MDA levels analysis:* MDA which causes membrane lipid peroxidation, is a final product of the accumulation of reactive oxygen species under salt stress (Hodges *et al.* 1999). The MDA content under the NaCl and $NaHCO_3$ treatments are shown (Fig. 6a,b). There was no significant variation of MDA content among the Line1, Line 2 and Line 3 treatments before and after soaking in NaCl and $NaHCO_3$ solutions. But there was a significant variation between the transgenic and WT *Populus*. Under salt stress conditions, the results indicated that *PutVHA-c* contributes to a reduction in membrane lipid peroxidation.

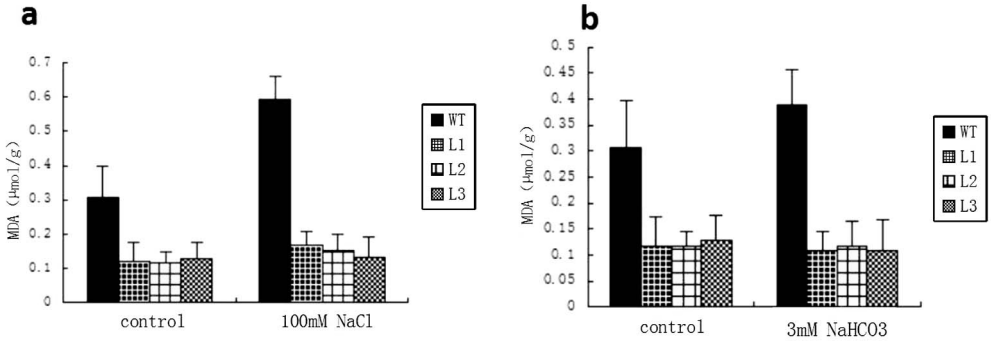


Fig. 6. MDA level of WT *Populus* and transgenic *Populus* leaves in (a) 100 mM NaCl and (b) 3 mM $NaHCO_3$.

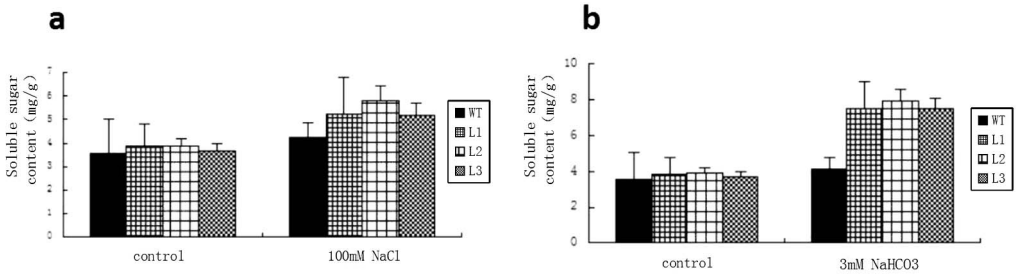


Fig. 7. Soluble sugar content of WT *Populus* and transgenic *Populus* leaves in (a)100 mM NaCl and (b) 3 mM $NaHCO_3$.

*Soluble sugar content analysis:* Soluble sugar content under the 100 mM NaCl and 3 mM $NaHCO_3$ treatments is presented in Fig. 7a,b, respectively. Compared to WT *Populus*, there was no significant variation of the soluble sugar content under normal growth conditions. Under salt-stress condition, transgenic and WT *Populus* soluble sugar content increased, and the WT *Populus* soluble sugar content were lower than transgenic *Populus*. These results indicated that *PutVHA-c* overexpression can increase the content of soluble sugar and improved the transgenic *Populus* for salt resistance.

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