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Salt tolerance conferred by over-expression of *OsNHX1* gene in Poplar 84K

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Abstract ***OsNHX1* gene (Na^+/H^+ antiporter gene of *Oryza sativa* L.) was introduced into Poplar 84K with *Agrobacterium tumefaciens*-mediated transformation. PCR, Southern and Northern blot analysis showed that *OsNHX1* gene was incorporated successfully into the genome of Poplar 84K and expressed in these transgenic plants. Salt tolerance test showed that three lines of transgenic plants grew normally in the presence of 200 mmol/L NaCl, while the Na^+ content in the leaves of the transgenic plants grown at 200 mmol/L NaCl was significantly higher than that in plants grown at 0 mmol/L NaCl. The osmotic potential in the transgenic plants with high salinity treatment was lower than that of control plants. Our results demonstrate the potential use of these transgenic plants for agricultural use in saline soils.**

Keywords: Poplar 84K, *OsNHX1*, transformation, salt tolerance.

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Salinity stress is one of the most serious factors limiting the development of agriculture and forestry. Today, nearly 20% of the world′s cultivated lands and nearly half of all irrigated lands are affected by salinity[1]. There are 37 million hectare saline lands in China, which cover 20% of cultivable areas approximately. Moreover, soil salinization is still increasing, which sustains seriously the development of agriculture. Cultivation of salt-tolerant plant, improving the adaptation of plant to high salinity is an effective way to decrease salt damage to agriculture and forestry.

Plants can use three strategies to remove Na^+ damage: Sodium exclusion, sodium compartmentation and sodium secretion. Na^+ transport out of cell and the compartmentation of Na^+ into the vacuole can be accomplished by the operation of Na^+/H^+ antiporters. The Na^+/H^+ antiporter that catalyzes the exchange of Na^+ for H^+ across membranes is widespread membrane protein, and found in animals, yeasts, bacteria and plants, and plays an important role in salt tolerance of plant[2].

Apse et al.[3] reported that the over-expression of *AtNHX1*, a vacuolar Na^+/H^+ antiporter from *A. thaliana*, allowed the transgenic plants to grow well in 200 mmol/L NaCl, but wild-type plants grown at a NaCl containing solution displayed a reduction in leaf size and chlorosis. Zhang and Blumwald[4] over-expressed *AtNHX1* in tomato plant, that the transgenic plants were able to grow, flower, and produce seeds in the presence of 200 mmol/L NaCl. Also, Zhang et al.[5] over-expressed *AtNHX1* in *Brassica* plants, that the growth of these plants was only marginally affected by the high salt concentration, in which the transgenic plants grown in high salinity accumulated sodium up to 6% of their dry weight. Moreover, seed yields and the seed oil quality were not affected by the high salinity of the soil. These results suggested the potential use of Na^+/H^+ antiporter gene for agricultural use in saline soils.

The *OsNHX1* gene (Na^+/H^+ antiporter gene of *Oryza sativa* L.) of 2330 bp containing an open reading frame of 1608 bp encodes for a protein of 535 amino acids[6]. Its expression product plays important roles in the compartmentation of Na^+ into the vacuoles, and the amount of the antiporter is one of the most important factors determining salt tolerance in rice[7].

Poplar 84K is a well-known hybrid between species of white poplar, and was introduced into China from South Korea in 1984. Now, it is popular in China because of its fast growth and high wood quality. But the distribution of Poplar 84K was confined by its limitation of salt-tolerance. In this study, the *OsNHX1* was transferred into Poplar 84K and the transgenic plants could grow normally at 200 mmol/L NaCl. We hope that this can help the studies on the mechanism of salt tolerance and the exploitation of saline soils.

1 Materials and methods

(ⅰ) Plant materials and bacterial strain. The sterile plantlets of Poplar 84K were obtained from Institute of Forestry, Chinese Academy of Forestry. LBA4404 and plasmid p3301 were kindly provided by Prof. Wang Guoying, College of Biological Sciences, China Agricultural University.

(ⅱ) Construction of the plant expression vector. *OsNHX1* gene was a gift from Dr. Fukuda, Department of Physiology, National Institute of Agrobiological Resources, Japan. The *gus* gene in plasmid p3301 was replaced by *OsNHX1* to form the plant expression vector, namely the plasmid pC3301-*OsNHX1* (Fig. 1). The plas-

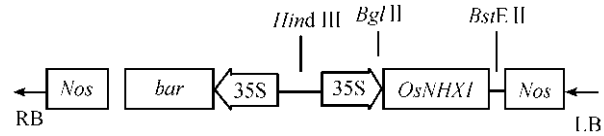

Fig. 1. Construction of plant expression vector pC3301-*OsNHX1*. RB, right border of T-DNA region; LB, left border of T-DNA region; 35S, 35S promoter; *bar*, *bar* gene; *OsNHX1*, *OsNHX1* gene; *Nos*, *Nos* terminal.

mid pC3301-*OsNHX1* was transformed into *A. tumefaciens* strain LBA4404.

(iii) Establishment of receptor system for transformation.

(1) Establishment of leaf regeneration system. Optimal shoot-inducing medium and root-inducing medium were screened by routine method.

(2) Sensitivity experiment of Basta. Leaf explants that cut into pieces were put in shoot-inducing media (MS supplemented with 1.0 mg/L 6-BA and NAA 0.1 mg/L) containing different concentrations of Basta for cultivation. Regenerated shoots were transferred to fresh medium biweekly. Two months later, the correct dose of Basta was determined according to the growth performance of leaf explants.

(iv) Gene transformation and screening of shoots resistant to Basta. According to the method of the leaf disk transformation[8], leaves were cut into pieces of 0.5 cm × 0.5 cm—1.0 cm × 1.0 cm, and put in pre-culture media (MS supplemented with 1.0 mg/L 6-BA and 0.1 mg/L NAA) to incubate for 2 d, and then submerged in the diluted solution containing *A. tumefaciens* for 10—20 min. After gentle shaking, it was ensured that all edges were infected. The leaf disks were blotted dry and incubated in MS medium for 3 d at 25℃ in the dark. After 3 d of co-cultivation, the leaf disks, which were dried by sterile filter paper, were transferred into the selection medium (MS supplemented with 1.0 mg/L 6-BA, 0.1 mg/L NAA, and 200 mg/L Cefotaxime and 5 mg/L Basta). About 20 d later, Basta-resistant shoots appeared at the edge of the leaf disks. When the Basta-resistant shoots were 1—2 cm tall, they were separated from the leaf disks and transferred onto rooting medium (1/2 MS supplemented with 0.05 mg/L NAA and 5 mg/L Basta). The Basta-resistant plants were regenerated at 25℃ with the intensity of illumination of 40—60 $E \cdot m^{-2} \cdot S^{-1}$.

(v) PCR analysis of *OsNHX1*. Total DNAs were extracted from leaves of transgenic plants and control plants by the CATB method [9] to be used as the templates. PCR amplifications were carried out with the forward and reverse primer of *OsNHX1*, 5′-taggatccaagccattgatcaggctgc-3′ and 5′-gcaagcttgtcttcctccatggctgctctg-3′ respectively, in reaction mixtures containing 2.5 μL 10 × PCR buffer, 2 μmol/L dNTPs, 0.2 μmol/L of each primer, 80 ng DNA templates and 1 U Taq polymerase in final volume of 25 μL. The PCR thermal cycles was performed at 94℃ 3 min; 94℃ 30 s, 58℃ 30 s, 72℃ 30 s for 30 cycles; and extended at 72℃ for 2 min. The PCR products were analyzed by electrophoresis on 0.8% agarose gel and photographed under UV light.

(vi) Southern and Northern blot analysis of *OsNHX1*. The total DNA from the transgenic plants and control plants were double-digested with restriction enzymes *Bgl* Ⅱ and *Bst*E Ⅱ. RNA extraction and nucleotide hybridizations were conducted according to the protocol described by Sambrook et al.[10].

(vii) Salt tolerance analysis of transgenic plants. Three transgenic lines of Poplar 84K, which were assessed by the molecule analysis, and control plants were transferred into the MS media containing NaCl in 0, 50, 100, 200 and 250 mmol/L, respectively.

(viii) Determination of Na^+ content of transgenic plants. Roots and leaves of the plants were harvested at the end of the salt treatment duration. Dry weight was measured after 24 h at 70℃ and Na^+ content was determined by atomic absorption spectrophotometry.

(ix) Determination of osmotic potential of transgenic plants. The leaves of transgenic and control plant in the same growth stage were collected and frozen immediately in liquid nitrogen for 30 min. After being thawed at room temperature for 20 min, the samples were transferred by 2.5 mL syringe with a filter into centrifuge tubes, and spun for 5 min at 4000 r/min. The supernatants were determined by an Osmomat 030.

2 Results

(i) Establishment of leaf explant regeneration system. The optimal shoot-inducing and rooting medium had been screened according to protocols described previously[11—13]. The optimal shoot-inducing medium was MS supplemented with 1.0 mg/L 6-BA and 0.1 mg/L NAA, with which the differentiation efficiency reached to 95%, and the optimal rooting medium was 1/2 MS plus 0.05 mg/L NAA.

(ii) Basta resistance test for leaf explants. Eight Basta concentrations were tested according to Brich et al.[14] and the results are shown in Table 1. In the absence of Basta, leaf explants were green and well differentiated. When Basta was added to the media, random shoot inductions were inhibited in different extents. In the media containing lower than 5 mg/L Basta, the buds came out slightly late and shoots were decrease, in which leaf explants turned yellow partially, but its growth was still normal. In the media containing 5 mg/L Basta or higher, callus induction and shoots differentiation were completely inhibited, in which no bud appeared. 20 d later, leaf explants turned yellow and died out. So we chose 5 mg/L Basta as the critical tolerant concentration to screen Basta-resistant shoots.

(iii) Gene transformation and screening of shoots resistant to Basta. After co-cultivation with *Agrobacterium*, the leaf explants were put in the selection medium containing 5 mg/L Basta and 200 mg/L Cefotaxime. 20 d later, Basta-resistant buds came out (Fig. 2), and leaf tissues surrounding the resistant bud turned yellow and died. When the Basta-resistant buds grew up, they were transferred into the rooting medium supplemented with 5 mg/L Basta. 10 d later, the Basta-resistant plantlets were regen-

erated. Through successive selections, 3 stable Basta-resistant plants were obtained.

Fig. 2. Shoots resistant to Basta.

(iv) PCR assay of Basta-resistant plants. DNAs were extracted from the 3 resistant plants and the wild-type plant to perform PCR amplification. Results of PCR assay showed that a 2.1 kb band was amplified in all 3 resistant plants (Fig. 3, Lane 1—3), but not the wild-type plant (WT). This indicated that the *OsNHX1* gene had been integrated into the genome of Poplar 84K.

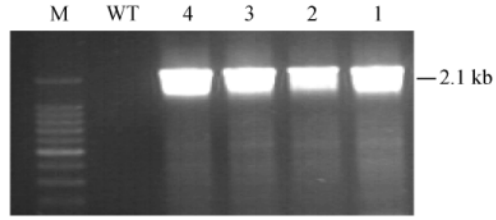

Fig. 3. PCR assay of the *OsNHX1* gene in transgenic plant.s. Lanes 1—3, transgenic plants; Lane 4, positive control (pC3301-*OsNHX1* plasmid containing *OsNHX1*); WT, wild-type plant; M, DL2000 Ladder.

Table 1 Results of Basta resistance test for leaf explants

Concentration of Basta (mg/L)	No. of leaf explants 20 d	Differentiation performance of leaf explants 60 d
0	20	calli , buds	emerald green, shoots normal
1	20	calli, buds	emerald green, shoots normal
2	20	calli, buds	emerald green, shoots normal
3	20	No. of calli and buds decreased	partial yellow, shoots inhibited
4	20	calli and buds decreased	partial yellow, shoots inhibited
5	20	no calli and buds	withered, died out
6	20	no calli and buds	died out
7	20	no calli and buds	died out
8	20	no calli and buds	died out

(v) Southern blot analysis of *OsNHX1*. Genomic DNAs from transgenic and wild-type plants were double-digested with restriction enzymes of *Bgl* II and *BstE* II for Southern blot analysis. The results in Fig. 4 showed that 2.1 kb DNA fragments from all 3 transgenic plants (Lane 1—3) and the positive control (Lane P) were hybridized by the probe, thus providing further evidence that *OsNHX1* had been integrated into the genome of Poplar 84K.

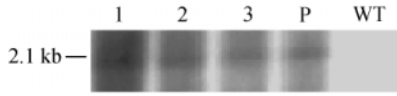

Fig. 4. Southern blot analysis of transgenic plants. WT, DNA from wild-type plant; Lanes 1—3, DNA from transgenic plants; P, positive control (pC3301-*OsNHX1*).

(vi) Northern blot analysis of transgenic plants. Total RNAs from leaves of wild-type plant and the transgenic plants after 3 d of NaCl-stress treatment were subjected to Northern blot analysis. Results in Fig. 5 showed that the *OsNHX1* gene was expressed in all 3 transgenic plants with clearly hybridized signals (Lane 1—3), but not in the wild type plant.

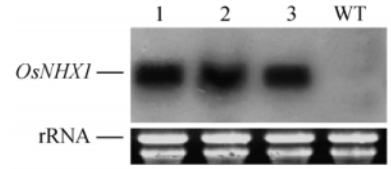

Fig. 5. Northern blot analysis of transgenic plants. Total RNA (30 μg) from the leaves of wild-type plants and three lines of transgenic plants respectively was analyzed by northern-blot analysis using the *OsNHX1* cDNA as probe. Ethidium bromide-stained rRNA bands were used as controls for equal loading. WT, wild-type; Lane 1—3, three lines of transgenic plants.

(vii) Salt tolerance of transgenic plants. The three transgenic lines and wild-type plants were transferred onto the MS media containing different salinity and cultivated for 20 d. On the medium were saline content was lower than 50 mmol/L, both transgenic plants and controls grew normally. On the medium containing 100 or 200 mmol/L NaCl, the transgenic plants still grew well, but the controls withered and died out. When the saline in medium was higher than 250 mmol/L, the growth of transgenic plants was inhibited slightly.

(viii) Na^+ content of transgenic plants. Na^+ content in roots and leaves of transgenic plants before or after salt-stress were analyzed. At lower salinity, no significant

differences of Na^+ content were observed on the leaf and root between wild-type and transgenic plants (Fig. 7 and 8). By treatment with 100 mmol/L and 200 mmol/L NaCl for 3 d, however, the Na^+ content in leaves of transgenic plants were markedly higher than that of wild-type plant (Fig. 7), while the Na^+ content in roots of transgenic plants

Fig. 6. Test for salt tolerance of transgenic plants with 200 mmol/L NaCl for 20 d. 1, transgenic plants; 2, wild-type plants.

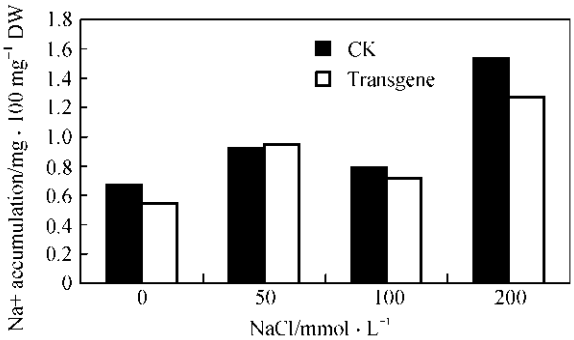

Fig. 7. Effect of NaCl stress on Na^+ accumulation in leaves of transgenic plant (white bar) and control (black bar).

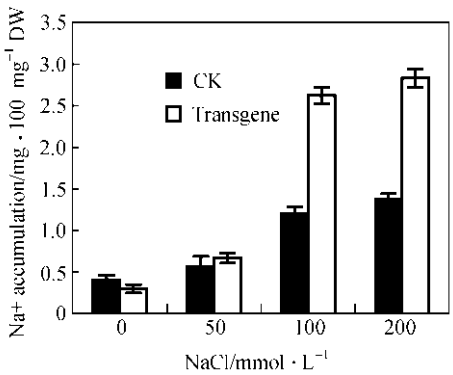

Fig. 8 Effect of NaCl stress on Na^+ accumulation in roots of transgenic plant (white bar) and control (black bar).

did not show any significant differences (Fig. 8).

(ix) Determination of osmotic potential of transgenic plants. The osmotic potential of wild-type and transgenic plants treated with different concentrations of NaCl for 3 d were determined. After treatment with saline lower than 50 mmol/L, no significant differences were observed between wild-type and transgenic plants. When treated with 100 mmol/L and 200 mmol/L, the osmotic potential of transgenic plants was significantly lower than that of wild-type plants (Fig. 9). These results show that the increasing accumulation of *OsNHX1* product induced by salt-stress can improve the salt tolerance of transgenic plants.

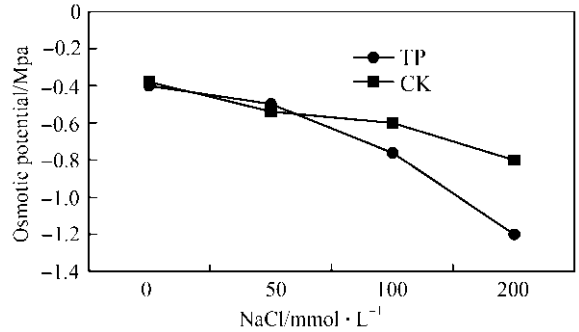

Fig. 9 Effect of NaCl stress on osmotic potential in leaves of transgenic plant and control. TP, transgenic plant; CK, control plant.

3 Discussion

We have used bar gene as a selectable marker gene in the transformation of Poplar 84K. The bar gene confers resistance to the herbicides Basta[15], with which the gene of target trait in co-transformed plants would be easy to be identified efficiently and fast. But different plant materials have variable sensitivity to Basta. Leaf herbicides test showed that growth of untransformed leaf explants incubated at medium containing 5 mg/L Basta was inhibited, turning yellow, wilted and died out, but transformed leaf explants could resist the selective pressure of Basta, grow and differentiate well. In addition, transformed plantlets could root well at medium containing 5 mg/L Basta, but the controls could not. PCR assay, Southern and Northern blot analysis indicated that the *OsNHX1* gene had been successfully integrated into the genome of Poplar 84K and expressed in high level.

After salt stress, Na^+ content in leaves of transgenic plant was higher than that of control plant, but growth of transgenic plants was not affected, this may be due to the compartmentation of Na^+ in transgenic plants to avert the deleterious effects of Na^+. In addition, under salt stress, the osmotic potential of transgenic plant was significantly lower than that of control plant. It was demonstrated that the increasing accumulation of *OsNHX1* product improved the salt tolerance of transgenic plants. From the assessment of salt tolerance, we know that transgenic plants grew well at 200 mmol/L NaCl, but wild-type plants died out. These results strongly suggest the feasibility of genetic engineering plants with improved salt tolerance.

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References

1. Yokoi, S., Bressan, R. A., Hasegawa, P. M., Salt stress tolerance of plant, JIRCAS Working Rep, 2002, 25—33.
2. Blumwald, E., Aharon, G. S., Apse, M. P., Sodium transport in plant cells, Biochem. Biophys. Acta, 2000, 1465: 140—151.
3. Apse, M. P., Aharon, G. S., Snedden, W. A. et al., Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in *Arabidopsis*, Science, 1999, 285: 1256—1258.
4. Zhang, H. X., Blumwald, E., Transgenic salt-tolerent tomato plants accumulate salt in foliage but not in fruit, Nat. Biotechnol., 2001, 19: 765—768.
5. Zhang, H. X., Hodson, J. N., Williams, J. P. et al., Engineering salt-tolerant Brassica plants: Characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation, Proc. Natl. Acad. Sci. USA, 2001, 98: 12832—12836.
6. Fukuda, A., Nakamura, A., Tanaka, Y., Molecular cloning and expression of the Na^+/H^+ exchanger gene in *Oryza sativa*, Biochim. Biophys. Acta**.**, 1999, 1446: 149—155.
7. Fukuda, A., Nakamura, A., Tagiri, A., Function, intracellular localization and the importance in salt tolerance of a vacuolar Na^+/H^+ antiporter from rice, Plant Cell Physiol., 2004, 45(2): 146—159.
8. Horsch, R. B., Fry, T. E., Hoffmann, N. L. et al., A simple and general method for transferring genes into plants, Science, 1995, 227: 1227—1231.
9. Murray, M. G., Thompson, W. F., Rapid isolation of high-molecular-weight plant DNA, Nucl. Acids. Res., 1980, 8: 4321—4325.
10. Sambrook, J., Fritsch, E., Maninatis,T., Molecular Cloning: A Laboratory Manual, 2rd ed., New York: Cold Spring Harbor Laboratory, 1989.
11. Fan, J. F., Li, L., Han, Y. F. et al., Establishment of leaf-explant regeneration system of Poplar 84K, J Northwest Forestry University, 2002, 17(2): 33—36.
12. Chun, Y. W., Morphogenic potential of leaf, internode, and root explants from *Populus alba×P. grandidentata* plantlets, Forest and Humanity, 1990, 3: 31—44.
13. Fan, G. Q., Zhai, X. Q., Zhai, C. J. et al., Callus induction from leaves of different *Paulownia species* and its plantlet regeneration ,Journal of Forestry Research, 2001, 12(4): 209—214.
14. Brich, R. G., Plant transformation: Problems and strategies for practical application, Annu. Rev. Plant Physiol. Plant Mol. Biol., 1997, 48: 297—326.
15. Denney, B. K., Petersen, W. L., Ford-Santino, C. et al., Comparison of selective agents for use with the selectable marker gene bar in maize transformation, Plant Cell, Tissue Organ Culture,1994, 36: 1—7.

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