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Full Length Research Paper

Overexpression *AtNHX1* confers salt-tolerance of transgenic tall fescue

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Saline soil is a serious problem worldwide, and it is necessary to improve the salt tolerance of plants so as to avoid the progressive deterioration of saline soil. Here we report that over-expression of *AtNHX1* improves salt tolerance in transgenic tall fescue. The *AtNHX1* gene driven with CaMV35S promoter was constructed into the plant expression vector pGreen0229, and introduced into the embryonic calli of hypocotyls of tall fescue (*Festuca arundinacea*) by particle bombardment. Regenerated plantlets were obtained by screening of herbicide (PPT, 2 mg/L), and the putative transformants were assayed by PCR and western blot analysis. 29 transgenic plants were obtained. The results indicated that the exogenous genes had been integrated into the genomes of transgenic plants, and *AtNHX1* is expressed in the plants. There was remarkable salt tolerance in transgenic plants compared to control plants.

Key words: *AtNHX1* gene, transgenic tall fescue, particle bombardment, salt-tolerance.

INTRODUCTION

Salt stress is a major problem in plant agriculture. High salinity causes ion imbalance, toxic levels of cytoplasmic sodium, and drought stress (Ward et al., 2003). Na+/H+ antiporters are ubiquitous membrane proteins that play major roles in cellular pH and Na+ homeostasis throughout the biological kingdom (Shi et al., 2002). NHX1 was identified in *Sacharomyces cerevisiae* and was localized to a late endosomal/prevacuolar compartment where it mediates intracellular sequestration of Na+ in a pH-dependent manner (Nass et al., 1997; Nass and Rao, 1998).This finding indicates a role for intracellularly localized Na+/H+ antiporters in mediating NaCl tolerance through prevacuolar compartmentation of Na+(Shi et al., 2002).

Overexpression of the Arabidopsis tonoplast membrane Na+/H+ antiporter, *AtNHX1*, under a strong constitutive promoter was reported to result in salt-tolerant Arabidopsis (Apse et al., 1999), *Brassica napus* (Zhang et al., 2001), and tomato (*Lycopersicon esculentum*) (Zhang and Blumwald, 2001). AgNHX1, an *AtNHX1* homologues from the halophytic plant *Atriplex gmelini* (Hamada et al., 2001), overexpression in rice (*Oryza sativa*) plants improved salt tolerance of the transgenic rice (Ohta et al., 2002). *AtNHX1* homologues from many plant species have been isolated; mostly based on their sequence homology to the *Arabidopsis* gene. Thus, the NHX1 system seems to be highly conserved between many different plant species and manipulation of this system in crop species will likely result in improved salt tolerance (Zhang et al., 2004).

In this study, *AtNHX1* was transferred into tall fescue by particle bombardment and transgenic plants obtained. Tall fescue recently has been recognized as turf species of choice in central-eastern China. Engineering salt tolerance in tall fescue might bring millions of acres of wounded or crippled land back into production in China.

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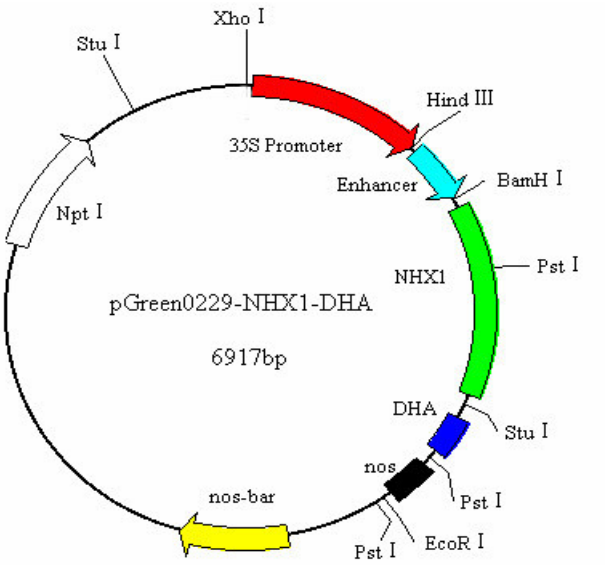

Figure 1. Transformation vector pGreen0229-NHX1-DHA. The *AtNHX1* gene with DHA tag driven by CaMV35S promoter was inserted into plasmid pGreeno229.

MATERIALS AND METHODS

Plant material

Tall fescue seeds of cultivar Crossfire II were obtained from Beijing Research and Development Center for Grass and Environment.

Plasmid for transformation

The empty vector pGreen0029 is from John Innes Center, UK, and *AtNHX1* gene is from Dr Roberto A. Gaxiola in University of Connecticut, USA. The *AtNHX1* gene with DHA tag driven by CaMV35S promoter was inserted into plasmid pGreeno229 (Figure 1).

Plant regeneration system

The tall fescue seeds firstly were sterilized by 70% alcohol for 2 min, and treated by 50% Javel water for 20 min. After being washed for 3-4 times by sterilized water, the seeds were inoculated into MS media with plant growth regulators (6-BA 1mg/L+ IAA 0.1 mg/L) for germination. The hypocotyls of 3-5 cm seedlings were induced to produce calli on MS + 2,4-D (10 mg/L) media, and one month later, the calli of hypocotyls were transferred into MS + 2,4-D (5 mg/L) media for another month to obtain the embryonic calli. The differentiation media of calli is MS + BA (1 mg/L) + IAA (0.1 mg/L) and the root inducing media is MS + NAA (0.5 mg/L).

Bombardment and screening

The shine and thick embryonic calli of hypocotyls with diameter 0.2 - 0.5 cm were bombarded with transformation vector pGreen0229-NHX1-DHA or empty vector pGreen0029 as earlier described (Cho et al., 2000). Approximately 80 embryonic calli were placed onto the center of 35-mm Petri dishes, and the dishes were placed in a Bio-Rad Biolistic PDS-1000/HE Particle Delivery System for particle bombardment. Optimized biolistic parameters are 26-in Hg of chamber vacuum, target distance of 15 cm, 1,300-psi particle acceleration pressure, and 1.6-µm gold microcarriers. The bombarded calli were placed on MS + 2,4-D (5 mg/L) media without light for one week, then cultured on selection media MS + BA (1 mg/L) + IAA (0.1 mg/L) + herbicide PPT (2 mg/L) to regenerate transformants. The developing shoots were transferred to the root inducing media with PPT (3 mg/L). The rooted plantlets of 3-5 cm were transplanted into vermiculite soil and grown in a growth chamber for further experiments.

PCR analysis

Genomic DNA was extracted from tall fescue leaves by CTAB method, and used as the template for PCR amplification. The primers were designed according to the sequences of *AtNHX1* and bar genes. The primers of *AtNHX1* gene were P1: 5' CAG GAT CCA TGT TGG ATT CTC TAG TGT 3' and P2: 5' ATA GGC CTA GCC TTA CTA AGA TCA GGA 3'. The primes of bar gene were P3: 5' ACT TTA TTG CCA AAT GTT TGA ACG A 3' and P4: 5' ATC TAC CAT GAG CCC AGA ACG AC 3'. PCR reactions were carried out in a 25 µl volume□ The PCR program of *AtNHX1* is initial denatured at 94□C for 3 min, and then subjected to 30 cycles of 94□C denaturation for 45 s, 56□C annealling for 1 min and 72□C extension for 2 min, plus a final extension 72□C for 7 min. The PCR program for bar gene is similar to that of *AtNHX1* except that annealing temperature is 58□C. The PCR products were separated on 1% agarose gel.

Proteins extracts and western blot

Total proteins were extracted from 0.3 g tall fescue leaves according to the EZ method described by Martinez-Garcia et al. (1999). The western blot analysis used rat monoclonal antibody anti-HA (Roche Applied Science, 3f10) as first antibody and peroxidase-conjugated goat anti-rat IgG(H+L) (Zhong Shan Golden Bridge Biotechnology Company, China) as second antibody.

Analysis of transgenic plant for salt tolerance

AtNHX1 transgenic and control (empty vector transformant) plants of similar age and height were assayed for salt tolerance after transfer to soil in the same pots containing 0, 50, 100, 200, 300 mM NaCl respectively. Plants were maintained in a growth chamber and irrigated daily with saline water containing the above-mentioned levels of salt for 1 month.

RESULTS AND DISCUSSION

Tungsten micro particles were coated with the DNA of vectors pGreen0229-NHX1-DHA and empty vector pGreen0229 only, respectively, and bombarded into embryonic calli of tall fescue hypocotyls. The bombarded calli were cultured on selection medium with 2 mg/L her-bicide for regeneration. The regenerated plantlets were subjected to another round of selection. The plant-lets were rooted on medium containing 2 mg/L herbicide, then transferred to the potted soil and grown in the green house. Eighty putative NHX1 and 21 putative empty vector only transformed tall fescue lines were obtained. Transformed plants were monitored by PCR. The expec-

Table 1. Tall fescue calli screening with phosphinothricin, plant regeneration and transformation efficiency.

Bombarded distance (cm)	Number of Bombarded calli	Number of resistant calli	Number of regenerated plants	Number of transgenic plants	Transformation efficiency
15	1686	290	80	29	1.7%

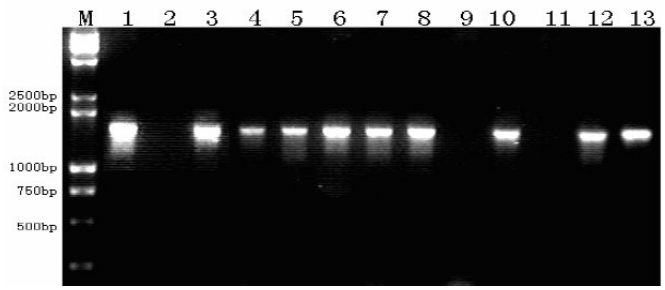

Figure 2. PCR analysis of *AtNHX1* transgenic tall fescue plants. M, DNA ladder; 1, plasmid pGreen0229-NHX1-DHA; 2, untransformed line; 3-13, putative transformed lines.

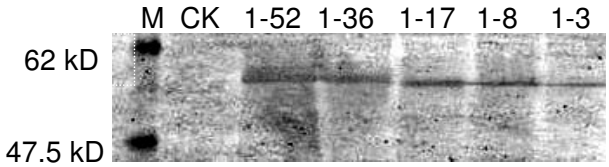

Figure 3. Western-blot analysis of *AtNHX1* transgenic plants. M, Protein ladder; CK, untransformed plant; 1-52, 1-36, 1-17, 1-8 and 1-3, different transgenic lines.

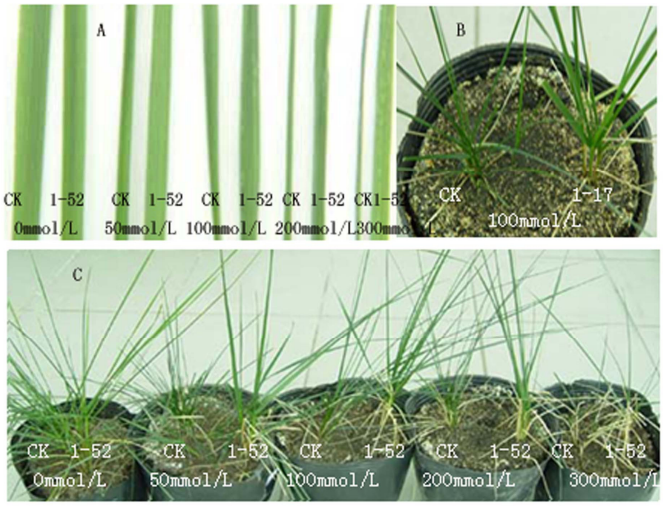

Figure 4. Effect of salt (50–300 mM NaCl) on empty vector transformed (CK) and *AtNHX1* transgenic (1-52, 1-17) tall fescue lines grown at different concentrations of NaCl. Plants were irrigated with water containing different concentrations of NaCl on alternate days for up to 1 month. A, Leaves of control (CK) and 1-52 plants under different NaCL treatment; B, 1-17 transgenic plant and control plant under 100 mM NaCL tratment; C, 1-52 transgenic plants and control under different NaCL treatment.

ted fragment (1617 bp) of *AtNHX1* was amplified from total DNA of 29 transformed lines. The results showed that the foreign gene *AtNHX1* had been integrated into the genome of these lines. The PCR results of other 51 putative transformed lines were negative, as shown in No.9 and No.11 of Figure 2. Empty vector transformant was identified by PCR of bar gene, 8 transformed lines were obtained (data not shown).

Taking the PCR positive results as a judge standard for transformed plants, 29 *AtNHX1* transgenic plants were obtained, and the tansformation efficiency of bombardment for tall fescue is about 1.7% (Table 1).

For further confirmation of *AtNHX1* integration into the tall fescue genome with protein expression, western blot was performed using 30 µg total protein isolated from the untransformed and random selected transformed tall fescue plants. According to Figure 3, the C-terminally DHA-tagged NHX1 protein was expressed in transgenic plant with the predicted molecular mass of 60 kDa, while there was no hybridization signal in the untransformed plant. Western blot showed the NHX1 protein expression in transgenic tall fescue, and indirectly confirmed the PCR results, reflecting the presence of *AtNHX1* transgene in tall fescue.

AtNHX1 transgenic plants and empty vector transgenic control plants were subjected to increasing degrees of salt stress, ranging from 50 to 300 mM NaCl for one month. Transgenic plants expressing the *AtNHX1* transgene thrive well up to 200 mM NaCl (Figure 4), whereas untransformed plants exhibited growth retardation at 100 mM NaCl. Three *AtNHX1* transgenic lines 1-52, 1-17, and 1-8 were assayed for salt tolerance; all of them appeared to grow better compared to control plants under the same salt stress. However, 1-52 and 1-17 transgenic lines exhibited much more tolerant to salinity than that of line 1-8. As shown in Figure 4, leaves of *AtNHX1* transgenic lines are much larger that that of control plants under the same concentration of salt treatment. Improved salinity tolerance has been achieved by overexpressing a vacuolar Na+/H+ antiport, up to 200 mM NaCl in tomato plants (Zhang and Blumwald, 2001). In this study, we report that expression the same gene in transgenic tall fescue also confers salt tolerance. Salt and draught are two big menaces to plants in many parts of the world. Salt stress is one of serious abiotic stresses that hampers the growth, developing, products of crops. Three different pathways are suggested to mediate

salinity tolerance in plants, which include maintenance of ion and osmotic homeostasis, regulation of cell division and growth, and detoxification of toxic byproducts and cellular repair (Zhu, 2002). The transgenic Arabidopsis plants of overexpressing *AtNHX1* could grow and develop continuously in soil when irrigated with 200 mmol/L NaCl solution (Apse et al., 1999). Transgenic *Brassica napus* plants overexpressing *AtNHX1* were able to grow, flower, and produce seeds in the presence of 200 mM NaCl without any obvious changes of products and quality (Zhang and Blumwald, 2001). In this study, the transgenic tall fescue are more salt-tolerance than control plants. However, different lines showed some difference in salt tolerance, which could be due to different protein expression level of NHX1. The tall fescue transgenic plants engineered for salt tolerance in this paper are implemented in T0 generation. The situations for T1 and T2 plants need to be further studied.

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