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Over-expression of the bacterial *nhaA* gene in rice enhances salt and drought tolerance

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Abstract

The *Escherichia coli nhaA* gene encodes a Na^+/H^+ antiporter, which plays a critical role in ion homeostasis. We transferred a bacterial *nhaA* gene into rice (*Oryza sativa* L. ssp. *japonica*) and detected high expression in the transgenic rice. The germination rate, growth, and average yield per plant of the transgenic lines were better than those of control lines under salt or drought stress. Moreover, the sodium and proline content of the transgenic lines under salt or drought stress was also higher than in control lines, implying that *nhaA* over-expression enhances osmoregulation by activating the biosynthesis of proline. Tolerance to both salt and drought was compared between transgenic rice over-expressing *nhaA* and that over-expressing Arabidopsis *δ-OAT* encoding ornithine-δ-aminotransferase. The transgenic plants with *nhaA* grew better than those with *δ-OAT* at high salinity, while the opposite was true for drought stress.

Keywords: *nhaA* Gene; *Agrobacterium*-mediated; Transgenic rice (*Oryza sativa* L.); Salt tolerance; Drought tolerance

1. Introduction

Osmotic stress due to salinity and drought seriously limits crop yields [1]. Plants have evolved a great variety of mechanisms to adapt to stress, including stomata adjustment, osmoregulation, selective uptake, compartmentation of ions, etc. [2]. Since plants respond to different stresses using a variety of mechanisms, traditional breeding strategies have met with limited success [3]. With the development of biotechnology, genetic engineering involving the transfer of a gene with a trait of interest can generate transgenic plants with modified traits. Recently developed strategies in functional genomics are providing more information on novel genes and their expression in response to stresses, which will provide a molecular base for achieving effective stress tolerance through genetic engineering [4,5].

Na^+ and H^+ are the ions most commonly involved in cell bioenergetics and proton concentration, which are critical to cell functioning. If concentrations of these two ions are too high or too low, the physiological activities of cells are inhibited [6]. To maintain the homeostatic balance in vivo, cells adopt different mechanisms, including enhancement of K^+ uptake, elimination of surplus Na^+, re-allocation of Na^+ into other intracellular compartments (such as vacuoles), and biosynthesis of compatible solutes in the cytoplasm to maintain osmotic equilibrium.

Na^+/H^+ antiporters, which exchange Na^+ or Li^+ for H^+, play a primary role in homeostasis and are found in every biological kingdom, from bacteria to humans to higher plants [6]. Na^+/H^+ antiporters are classified into several families: NhaA, NhaB, NhaC, NhaD, and NapA in prokaryotes, SOD2 and Nha1 in fungi, and AtNHX1 and SOS1 in Arabidopsis. Of these, *Escherichia coli* NhaA has been studied extensively. NhaA is the key Na^+/H^+ antiporter in both the plasmalemma and tonoplast of *E. coli*, and it plays a major role in maintaining cell pH and Na^+ homeostasis [7]. The *nhaA* gene has been cloned [8] and expressed in yeast, resulting in enhanced salt tolerance [9]. Moreover, increasing the Na^+ or Li^+ concentration, rather than the osmolarity or ionic strength, turns on expression of *nhaA*, suggesting that there is a unique mechanism in *E. coli* that responds

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to Na^+ or Li^+ specifically, and that the cytoplasm Na^+ concentration serves as a direct signal for inducing *nhaA* [10].

Rice (*Oryza sativa* L.) is an extremely important crop. It feeds more than half of the world's population and is the primary source of calories for people in Asia. Increasing rice yields has been a goal of agricultural scientists for several decades, and many technologies have been developed to achieve this. However, rice is a nonhalophyte, with weak ion-compartmentation ability. To improve salt and drought tolerance in rice, recent research has focused on the use of genetic engineering to modify osmolytes such as proline. Some genes encoding key enzymes in proline biosynthesis have been over-expressed in transgenic plants and enhanced salt tolerance was observed [11–15]. Na^+/H^+ antiporter genes were not seriously considered for improving salt and drought tolerance in plants until their potential roles in maintaining cell homeostasis were recently elucidated [6]. Over-expression of the *Arabidopsis thaliana AtNHX1* gene, which encodes a tonoplast Na^+/H^+ antiporter, enhanced salt tolerance in transgenic Arabidopsis plants, which were able to survive 0.2 mol/L NaCl [16]. However, potential enhancement of salt and drought tolerance by transferring a bacterial Na^+/H^+ antiporter gene has not been examined.

To increase osmotolerance in rice and to evaluate the contribution of the Na^+/H^+ antiporter to salt and drought tolerance in plants, we transferred a bacterial *nhaA* gene into rice (*O. sativa* L. ssp. *japonica* cv. Zhongzuo 321) using an *Agrobacterium*-mediated method. This study evaluated the effects of *nhaA* over-expression on the seed germination rate, growth performance, and proline content of the transgenic plants under normal and osmotic stress conditions. Furthermore, we compared the growth performance and average yield per plant of transgenic rice plants that over-expressed either bacterial *nhaA* or Arabidopsis *δ-OAT*, the gene encoding ornithine-δ- aminotransferase that is the key enzyme involved in proline biosynthesis.

2. Materials and methods

2.1. Materials

Rice (*O. sativa* L. ssp. *japonica* cv. Zhongzuo 321) seeds were provided by the Institute of Saline and Alkaline Land Exploitation, Liaoning Province, China. Plant expression vectors pRS-133 and pCAM-GUS, containing the *nhaA* and *GUS* genes, respectively, were provided by Prof. Michel Jacobs of Vrije Universiteit, Brussels. The *E. coli nhaA* gene and *GUS* gene were released and cloned into pCAMBIA1300 to construct plant expression constructs driven by an enhanced CaMV 35S promoter with a nuclear matrix attachment region (MAR) from the tobacco *Rb7* gene and a 3′ Nos terminator (Fig. 1A). Two regenerated plant lines were used as controls: Zhongzuo 321 (CK1) and transgenic Zhongzuo 321 with pCAM-GUS (CK2, Fig. 1B). The *Agrobacterium tumefaciens* strain EHA105 was provided by the National Laboratory of Protein Engineering and Plant Genetic Engineering, Peking University.

2.2. Production of transgenic rice plants

Dehusked rice seeds were sterilized with 75% ethanol and 20% NaClO, and then spread on callus-induction medium. After 7–14 days at 25 °C in the dark, the calli were peeled off and placed on callus growth medium for 2 weeks at 25 °C in the dark. Fresh calli were transferred onto the *Agrobacterium* infection medium for 48 h at 25 °C in the dark. Simultaneously, *Agrobacterium* EHA105 transformed with pRS-133 was grown in 10 ml of liquid medium, with shaking at 200 rpm, at 28 °C for 24 h, before 2.5 ml of the culture was inoculated into 25 ml of medium, and shaken at 200 rpm and 28 °C for 12 h. The bacteria were placed in 50-ml tubes and spun down for 10 min at 4 °C. The bacteria pellet was resuspended in 10 ml of bacterial resuspension solution. The rice calli were dipped into the bacterial solution for 0.4 h, and dried on sterilized filter paper in the

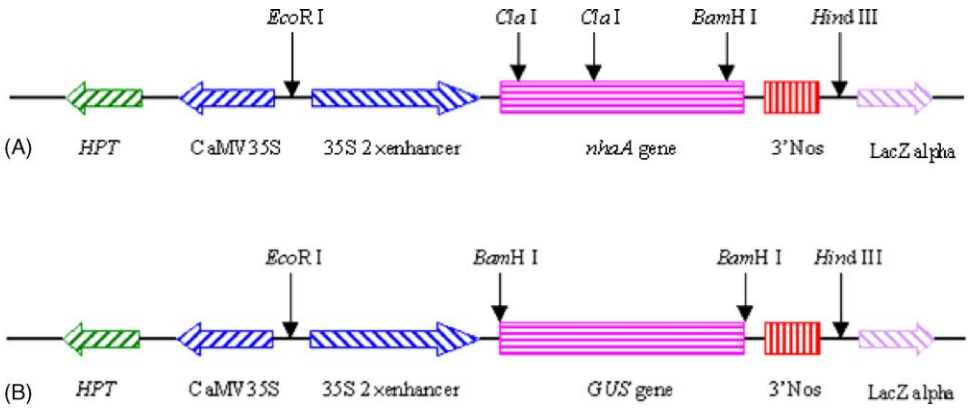

Fig. 1. Schematic of the expression vectors pRS-133 (A) and pCAM-GUS (B). CaMV 35S, cauliflower mosaic virus 35S promoter; 35S 2 × enhancer, CaMV 35S contained a nuclear matrix attachment region from the tobacco *Rb7* gene; Nos, nopaline synthase terminator; *nhaA*, Na^+/H^+ antiporter gene; *GUS*, β-glucuronidase gene; *HPT*, hygromycin phosphotransferase gene.

shade before they were placed on the medium for 2–3 days at 25 °C in the dark. The recovered calli were washed with 300 mg/L cefotaxime four times and with sterilized water once, and dried on sterilized filter paper for 3 days in the shade. The calli were cultured on selection medium for 6–8 weeks at 25 °C in the dark, on differentiation medium for 20 days at 25 °C in the dark, and on callus regeneration medium for 3–4 weeks at 25 °C in the light. The regenerated seedlings were transferred onto plant growth medium until the plantlets were 6–8 cm tall before they were transferred into soil and grown until harvest.

2.3. Molecular analysis of transgenic rice plants

Total DNA was extracted using the CTAB protocol [17]. PCR amplification and Southern and Northern blotting were performed using existing protocols [15]. For PCR analysis, the primers used were 5′-ATT CAG CGA GAG CCT GAC CTA TTG-3′ and 5′-AAG ATG TTG GCG ACC TCG TAT TGG-3′, which amplified a 450-bp fragment of the *HPT* (hygromycin phosphotransferase) gene. The probe used for Southern and Northern blotting was a 523-bp-long *Cla*I restriction fragment of the *nhaA* gene. For RT-PCR analysis, mRNA was extracted using a Quickprep mRNA Purification kit (Pharmacia) and the cDNA was synthesized using a Time-saver^{TM} cDNA synthesis kit (Pharmacia), according to the manufacturer's instructions. The two primers used for RT-PCR were 5′-ATG CAA GGA TCG CTA GCC AGC TTA-3′ and 5′-AAC GAA CGC GTA ACC AGC TGT ATC-3′, which amplified a 900-bp fragment of the *nhaA* gene.

2.4. Biochemical and physiological analysis of transgenic rice

T_2 seeds from transgenic plants and the controls were germinated and grown at 25 °C in water or 0.1–0.4 mol/L NaCl solution in light for 3 weeks before the germination rate and growth performance were determined. In addition, seeds were germinated in water at 25 °C for 10 days in light and treated with 0.1–0.4 mol/L NaCl solution in light for 5 days before the growth performance was checked.

Na^+ contents of transgenic and control plants were measured in shoots by an inductive argon plasma emission spectrophotometer (Jobin-Yvon JY 48) after digestion of dry matter in a 3:1 nitric:perchloric acid mixture.

To monitor the growth of *nhaA* transgenic plants under osmotic stress, T_2 seeds were germinated and grown in MS medium or MS with 10–30% PEG-6000 for 15 days, and the germination rate and growth were checked using reported procedures [12].

Fifteen-day-old T_2 seedlings were treated with 10–30% PEG-6000 starting with germination of the seeds. The leaves were collected and proline was extracted with 3% sulfosalicylic acid and determined as reported previously [18].

3. Results and discussion

3.1. Expression of nhaA is affected by salt stress

The bacterial *nhaA* gene was transferred into rice (*O. sativa* L ssp. *japonica* cv. Zhongzuo 321) using an *Agrobacterium*-mediated method and 36 transgenic rice lines were obtained and confirmed by PCR analysis (data not shown). Southern blot analysis showed that the *nhaA* gene integrated into the rice genomes successfully, and transgenic lines 133-1 and 133-2 each possessed a single copy of the bacterial *nhaA* (data not shown).

The expression of the *nhaA* gene in line 133-1 was determined under different salt stress conditions. The detected level of *nhaA* transcripts varied with the salt concentration, highest at a concentration of 200 mM, but lower as the salt concentration continued to increase (Fig. 2). This phenomenon may be the result from the tobacco Rb7 MAR that is attached to the 35S promoter in the *nhaA* over-expression construct. The tobacco Rb7 MAR is capable of enhancing transcription level of promoters and stabilizing the expression of the transgenes [19,20]. When severely stressed, the rice growth is retarded and the enzyme systems and the metabolic systems in rice cells are inhibited, resulting reduction of total RNA. However, transcription of the transgene *nhaA* in the transgenic rice is sustained due to the function of 35S promoter and the tobacco Rb7 MAR, making the transcription level of *nhaA* detected is relatively high. Moreover, when the salt concentration was low (≤200 mM NaCl), more *nhaA* was expressed in young seedlings (5 weeks old) than in mature plants (10 weeks old). Conversely, as the salt concentration increased further (300 or 400 mM NaCl), expression of *nhaA* was greater in mature plants than in seedlings (Fig. 2). It is possible that the high salt concentration severely retarded the growth of transgenic rice, and that the young seedlings were more sensitive to this (Fig. 2).

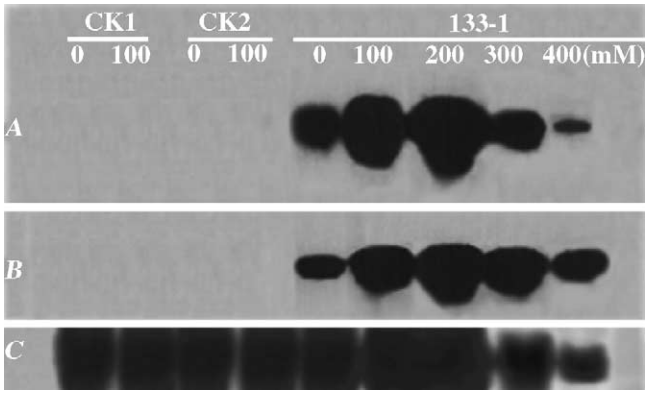

Fig. 2. Northern analysis of transgenic rice line (133-1) with the *nhaA* gene. CK1 and CK2 are two control lines. (A) 3-Week-old rice plants treated with 0–0.4 mol/L NaCl for 2 weeks; (B) 8-week-old rice treated with 0–0.4 mol/L NaCl for 2 weeks. (C) replica filter of *B* probed with a rice actin gene, *Act1*. Each lane contained 20 μg of total RNA except that about 10 μg was loaded in 300 and 400 mM-treated lanes.

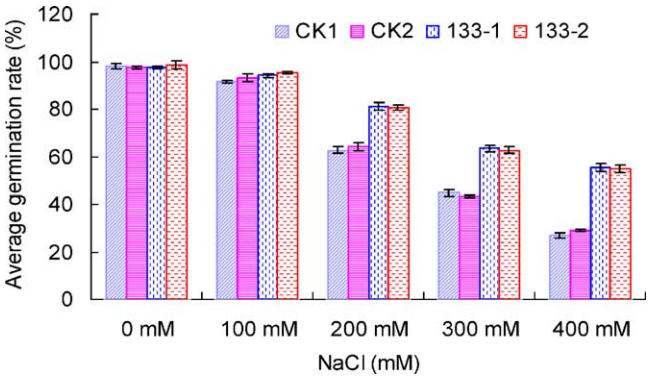

Fig. 3. Average germination rate of 50 seeds under NaCl stress. CK1 and CK2 are the control lines; 133-1 and 133-2 are the transgenic lines with the *nhaA* gene. Vertical bars represent the S.D. ($n = 3$).

3.2. The tolerance of transgenic rice to salt and drought stress is enhanced

The germination rates of transgenic and control lines were compared. Under slight stress (<100 mM NaCl) or normal conditions, the germination rates of the transgenic and control lines were similar. When the salt concentration exceeded 200 mM, the germination rates of the transgenic lines were significantly higher than those of the control lines (Fig. 3 and Table 1). For instance, when treated with 400 mM NaCl, the germination rate of transgenic lines was twice that of the controls (Fig. 3).

The growth of transgenic rice plants was evaluated under a series of stress conditions, as in our previous report [13]. The growth of all rice plants was inhibited more severely with increasing NaCl concentration. However, the transgenic rice plants grew faster than the control lines under the same stress conditions (Fig. 4). The relative average growth rate of the stem, shoot weight, and root weight of transgenic rice plants were 2–4 times those of the control plants when treated with NaCl exceeding 0.3 mol/L. Moreover, the resistance of the transgenic rice plants to KCl and $MgSO_4$ was also enhanced (data not shown).

Table 1
t-Test of the numbers of sprouting transgenic and control line plants

NaCl (mol/L)	133-1:CK1	133-2:CK1	CK2:CK1	
0	−0.50	0.50	−0.32	
0.1	2.00	2.12	2.12	
0.2	7.48	7.22	0.63	
0.3	7.48	9.55	−0.53	
0.4	12.66	18.34	0.60	
	$\alpha = 0.05$	$\alpha = 0.01$	$\alpha = 0.005$	$\alpha = 0.001$
Critical value of the single tail test	2.13	4.54	5.84	10.21
Critical value of the double tail test	2.78	5.84	7.45	12.92

The data in the second table are the *t*-stats, which adopt a different variance hypothesis for double samples. CK1 and CK2 are control lines; 133-1 and 133-2 are transgenic rice lines with the *nhaA* gene.

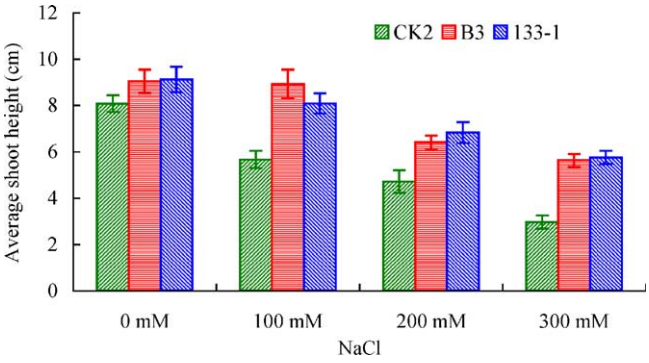

Fig. 4. Average shoot height of transgenic rice plants. All the rice plants were cultured in water for 10 days and treated with NaCl for 5 days. CK2 is the control line with pCAM-GUS; B3 is the transgenic line with the *δ-OAT* gene; 133-1 is the transgenic line with the *nhaA* gene. Vertical bars represent the S.D. ($n = 20$).

The transgenic plants with the *nhaA* gene grew better than control plants under drought conditions (treated with PEG-6000 or water withheld), suggesting that *nhaA* over-expression enhances the osmoregulation ability of plants. Analysis of the Na^+ contents in both control (CK2) and transgenic (133-1) plants showed that the Na^+ content increased with exposure to NaCl, and that those of the transgenic plants were higher than those of the control plants (Fig. 5). The resulting accumulation of NhaA likely facilitates Na^+ transportation into vacuoles and out of the cell, which enhances the ion compartmentation in transgenic rice cells. Moreover, with the accumulation of Na^+ in vacuoles, the transgenic rice increases the biosynthesis of proline in order to balance the osmotic potential. This has been confirmed by measuring the proline content in the leaves of transgenic and control rice plants. The proline contents of the transgenic lines with *nhaA* were about 1.72, 1.61, and 1.92 times those of control plants in 10, 20, and 30% (w/v) PEG-6000, respectively (Fig. 6). This

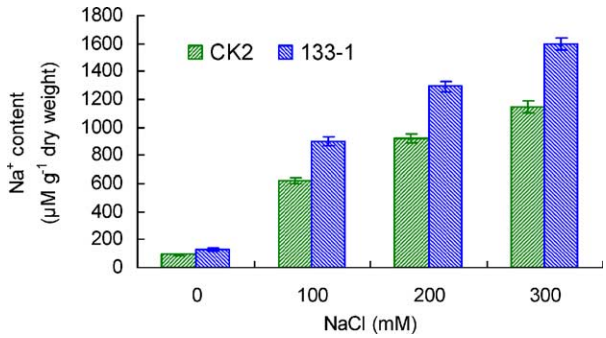

Fig. 5. The Na^+ contents in CK2 and transgenic plants (133-1) grown in the absence or presence of NaCl. The above-ground parts of the plants grown for 10 days under normal condition were harvested after 5 days of exposure to 0, 100, 200 or 300 mM NaCl. Dry weight was measured after 48 h at 70. Vertical bars represent the S.D. ($n = 5$).

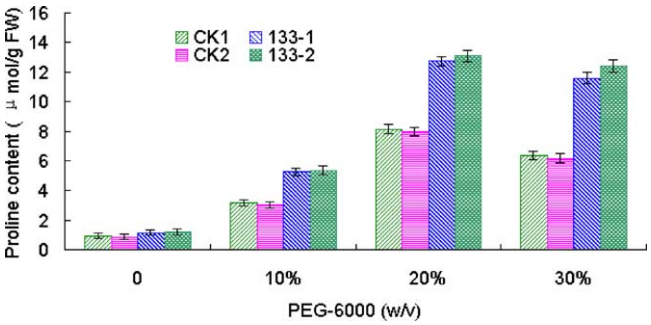

Fig. 6. Proline content of transgenic rice and controls treated with PEG-6000 for 15 days. CK1 and CK2 are the controls; 133-1 and 133-2 are the transgenic lines with the *nhaA* gene. All the rice plants were germinated on MS medium and cultured for 5 days before transfer to callus growth medium containing PEG-6000.

suggests that the transgenic plants with *nhaA* enhance their osmoregulation ability by activating proline biosynthesis.

Interestingly, measurements of the shoot and root biomass showed that constitutive over-expression of *nhaA* did not adversely affect the growth of transgenic plants when no stress was applied, but resulted in better growth than controls (Fig. 4). Under both salt and drought stresses, the growth of the transgenic plants improved significantly. Similarly, under either non-stress or osmotic stress conditions, *nhaA* over-expression did not affect the blooming, tasseling, or seeding of the transgenic plants, and it improved the rice yield compared with that of controls (Fig. 7). For instance, the average yield per plant for the transgenic lines was 1.22, 2.94, and 24.64 times that of control lines when plants were stressed with 0.1, 0.2, and 0.3 mol/L NaCl following germination, respectively, and was 1.12, 1.68, and 7.08 times that of control lines when the plants were grown under normal conditions for 8 weeks before being stressed with 0.1, 0.2, and 0.3 mol/L NaCl, respectively (Fig. 7).

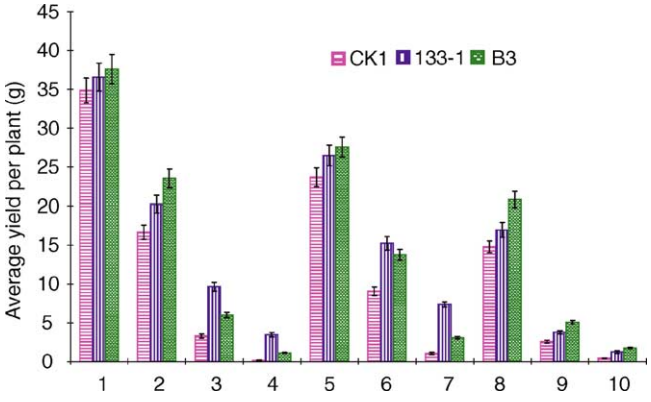

Fig. 7. Average yield per plant of transgenic rice lines 133-1, B3, and control CK1 under different stress conditions. Abscissa: (1) no stress; (2–4) rice stressed with 0.1–0.3 mol/L NaCl since germination; (5–7) 8-week-old rice plants stressed with 0.1–0.3 mol/L NaCl; (8–10) rice plants stressed with 10–30% PEG-6000 for 15 days, and then grown in soil without stress for 4 weeks, with three cycles of withholding water. Ten plants for each condition were calculated. Average yield per plant = (average spike number × average grain number per spike × average weight per 100 grains)/100.

3.3. The enhanced tolerance by over-expressing nhaA is different from that by δ-OAT

The comparison of transgenic rice plants with *nhaA* and those with *δ-OAT* under the same stress conditions showed that they were not identical. Both transgenic rice plants grew better than control plants when treated with 0.1–0.3 mol/L salt (Table 2). Nevertheless, the difference between the two transgenic plants was significant. When no stress was applied, both transgenic plants were significantly taller than controls; the average shoot height of transgenic rice plants with *nhaA* was 9.13 cm, which was similar to that of the transgenic rice plants with *δ-OAT* (9.04 cm). The growth of control plants might have been hampered by flooding stress, which induces the closure of leaf stoma, depresses photosynthesis, and decreases carbohydrate transportation. This also suggests that *nhaA* over-expression enhances tolerance of flooding stress.

When treated with 0.1 mol/L NaCl, the shoots of the transgenic rice plants with *nhaA* (8.09 cm) were significantly shorter than those with *δ-OAT* (8.93 cm) (Table 2), indicating that these rice plants suffered from osmotic stress at this NaCl concentration, and that the accumulated proline in lines with *δ-OAT* played an important role in osmoregulation to maintain relatively healthy growth. The lines with *nhaA* eliminated Na^+ from the cells via NhaA, which is probably not sufficient to maintain the osmoregulation ability necessary for growth.

A different picture was seen when the NaCl concentration exceeded 0.2 mol/L: the transgenic plants with *nhaA* grew much better than those with *δ-OAT* when 0.2 and 0.3 mol/L NaCl were applied (Fig. 4). This indicates that at high Na^+ concentrations, the effect of Na^+ toxicity exceeds the effect of osmotic stress, and that the osmoregulation activity

Table 2
t-Test of the shoot height of transgenic and control rice plants cultured in water for 10 days and then treated with NaCl for 5 days

NaCl (mol/L)	B3:CK2	133-1:CK2	B3:133-1	
0	3.41	3.55	0.27	
0.1	9.97	9.37	2.47	
0.2	6.66	7.09	1.73	
0.3	15.16	15.41	0.76	
	$\alpha = 0.05$	$\alpha = 0.01$	$\alpha = 0.005$	$\alpha = 0.001$
Critical value of the single tail test	1.69	2.44	2.73	3.35
Critical value of the double tail test	2.03	2.73	3.00	3.60

The data in the second table are the *t*-stats, which adopt a different variance hypothesis for double samples. CK2 is the control line; 133-1 is the transgenic rice line with the *nhaA* gene and B3 is the transgenic rice line with *δ-OAT*.

of proline is high only at low osmotic pressures. The difference between lines B3 (with *δ-OAT*) and 133-1 (with *nhaA*) decreased at 0.3 mol/L NaCl, as both plants were stressed severely and grew slowly at this high salt concentration. Moreover, the average shoot height of the control CK2 plants was even shorter (Fig. 4).

The transgenic rice plants were better adapted to drought than the control plants, and the trend in the growth change was consistent with that of the salt tolerance assay. However, the transgenic plants with *δ-OAT* grew better than those with *nhaA* under different drought stress conditions (data not shown). The yields of *nhaA* transgenic plants were higher than those of *δ-OAT* plants when stressed with NaCl concentrations exceeding 0.2 mol/L, and vice versa at low NaCl concentrations and drought conditions.

In conclusion, over-expression of the bacterial *nhaA* gene in rice plants enhanced their tolerance to salt and drought, probably by activating proline biosynthesis, and it also improved rice yield. Under high salt conditions, *nhaA* is more useful than *δ-OAT* in terms of tolerance enhancement. The transfer of both these genes into rice is predicted to generate more salt- and drought-tolerant rice plants, which would be of great benefit to agriculture.

Acknowledgements

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