A chimeric vacuolar Na⁺/H⁺ antiporter gene evolved by DNA family shuffling confers increased salt tolerance in yeast

Guangxia Wu^{a,b}, Gang Wang^{a,*}, Jing Ji^a, Yong Li^c, Hailing Gao^a, Jiang Wu^{b,d}, Wenzhu Guan^{a,b}

^a School of Environmental Science and Engineering, Tianjin University, Tianjin 300072, People's Republic of China

^b School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, People's Republic of China

^c Key Laboratory of Ecosystem Network Observation and Modeling, Institute of Geographic Sciences and Natural Resources Research, Chinese Academy of Science, Beijing 100101, People's Republic of China

^d Department of Agronomy, Tianjin Agricultural University, Tianjin 300384, People's Republic of China

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ABSTRACT

The vacuolar Na⁺/H⁺ antiporter plays an important role in maintaining ionic homeostasis and the osmotic balance of the cell with the environment by sequestering excessive cytoplasmic Na⁺ into the vacuole. However, the relatively low Na⁺/H⁺ exchange efficiency of the identified Na⁺/H⁺ antiporter could limit its application in the molecular breeding of salt tolerant crops. In this study, DNA family shuffling was used to create chimeric Na⁺/H⁺ antiporters with improved transport activity. Two homologous Na⁺/H⁺ antiporters from halophytes *Salicornia europaea* (SeNHX1) and *Suaeda salsa* (SsNHX1) were shuffled to generate a diverse gene library. Using a high-throughput screening system of yeast complementation, a novel chimeric protein SseNHX1 carrying 12 crossover positions and 2 point mutations at amino acid level was selected. Expression of SseNHX1 in yeast mutant exhibited approximately 46% and 22% higher salt tolerance ability in yeast growth test than that of SsNHX1 and SeNHX1, respectively. Measurements of the ion contents demonstrated that SseNHX1 protein in yeast cells accumulated more Na⁺ and slightly more K⁺ than the parental proteins did. Furthermore, this chimera also conferred increased tolerance to LiCl and a similar tolerance to hygromycin B compared with the parental proteins in yeast.

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1. Introduction

Soil salinity is one of the most serious threats to world agriculture, particularly in irrigated lands. The relatively high solute concentrations in the soil would seriously disrupt the normal metabolism of plants. To cope with salt stress and the toxic effect of sodium, plants have developed several adaptive mechanisms and one of the most effective strategies is to regulate cellular ion homeostasis involving excessive cytosolic Na⁺ exclusion and vacuolar compartmentalization of toxic ions from cytosol mediated by Na⁺/H⁺ antiporters (Quintero et al., 2002; Yokoi et al., 2002). In plants, Na⁺/H⁺ antiporters are located both in the plasma membrane (SOS1-type) and tonoplast (NHX1-type) where they function to catalyze the exchange of Na⁺ and H⁺ across membranes using a H⁺-motive force created by proton pumps as the driving power (Blumwald et al., 2000; Fukuda et al., 2004).

The compartmentation of Na⁺ into the vacuole is a critical process in salt tolerance, which provides an efficient mechanism to avert the deleterious effects of Na⁺ in the cytosol, while mediating vacuolar osmotic adjustment that facilitates water uptake into cells (Leidi et al., 2010). The ability to compartmentalize Na⁺ is considered to be a salt adaptation not only conserved in halophytes but also in glycophytes (Hasegawa et al., 2000). To date, a large number of cDNAs encoding vacuolar Na⁺/H⁺ antiporter have been isolated and increasing evidence has demonstrated that overexpressing vacuolar Na⁺/H⁺ antiporter gene improved salt tolerance in various plant species (Chen et al., 2007a, 2007b; Fukuda et al., 2011; Li et al., 2011; Zhang, 2001). All these studies indicate that an improved capacity for vacuolar Na⁺ sequestration is vital to salinity tolerance and overexpression of vacuolar Na⁺/H⁺ antiporters provides an approach that can contribute to the molecular breeding of salt-tolerant plants. However, the relatively low Na⁺/H⁺ exchange efficiency of the cloned Na⁺/H⁺ antiporters is a limiting factor in the application of these antiporter genes for crop breeding. One of the reasons for this may be that salt-sensitive plants showed weak induction of both Na⁺/H⁺ antiporter expression and activity

* Corresponding author. Tel.: +86 022 87402200; fax: +86 022 87402200.
E-mail address: gangwang@tju.edu.cn (G. Wang).

in response to NaCl treatment. Therefore, creating new vacuolar Na⁺/H⁺ antiporter gene from halophytes and molecular evolution of the identified antiporter with high strong salt tolerance ability are urgently needed for plant molecular breeding of salt tolerance.

DNA family shuffling is a developed technique for molecular directed evolution in vitro, which generates a library of chimeras by recombination of homologous sequences and combing useful mutation for individual genes. It mimicks and extends traditional breeding methods by recombining the same gene from related species or related genes from a single species in a single DNA shuffling reaction. Compared with other molecular breeding technology, DNA family shuffling can not only significantly accelerate the accumulation of beneficial mutations, but also efficiently increase the diversity of the target protein (Kikuchi and Harayama, 2002). In recent years, DNA family shuffling has widespread practical applications. For example, DNA family shuffling has been successfully used to improve activity and thermostability in lipase (Akbulut et al., 2013; Suen et al., 2004), improve activity and stability in endo-β-1,4-mannanase (Wang et al., 2013a), extend diversity in cytochrome P450 (Rosic et al., 2007) and alter substrate specificity in avidin (Niederhauser et al., 2012; Wang et al., 2013b). At the same time, the theoretical and technics of DNA shuffling have also been applied to improve the activity of Na⁺/H⁺ antiporters. It was reported that a novel plant vacuolar Na⁺/H⁺ antiporter AtNHXS1 was demonstrated about 1-fold improvement in salt tolerance via DNA shuffling of AtNHX1 from *Arabidopsis thaliana* (Xu et al., 2010). Subsequently, their laboratory conducted DNA family shuffling of AtNHX1 with wide type vacuolar Na⁺/H⁺ antiporters from *oryza sativa* (OsNHX1) and *Dendranthema morifolium* (DmNHX1) (Zhang et al., 2012). However, previous works focused on a single gene or multiple genes from glycophytes which have low salt tolerance range and limited the amount of improvement available.

In this study, we chose two strong Na⁺/H⁺ antiporter genes from halophytes *Salicornia europaea* (SeNHX1, GenBank accession no. AY131235) and *Suaeda salsa* (SsNHX1, GenBank accession no. AY261806) as templates (parents) to conduct DNA family shuffling. Chimeric Na⁺/H⁺ antiporters with higher ability of salt tolerance were identified by yeast complement system screening. Finally, one novel chimera (named SseNHX1) was obtained and demonstrated improvement salt tolerance in yeast.

2. Materials and methods

2.1. Yeast strains and medium

Saccharomyces cerevisiae wild type strain W303-1B (*MATα,ra3-1,leu2-3,trp1-1,his3-11,ade2-1,can1-100*) and the salt-sensitive mutant strain AXT3 (*Δena1-4::HIS3 Δnha1::LEU2Δnhx1::TRP1*) (Quintero et al., 2000), derived from W303-1B were used. Yeast cells were grown in YPD (1% yeast extract, 2% peptone, 2% glucose), and selected on SD media (0.67% yeast nitrogen base, 2% glucose, and the appropriate amino acid). Cation tolerance tests were performed in the alkali cation-free medium AP (10 mM arginine, 8 mM phosphoric acid, 2 mM MgSO₄, 1 mM KCl, 0.2 mM CaCl₂, 2% glucose, trace vitamins and minerals, at pH 5.6 with arginine and phosphoric acid) supplemented with or without NaCl or LiCl as indicated. Resistance to KCl or hygromycin B (Hyg B) was assayed in YPD medium. For induction of antiporter expression in cells carrying plasmids with antiporter genes under control of the GAL1 promoter, glucose (2%) was replaced by galactose (2%) in the growth medium.

2.2. DNA family shuffling and library construction

DNA family shuffling was performed as previously described with slight modifications (Stemmer, 1994; Zhao and Arnold, 1997).

Table 1
Primers used in the present study.

Primer	Oligonucleotide sequences (5′-3′)	Restriction site
ssF	CGCGGATCCATGTGGTCACAGTTAAGCTC	<i>Bam</i> H I
ssR	ACGCGTCGACTTATGTTCTGTGACAAATTAGTGG	<i>Sal</i> I
seF	CGCGGATCCATGTGTGACAAATTGAGCTC	<i>Bam</i> H I
seR	ACGCGTCGACTTGTCTGTAGCAAATTGTC	<i>Sal</i> I

The introduced restriction sites are underlined.

All the assembly and DNA amplification steps in the whole shuffling process were performed with Taq Plus DNA Polymerase (Tiangen, China), which is a mixture of Pfu and Taq DNA polymerase. Briefly, the DNA family shuffling procedure consists of four steps, that is parental templates preparation, DNase I digestion, primer-less PCR and primer PCR. Firstly, two DNA fragments containing the Na⁺/H⁺ antiporter genes were amplified by conventional PCR from plasmids pGH-SsNHX1 and pMD18T-SeNHX1 using the primers ssF/ssR and seF/seR, respectively (Table 1). Fragments of ≈1.7 kb used as substrates for shuffling were purified from 0.8% agarose gels using QIAEX II gel extraction kit (QIAGEN, Chatsworth, CA). Equal amounts of the two gene preparations were mixed for a total of ≈3 μg. This mixture was equilibrated for 5 min at 15 °C before 0.3 U DNase I (10 U/μL; order No. EN0525 Fermentas, St. Leon-Rot, Germany) was added. The digestion was done in the presence of 2 mM Mn²⁺ for 2 min at 15 °C and terminated by heating at 90 °C for 10 min. The digested DNA was electrophoresed as a smear in a 2% agarose gel. That the desired DNA fragments between 100 and 200 bp were isolated and purified as described above. Primer-less PCR was performed in mixture containing 2.5 U Taq Plus DNA Polymerase, 1× Taq Plus buffer, 1 μg of the fragments mixture, 0.2 mmol/L dNTP with a total volume of 50 μL. The reaction was carried out under the conditions of 94 °C for 1 min, 40 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s, and a final incubation at 72 °C for 8 min. One microlitre of this reaction was used as a template to amplify the full-length genes using a parallel set of primers (ssF/ssR or seF/seR) or a “skew” set of primers (ssF/seR or seF/ssR). The PCR program was 4 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 56 °C, 1 min 40 s at 72 °C, and finally 8 min at 72 °C. A single product of the correct size was obtained.

To generate the mutant library, the mutated PCR products of the full-length gene were digested by *Bam*H I and *Sal* I before purification and ligated into the yeast-*E. coli* shuttle vector pYES2 (Invitrogen, Carlsbad, USA) which was digested by the same enzymes. The ligation products were used to transform competent *E. coli* DH5α cells by electroporation. All the resulting colonies were washed down with sterile water, then plasmid DNA was extracted from the pooled *E. coli* library and subsequently transformed into the triple-deletion yeast mutant AXT3 using lithium acetate method (Gietz and Woods, 2002).

2.3. Screening for high salt tolerance yeast clones

Transformed yeast colonies harboring shuffled Na⁺/H⁺ antiporter genes were primarily screened on the AP selective medium supplemented with 100 mM NaCl. Plates were then incubated for 4 days at 30 °C. The selected yeast clones from the first screening were cultured in liquid AP to OD600=0.5, 5 μL of the cells were loaded onto AP medium supplemented with 125 and 150 mM NaCl for the second selection.

2.4. Salt tolerance test

To compare salt tolerance of the hybrid Na⁺/H⁺ antiporter gene with that of the parental genes, the ORF of SsNHX1 and SeNHX1 were also constructed into the yeast express vector pYES2 (driven

by a GAL1 promoter) using *Bam*H I and *Sal* I. The constructs were also introduced into the AXT3 mutant strain by separate transformation. As control, the wild type W303-1B and mutant AXT3 were all transformed with empty pYES2. The transformants were selected on SD medium lacking uracil.

For drop tests, transformed yeast cells were pre-cultured overnight in liquid SD media, and protein expression was induced by addition of 2% galactose. Six hours later, cells were adjusted to the same initial OD₆₀₀ = 1.0. Serial tenfold dilutions were prepared, and 5 μ L of each dilution was spotted on AP agar plates supplemented with different concentrations of NaCl. Growth was recorded over a period of 4 days.

For growth assays, 50 μ L of the same strain seed culture (OD₆₀₀ = 1) were grown in 4 ml of AP medium supplemented with 0, 25, 50, 75, 100, 150, 175 or 200 mM NaCl at 30 °C for 48 h with shaking (200 rpm). Cell growth was then determined by OD₆₀₀ measurements. There were three replications for each treatment.

For ion content measurements, yeast cells were grown in AP medium to an OD₆₀₀ of approximately 0.3, then resuspended into the same medium supplemented with 70 mM NaCl and continued to culture 6 h. Yeast cells were harvested by centrifugation, washed twice with ice-cold 10 mM MgCl₂, 10 mM CaCl₂, 1 mM HEPES, and extracted with acid as described before (Montiel and Ramos, 2007). Ion contents of the samples were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES, Thermo).

2.5. K⁺, Li⁺ and Hyg B tolerance test

Yeast cells were prepared under the same conditions for drop test of salt tolerance experiment. 5 μ L of each dilution was spotted on AP or YPD agar plates supplemented with or without KCl, LiCl or Hyg B as indicated. Growth was recorded over a period of 3 days.

2.6. Sequence analysis

In order to identify the substituted residues, the sequence alignments of SseNHX1 with SsNHX1 and SeNHX1 were performed by the CLUSTAL X program (Thompson et al., 2002). Protein secondary structure was predicted and two dimensional models of alpha helical transmembrane proteins was created by using online available software TMRPres2D (Omasits et al., 2014).

2.7. Statistical analysis

The statistical differences between the controls and shuffled gene SseNHX1 were assessed based on the analysis of variance ANOVA using SPSS (Ver. 15, Chicago, USA). Differences were considered significant at a probability level of $P < 0.05$.

3. Results

3.1. DNA family shuffling and screening of the library

The parental genes SsNHX1 and SeNHX1 were amplified by regular PCR and digested by DNase I into small fragments. Gel-purified 100–200 bp fragments were reassembled to a faintly visible single PCR product of the correct size. Reassembled products from primerless PCR were used as templates for primer PCR. As a result, the clear main gene bands on agarose gel corresponding to the expected size were obtained with different primers. The detection of products in the process of DNA family shuffling was shown as Fig. 1.

DNA family shuffling of SsNHX1 and SeNHX1 resulted in a library of 10⁵ colonies in *E. coli*. Plasmid DNA was isolated from this *E. coli* library and used to transform salt sensitive yeast strain AXT3 for screening improved forms of the vacuolar antiporter with the NaCl selective pressure. Approximately 200 colonies with better growth (as judged by eye) were selected for the second screening step, in which higher salt concentrations for yeast growth were applied. From these, one clone, named SseNHX1, showed more resistant to salt than all the other selective clones (Fig. 2a).

3.2. Increased salt tolerance conferred by shuffled SseNHX1

To compare the capacity of mediating salt tolerance in yeast expressing the shuffled SseNHX1 gene with that of the parental genes, the three Na⁺/H⁺ antiporter genes were expressed separately in the salt-sensitive AXT3 yeast mutant. The different yeast strains were grown on the AP medium supplemented with different NaCl concentrations. As shown in Fig. 2b, no differences in yeast cell growth were detected given the presence or absence of Na⁺/H⁺ antiporter genes on a NaCl free medium. Since Na⁺ sensitivity of the yeast mutant strain AXT3, it was seen to be noticeably suppressed with increasing salt concentration compared with that of the wild type W303-1B. Expression of SsNHX1 or SeNHX1 in AXT3 partially recovered the mutant phenotype, but the recovery was not as great as SseNHX1. Complementation with SseNHX1 expression was observed up to 10⁻² in the presence of 150 mM NaCl, whereas complementation with the parental gene expression were only able to increase population growth rates at 10⁻¹ in the same growth medium. These results accorded with the liquid salt tolerance test which showed that the salt tolerance of yeast expressing SseNHX1 was improved about 46% and 22% as compared to the yeast carrying SsNHX1 and SeNHX1, respectively (Fig. 2c). To determine whether SseNHX1 sequester greater Na⁺ to yeast cells, the total intracellular ion contents of the yeast cells grown in AP medium with 70 mM NaCl and 1 mM KCl were measured. As shown in Fig. 2d and e, the yeast mutant harboring SseNHX1 was able to accumulate 1.2 and 1.4-fold Na⁺ than that of yeast harboring the parents,

Fig. 1. Process of DNA family shuffling of SeNHX1 and SsNHX1 (a) Two 1.7 Kb fragments encoding Na⁺/H⁺ antiporter gene were amplified by PCR. (b) Fragments of 100–200 bp were purified from an agarose gel. (c) Purified fragments were reassembled into a full-length gene. (d) A single PCR product of the correct size is typically obtained in the presence of different primers. Lane 1 (ssF/ssR), Lane 2 (seF/seR), Lane 3 (ssF/seR), Lane 4 (seF/ssR).

Fig. 2. NaCl tolerance of SsNHX1, SeNHX1 and shuffled SseNHX1 genes in expressed in AXT3. (a) The second selection of shuffled genes. Several yeast clones obtained from the first screening were grown on AP plates containing 100, 125 and 150 mM NaCl for 3 days. (b) Complementation of yeast with SsNHX1, SeNHX1 and shuffled SseNHX1. Five microliters of serial decimal dilutions (10^0 , 10^{-1} , 10^{-2} , 10^{-3}) were spotted onto plates of the same medium supplemented with the indicated NaCl concentrations. Cells of strain W303-1B were used as control as indicated. Plates were incubated at 30 °C and photographed after 4 days. (c) Growth of the yeast strains in liquid medium. Absorbance measured at 600 nm after culturing for 48 h at 30 °C. Results are the Mean $\pm$ SD ($n=3$). (d) and (e) Na⁺ and K⁺ content of cells grown in the presence of NaCl. Intracellular Na⁺ and K⁺ contents were determined in cells grown in AP medium with 70 mM NaCl until mid-exponential growth phase. Results are the Mean $\pm$ SD ($n=3$).

while there were no noticeable differences between SseNHX1 and parental proteins with regard to K⁺ content. These results would suggest that the yeast strain expressing SseNHX1 have a higher ability to sequester Na⁺ into the vacuole than that from parental genes, and subsequently a enhanced NaCl tolerance.

3.3. SseNHX1 conferred resistance to KCl, LiCl and Hyg B in yeast mutant

In an attempt to characterize other cation (Li⁺ and K⁺) sensitivity of AXT3 expressing with SseNHX1 and parental NHX1 genes, similar complementation studies were carried out for each one of these genes. As shown in Fig. 3a, AXT3 mutant was hypersensitive to LiCl and failed to grow in the AP medium containing 3 mM LiCl while the wild type strain grew vigorously. Although expression of SsNHX1 and SeNHX1 also partly recovered tolerance to Li⁺, the recovery capacities of SsNHX1 and SeNHX1 were not as great as that of SseNHX1, suggesting that SseNHX1 also conferred Li⁺ tolerance. Overexpression of all NHX1 genes can, to a certain extent, increase the tolerance to KCl (Fig. 3b). These results suggested that SseNHX1 not only enhanced Na⁺ tolerance of yeast but also, to a relatively lesser extent, facilitated K⁺ and Li⁺ homeostasis.

Vacuolar Na⁺/H⁺ antiporters have function for resistance to various toxic cations, including Hyg B and Hyg B sensitivity of yeast nhx1 mutant has been widely used in functional complementation studies to identify Na⁺/H⁺ antiporters (Gaxiola et al., 1999; Shi et al., 2002). Clearly, the growth of the mutant yeast strain complemented with the different Na⁺/H⁺ antiporter genes was significantly different than that with the control plasmids (Fig. 3b). However, there appears to be no obvious difference among the Na⁺/H⁺ antiporter genes in the degree of the complementation with Hyg B, which indicated that the introduced mutations did not change the tolerance of Na⁺/H⁺ antiporter to Hyg B (Fig. 3b).

Table 2

DNA and amino acid substitutions in SseNHX1 mutant compared with SsNHX1.

DNA substitution	Amino acid substitution	Property
GCA→TAT	10A→10Y	Random mutation
GTT→CTA	16V→16L	Recombination
GTT→GCG	24V→24A	Recombination
TTT→TCT	117F→117S	Random mutation
CTT→TAT	150L→150Y	Recombination
CAC→AAT	206H→206N	Recombination
GCT→ATG	402V→398M	Recombination
TCA→ACG	410S→406T	Recombination
TTT→TAC	540F→536Y	Recombination
ACC→ATT	546T→542I	Recombination
AGC→AAC	549S→545N	Recombination
ACT→GAC	551T→547D	Recombination
TCA→CTA	554S→550L	Recombination
CAG→GAC	555Q→551D	Recombination
AGAATTGGCTAC	210-213RIAY	Deletion

3.4. Sequence analysis of shuffled SseNHX1

The shuffled SseNHX1 clone was sequenced and compared to those of the parental genes. Nucleotide sequence alignment identified numerous nucleotide substitutions (data not shown). Most of these nucleotide substitutions resulted in silent mutations, but a few resulted in amino acid residue substitutions (mutations). The amino acid sequence alignment with its parents revealed that the most amino acid sites were conserved (Fig. 4a). Based on sequence analyses, chimeric SseNHX1 was found to be well-shuffled and the different amino acid elements from two parents were presented in Fig. 4a. The mutant DNA sequences and the identities of the deduced amino acid sequence changes in the SseNHX1 are listed in Table 2. Sequencing revealed that this chimera with up to 17 crossovers, producing up to 12 amino acid substitutions compared with the more similar parent SsNHX1 (Table 2). The 12 amino acid

Fig. 3. Effect of LiCl, KCl and Hygromycin B on the salt-sensitive AXT3 yeast mutant, either with or without SsNHX1, SeNHX1 or SseNHX1. Ten-fold serial dilutions are as in Fig. 2b. Strains (5 µL) were spotted onto AP plates containing 3 mM LiCl or YPD plates supplemented with the indicated Hygromycin B or KCl concentrations. Yeast strain W303-1B was used as a positive control. Plates were incubated at 30 °C and photographed after 3 days.

substitutions were all derived from SeNHX1, and two new mutations (A10Y and F117S) were introduced into the mutant SseNHX1 protein sequence. In addition, amino acid residues RIAY (from 210 to 213) existing in the corresponding positions of parental SsNHX1 were deleted. The SseNHX1 protein shares 97% and 90% identity to the SsNHX1 protein and SeNHX1 protein respectively, suggesting SseNHX1 is a result of a shuffled Na^+/H^+ antiporter gene and could have the inherent functions. Two dimensional secondary structure of SseNHX1 protein sequence predicted by TMRPres2D showed 12 transmembrane domains (Fig. 4b). In addition, the binding site of amiloride ($^{86}\text{LFFIYLLPPI}^{95}$) that inhibits eukaryotic Na^+/H^+ antiporters was present in the third domain while cation binding domains was predicted in fifth and sixth domain (Fig. 4b).

4. Discussion

DNA family shuffling is a powerful tool for directed molecular evolution in vitro which provides an effective method to obtain chimeric proteins with improved properties. In view of plant vacuolar Na^+/H^+ antiporters playing important roles in cellular ion homeostasis and salt tolerance, the molecular evolution of Na^+/H^+ antiporters could potentially increase the application of these genes in salt tolerance-crop breeding and help us to understand the mechanism of transport and regulation of the Na^+/H^+ antiporters. In this study, the templates SsNHX1 and SeNHX1 share a sequence identity of 86% and 87% at the DNA and protein levels, respectively. This level of identity is beneficial to the occurrence of crossover annealing events. Xu et al. (2010) reported that using AtNHX1 from *Arabidopsis thaliana* as the substrate for DNA shuffling resulted in the salt tolerance range not remarkable. Moreover, previous studies demonstrated that the effect of DNA shuffling by a single gene was significantly lower than that of DNA family shuffling (Cramer et al., 1998). *S. salsa* and *S. europaea* are two succulent euhalophyte in *Chenopodiaceae*. Unlike some other halophytic plants, *S. salsa* and *S. europaea* lack salt glands or salt bladders on their leaves. Thus, these plants must compartmentalize the toxic Na^+ in the vacuoles. Furthermore, *S. europaea* plants can accumulate up to 50% NaCl of dry weight in shoot (Lv et al., 2012; Ushakova et al., 2005). Therefore, we used *S. salsa* and *S. europaea* as excellent candidates for the

design of improved novel proteins by DNA family shuffling, which promotes the range of salt tolerance, thereby yielding chimeras with higher salt tolerance than parents.

Primer-less PCR is the key step in the process of DNA family shuffling. Taq Plus DNA polymerase was used in our protocol, which can effectively prevent the accumulation of spontaneous mutations in each of DNA family shuffling steps and contribute to fragments recombination in primer-less PCR. In addition, for expanding the pool of recombinants and reduction of parental background genes, skew primers consisting of one gene at the 5'-end and the other gene at the 3'-end were added to amplify the parental genes (Fig. 1d). Another important factor of a successful DNA family shuffling lies in constructing a large enough mutant library ($\approx 10^5$ shuffled gene mutants by a one-time operation) and finding an appropriate screening method. We applied yeast mutant as a heterologous expression system (Dreyer et al., 1999) to screen novel Na^+/H^+ antiporter shuffled genes with higher NaCl tolerance. In terms of the establishment of the mutant library, we firstly establish a mutation plasmid library through the electric transformation of *E. coli*, and then transformed yeast with mutation plasmid library. Approximately 200 clones were obtained after the first screening, and finally one desired clone was chosen to be further characterized by comparison with the two parent Na^+/H^+ antiporters after the second selection test (Fig. 2a). Yeast complementation tests showed that this shuffled clone exhibited higher tolerance to NaCl compared with that of the parents (Fig. 2b).

The sequencing results revealed a high degree of chimera genesis and a low rate of spontaneous mutation. In fact, a high proportion of parental sequences occur frequently in family shuffling libraries. At the amino acid level, the crossovers resulted in 14 mutations away from the most similar parent. Two-dimensional structure showed that cation-binding and amiloride-binding sites of the shuffled Na^+/H^+ antiporter remained in transmembrane domains and were strictly conserved. It is important to notice that none of the random mutations and substitutions was found to locate in those strictly conserved regions. Our previous study demonstrated that the deletion of a part of the long C-terminal hydrophilic tail of SeNHX1 significantly inhibited the antiporter activity (Wu et al., 2014). Thus, it appeared that the six amino

Fig. 4. Sequence analysis of SseNHX1. (a) Protein sequence alignment of the shuffled chimera SseNHX1 with the two parental proteins. Conserved residues are indicated in white on a black background. Sequence data were obtained from GenBank, and aligned using ClustalX and GeneDoc. The accession numbers of two parental Na^+/H^+ antiporters are as follows: *S. salsa* (SsNHX1; AAP15178), *Salicornia europaea* (SeNHX1; AAN08157). (b) Proposed topological model of SseNHX1. The protein secondary structure was created by the program PROTTER, which was available online at <http://wlab.ethz.ch/protter/start/>.

acid substitutions, localized in the long C-terminal hydrophilic tail, might favor Na^+/H^+ exchange. Some progresses had been made regarding the regulation of the C-terminal region of the Na^+/H^+ antiporters in recent years. There are plenty of compelling precedents for the regulation of transport characteristics for Na^+/H^+ antiporter by modification of the transporter C terminus. In plasma membrane Na^+/H^+ antiporter salt-overly-sensitive 1 (SOS1), the C-terminus contains an auto-inhibitory domain and the interaction of this region with another domain of SOS1 kept the transporter in a resting state with basal activity (Quintero et al., 2011). Hamada et al. (2001) also found that the deletion of a part of the long C-terminal hydrophilic tail significantly inhibited the carrier activity of *Synechocystis* Species PCC 6803 which homologous to SOS1. Li et al. (2009) reported that deletion of the hydrophilic C-terminus of AtNHX4 dramatically increased the hypersensitivity of transformants. A similar phenomenon was found in *Caragana korshinskii* Na^+/H^+ exchanger 1 (CkNHX1), indicating that the hydrophilic C-terminus should be required for the activity of CkNHX1 (Yang et al., 2012). By contrast, the deletion of the hydrophilic C terminus of AtNHX1 from *Arabidopsis thaliana* resulted in a dramatic increase in the relative rate of Na^+/H^+ transport (Xu et al., 2010). Further studies indicated that the cation selectivity is regulated by calmodulin from within the vacuole in a Ca^{2+} - and pH-dependent manner (Yamaguchi et al., 2005). Coincidentally, the C-terminus deletion of a novel plant vacuolar Na^+/H^+ antiporter AtNHX51 evolved by DNA shuffling improved Na^+/H^+ exchange activity in yeast also suggesting a regulatory role of the C-terminus on the antiporter activity (Xu et al., 2010).

Several studies have appeared that plant Na^+/H^+ antiporters have ion specificity. Transgenic tomato overexpressing AtNHX1 showed increased antiport activity with both Na^+ and K^+ , albeit with lower specificity for K^+ (Zhang and Blumwald, 2001). By contrast, Qiu et al. (2004) reported that in tonoplast vesicles isolated from *Arabidopsis*, the vacuolar Na^+/H^+ exchange was shown significant transport activity with Na^+ , and to a lesser extent with Li^+ transport, but not K^+ transport. In the halophytes *Suaeda salsa*, *Populus euphratica* (Ye et al., 2009), and *Salicornia europaea*, the Na^+/H^+ antiport activity was greater than the K^+/H^+ exchange. Our data showed that the shuffled antiporter SseNHX1 had greatly selectivity to Na^+ , and to a relatively lesser extent to K^+ and Li^+ , which was consistent with the selectivity of halophytes.

In conclusion, the utility of DNA family shuffling of two halophytic Na^+/H^+ antiporter genes with strong tolerance to create Na^+/H^+ antiporter variants is an effective method in the enhancement of salt transport activity. One chimeric variant (SseNHX1) with 12 amino acid substitutions and 2 new mutations was obtained and demonstrated 1.2- and 1.4-fold improvement in sequestering Na^+ from the cells than parental genes, and is therefore a promising protein for the future development of transgenic crops resistant to high salt salinity. Further study of this hybrid antiporter, such as site-directed mutagenesis and modeling studies of molecular dynamics are needed to better understand the effects of the mutations present in the SseNHX1 variant on the transport activity.

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