Molecular Cloning and Expression Analysis of *AtNHX2* Promoter*

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Abstract The *Arabidopsis thaliana AtNHX2* gene is one member of the *Arabidopsis* NHX Na^+/H^+ antiporter gene family and play an important role in salt tolerance. A sequence-analyzed 2.8 kb DNA fragment on upstream of ATG start codon of *AtNHX2* gene was cloned into pCAMBIA1301-1. The promoter activity was detected by transient expression in onion epidermis. The reconstructed vector pCAMBIA1301-1/ *AtNHX2* promoter was transformed into *Arabidopsis thaliana* by Floral Dip method. *AtNHX2* promoter-GUS analysis in transgenic *Arabidopsis* showed that *AtNHX2* was expressed in all tissues. Strong GUS expression was detected in guard cells suggesting the possibility that it is involved in stomatal regulation. *AtNHX2* promoter activity was decreased by NaCl and up-regulated by KCl, demonstrating that NaCl and KCl regulation to *AtNHX2* expression occurs at the transcriptional level. GUS activity in old leaves was higher than in new leaves, which reveals a role of *AtNHX2* in salt tolerance. Strong GUS activity in root hair cells was observed. It suggests that *AtNHX2* may play an important role in partitioning of Na^+ into the enlarged vacuoles of root hair cells.

Key words *AtNHX2* promoter, transient expression, expression analysis, salt stress

Soil salinity is a major limit factor to productivity of crops. High salinity causes ion imbalance and hyperosmotic stress in plants. Excessively poisonous ion should be excluded from cytoplasm or partitioned into vacuolar and therefore ion homeostasis was reestablished. Na^+/H^+ antiporters are ubiquitous membrane proteins that play major roles in cellular pH and Na^+ homeostasis throughout the biological kingdom.

The plasma membrane Na^+/H^+ antiporters *SOS1* gene was cloned from the salt overly sensitive *Arabidopsis* mutant[1]. *SOS1* encodes a plasma membrane Na^+/H^+ antiporter and has functions of controlling long distant Na^+ translocation from root to shoot in *Arabidopsis*[2]. Overexpression of *SOS1* improves salt tolerance in transgenic *A. thaliana* and increased salt tolerance is correlated with reduced Na^+ accumulation under salt stress[3].

A Na^+/H^+ exchanger that is energized by the pH across the tonoplast facilitates vacuolar compartmentalization of the cation. The *Arabidopsis AtNHX1* was isolated by functional genetic complementation of a yeast mutant defected for the endosomal Na^+/H^+ antiporter yeast (ScNHX1) and has sequence similarity to mammalian NHE transporters[4~6]. Transgenic *Arabidopsis* and tomato plants that overexpress *AtNHX1* accumulate abundant quantities of the transporter in the tonoplast and exhibit substantially enhanced salt tolerance[4,6,7]. The transgenic rice plants overexpressing the *AgNHX1* gene displayed a significantly increased level of salt tolerance under conditions of salt stress[8]. *AtNHX1* expression was detected in all tissues except the root tip by *AtNHX1* promoter-GUS analysis in transgenic *Arabidopsis*. *AtNHX1* promoter activity was up-regulated by NaCl, KCl or ABA[9]. *AtNHX2* localizes to the vacuole of plant by transient expression in onion epidermal cells of an *AtNHX2*: GFP translational fusion and has a major function in vacuolar compartmentalization of Na^+[10].

Here we reported about the pattern of expression and salt stress regulation of *AtNHX2* in *Arabidopsis*. *AtNHX2* expression was detected in all tissues including the root tip. *AtNHX2* promoter activity was down-regulated by NaCl and up-regulated by KCl, which will suggest that *AtNHX2* expression is regulated by NaCl and KCl at the transcriptional level.

1 Materials and methods

1.1 Plant materials and growth conditions

Seeds (Columbia) were surface-sterilized in a solution of 10% Clorox for 5 min, washed with sterilized water for five times, and then planted onto agar medium containing 1/2 Murashige and Skoog (MS) salts, 10% sucrose and 0.7% agar, pH 5.7, with or without antibiotics. Seeds were treated at 4℃ for two days to synchronize germination. Plates were then incubated at 22℃ under programmed for a photoperiod of 16 h day and 8 h night. For the growth of mature plants, 10-day young seedlings were transferred from MS agar plates into pots which contained a soil mixture of vermiculite/ perlite (3∶1)

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and grew under normal growth conditions.

1.2 Plant treatments

Ten-day old seedlings grown in MS agar plates were used for different treatments. For NaCl, KCl, and cold treatments, the seedlings were transferred to a filter paper soaked with MS salts solution containing different concentrations NaCl (100, 200, 300 and 400 mmol/L) or 300 mmol/L KCl for 5 h, or stored at 4℃ for 24 h as described before[1]. For dehydration treatment, seedlings were transferred from MS agar medium to a filter paper and kept in a hermetic container for 30 min.

1.3 Construction of *AtNHX2* promoter-GUS

Two primers were designed according to *Arabidopsis* genomic sequence (GI: 5932531), P1: 5′ ATCAGAATTCTGGTGAAATCTTTCTGCAGC 3′; P2: 5′ CGATGAGCTCCCCTTTTTCTTCACCAATGA 3′. Underlined sequences are digested sites. A 2.8 kb promoter region just upstream of ATG start codon of *AtNHX2* gene was amplified from genomic DNA by PCR and verified by sequencing. The PCR fragment was cloned into the *Eco*R Ⅰ/*Sac* Ⅰ site of binary vector pCAMBIA 1301-1 (35S promoter was cut from pCAMBIA 1301) to obtain a transcriptional fusion of the *AtNHX2* promoter and the GUS coding sequence. The activity of promoter was tested by transient expression in onion epidermis, and then the constructs were introduced into *Agrobacterium* and transferred into *Arabidopsis* Columbia wild-type plants using the Floral Dip Method[11]. Two testing primers (Test P1 5′ GTTGGAATGGAGGGTCCGGAAAG 3′; Test P2: 5′ TGGCACAGCAATTGCCCGGC 3′) were used to validate transgenic plant by PCR. Transgenic lines in the T_2 generation were grown on MS medium containing 35 mg/L hygromycin and were subjected to β-glucuronidase (GUS) assays.

1.4 GUS assays

Young seedlings at different developmental stages and different parts from mature transgenic plants were collected and used for histochemical detection of GUS expression. For general detection of GUS expression patterns in seedlings and different cells and organs, these materials were stained at 37℃ overnight in 0.5 g/L X-gluc, 0.03% Triton X-100, and 0.1 mol/L sodium phosphate buffer (pH 7.0). To test the induction of GUS expression by NaCl, KCl, cold and dehydration, the treated and control transgenic seedlings were stained for 5 h for histochemical detection as described before[9].

2 Results

2.1 Cloning of *AtNHX2* promoter and construction of *AtNHX2* promoter-GUS

P1 and P2 were used to amplify a 2.8 kb promoter region upstream of the *AtNHX2* ATG start codon from genomic DNA and the product was tested by 1% agarose gel electrophoresis (Figure 1). An about 2 800 bp fragment was obtained and purified. This purified fragment was cloned into pCAMBIA1301-1 vector and was fused with the β-glucuronidase reporter gene (GUS). The recombined vector was digested with *Eco*R Ⅰ and *Sac* Ⅰ and its sequence was analyzed (Figure 2).

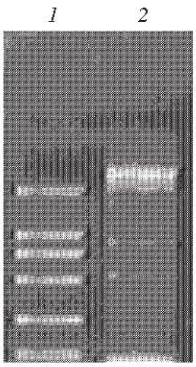

Fig. 1 Amplification of *AtNHX* promoter by PCR from genomic DNA of *Arabidopsis*
1: DL2000 marker; *2*: *AtNHX2* promoter.

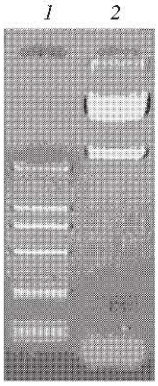

Fig. 2 Restrictive enzymic analysis of the pCAMBIA1301-1 /*AtNHX2* promoter
1: DL2000 marker; 2: Digested pCAMBIA1301-1/*AtNHX2* promoter by *Eco*R Ⅰ and *Sac* Ⅰ.

2.2 Transient expression

Constitutional plasmid was enwrapped with golden powder and the chimeric construct was delivered into onion epidermal cells by particle bombardment. Transient expression of *AtNHX2* promoter-GUS was monitored by GUS assays. The result shows that *AtNHX2* promoter have activity (Figure 3).

2.3 Test transgenic *Arabidopsis*

Transgenic plants were validated by PCR. Test P1 and Test P2 were used to amplify 450 bp fragment from genomic DNA of positive transgenic or wild *Arabidopsis* and the product was tested by 1% agarose gel electrophoresis (Figure 4). A 450 bp fragment was

obtained from transgenic *Arabidopsis* and control was not.

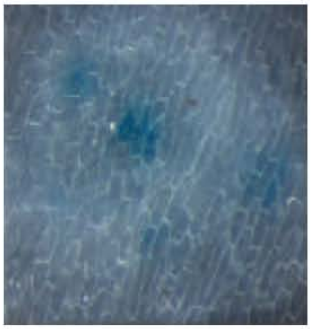

Fig. 3　Transient expression in onion epidermis

Fig. 4　Testing transgenic *Arabidopsis* by PCR
1: DL2000 marker; *2*: Transgenic *Arabidopsis*; *3*: Wild *Arabidopsis*.

2.4 The expression of *AtNHX2* is detected throughout plant development

A promoter-GUS analysis was employed to check the tissue expression pattern of *AtNHX2* during plant development. The construct of *AtNHX2* promoter-GUS was introduced into wild-type *Arabidopsis* plants. As shown in Figure 5, GUS expression was examined at all developmental stages by GUS staining, from seed germination to flowering and seed setting, confirming that *AtNHX2* is expressed at all developmental stages and throughout the *Arabidopsis* plant. GUS expression was detected in two-day old germinated seed, it had strong GUS staining in the hypocotyl, but relatively weak in the emerging radicle. The root tip at this stage had GUS expression (Figure 5a). Similarly, GUS activity was checked in the root tip of ten-day old seedlings (Figure 5b). GUS activity was also observed in the vascular strands of root (Figure 5c). Strong GUS expression was observed in the root hair of young seedlings. It suggests that *AtNHX2* may play an important role in partitioning Na^+ into the enlarged vacuoles of root hair cells (Figure 5d). *AtNHX2* was expressed at a high level in leaves and the expression was significantly strong in older leaves (Figure 5e). When observed under microscope at high magnification, a high level of GUS expression was detected in guard cells which appearing as dark blue

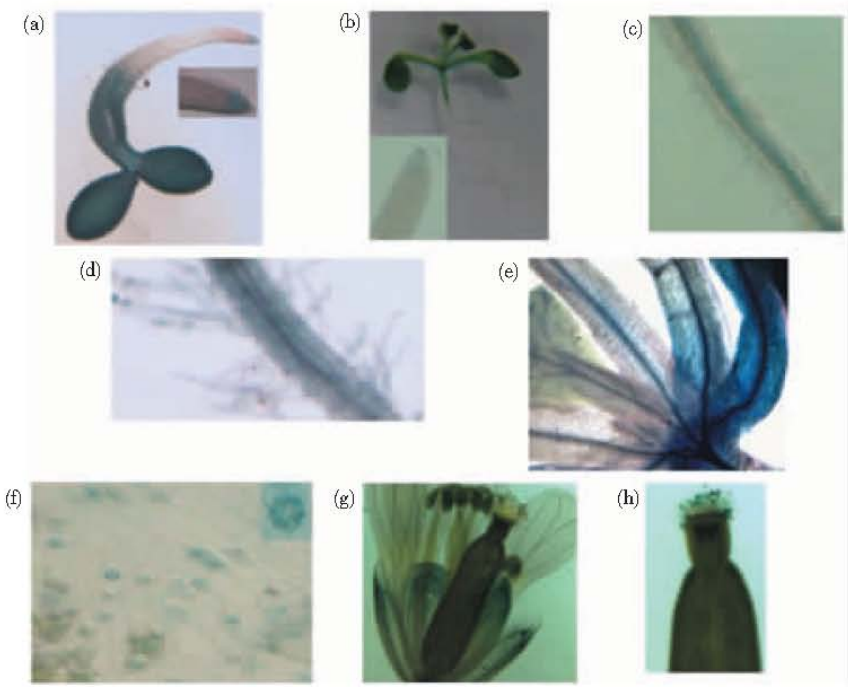

Fig. 5　Expression pattern of *AtNHX2* promoter-GUS in transgenic *Arabidopsis* plants
(a) Two-day old germinating seed and amplified picture of root tip. (b) Ten-day old seedling and amplified picture of root tip. (c) The vascular tissue of root. (d) Root hair. (e) Leaves. (f) GUS activity in guard cells and amplified picture of guard cells. (g) Flower. (h) Silique.

spots of GUS staining in the leaf (Figure 5f). Strong GUS staining in flowers was viewed in sepals and in pollens within anthers (Figure 5g). In siliques, GUS staining was obvious (Figure 5h).

2.5 *AtNHX2* promoter activity is regulated under NaCl and KCl treatments

The activity of *AtNHX2* promoter was decreased by treatment with different concentration of NaCl. The decrease level of the GUS activity was correlated with the NaCl concentration used in the treatments (Figure 6). *AtNHX2* promoter activity was up-regulated in leaf and root by treating with 300 mmol/L KCl, but was not impacted by cold and dehydration (Figure 7).

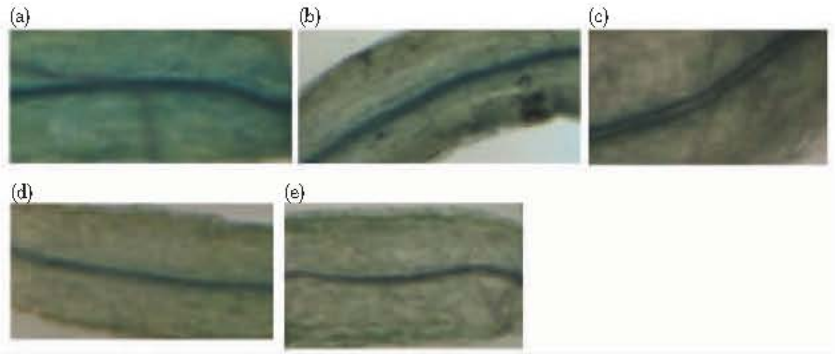

Fig. 6 GUS activity under salt stresses in different NaCl concentrations
(a) Control. (b) 100 mmol/L NaCl. (c) 200 mmol/L NaCl. (d) 300 mmol/L NaCl. (e) 400 mmol/L NaCl.

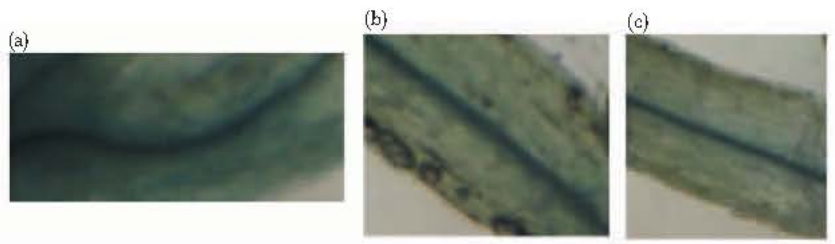

Fig. 7 GUS activity under different stresses
(a) KCl stress. (b) Cold stress. (c) Dehydration stress.

3 Discussion

AtNHX2 gene, one member of the *Arabidopsis* NHX Na^+/H^+ antiporter gene family, was localized to the vacuole of plant same as *AtNHX1* gene[4,10]. A 2.8 kb promoter region upstream of the *AtNHX2* ATG start codon was amplified by PCR. *AtNHX2* promoter-GUS was constructed and transformed into *Arabidopsis thaliana*. GUS expression and distribution were monitored via histochemical staining. Strong GUS expression was observed in the vascular strands of root and root tip of ten-day seedlings (Figure 5a, b). This pattern is reverse to that of *AtNHX1*[9]. And we speculated that their functions may be compensative. Stronger GUS staining in old leaves than in new leaves were observed in this experiment. The result shows a potential role that *AtNHX2* accumulates poisonous ions into old leaves preferentially and avails natural development of plant (Figure 5e). The expression of *AtNHX2* in guard cells suggests the possibility that it, like *AtNHX1*[7], is involved in stomatal regulation.

GUS expression could be depressed by NaCl treatments and its depressed level was correlated with the NaCl concentrations (Figure 6). This result was different from another study. Shuji *et al.*[10] reported that seedling steady-state mRNA levels of *AtNHX2* increase after treatment with NaCl. It may be due to the promoter region is not long enough to include the up-regulated boxes or there may be some boxes in the gene to increase the gene expression. However, *AtNHX2* promoter activity is up-regulated by KCl treatment. In salt stress, *AtNHX2* may improve the salt tolerance of plant by absorbing K^+ and this may be the reason of high *AtNHX2* expression at root tip consistently. Venema *et al.*[12] reported that *AtNHX1* reconstituted in artificial liposomes was able to mediate low affinity Na^+ as well as K^+ transport. Therefore, excepting the Na^+ transport, *AtNHX2* in company with *AtNHX1* may be involved in K^+ accumulation in the vacuole and in pH regulation in guard cells and other

cells. It would be interesting to identify these up-regulated boxes or reduced boxes which regulated the expression of *AtNHX*2 and to elucidate further *AtNHX*2 function *in vivo* by future identification of the location of *AtNHX*2 in the root meristematic cells.

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References

1 Shi H, Ishitani M, Kim C, *et al*. The *Arabidopsis thaliana* salt tolerance gene *SOS*1 encodes a putative Na^+/H^+ antiporter. Proc Natl Acad Sci USA, 2000, **97** (12): 6896 ~ 6901

2 Shi H, Quintero F J, Pardo J M, *et al*. The putative plasma membrane Na^+/H^+ antiporter SOS1 controls long-distance Na^+ transport in plants. Plant Cell, 2002, **14** (2): 465 ~ 477

3 Shi H, Lee B, Wu S, *et al*. Overexpression of a plasma membrane Na^+/H^+ antiporter gene improves salt tolerance in *Arabidopsis thaliana*. Nature Biotechnology, 2003, **21** (1): 81 ~ 85

4 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** (5431): 1256 ~ 1258

5 Gaxiola R A, Rao R, Sherman A, *et al*. The *Arabidopsis thaliana* proton transporters, *AtNHX*1 and Avp1, can function in cation detoxification in yeast. Proc Natl Acad Sci USA, 1999, **96** (4): 1480 ~ 1485

6 Quintero F J, Blatt M R, Pardo J M. Functional conservation between yeast and plant endosomal Na^+/H^+ antiporters. FEBS Lett, 2000, **471** (2 ~ 3): 224 ~ 228

7 Zhang H X, Blumwald E. Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. Nat Biotechnol, 2001, **19** (8): 765 ~ 768

8 Ohtaa M, Hayashia Y, Nakashimaa A. Introduction of a Na^+/H^+ antiporter gene from *Atriplex gmelini* confers salt tolerance to rice. FEBS Lett, 2002, **532** (3): 279 ~ 282

9 Shi H, Zhu J K. Regulation of expression of the vacuolar Na^+/H^+ antiporter gene *AtNHX*1 by salt stress and abscisic acid. Plant Molecular Biology, 2002, **50** (3): 543 ~ 550

10 Shuji Yokoi, Quintero F J, Cubero B, *et al*. Differential expression and function of *Arabidopsis thaliana* NHX Na^+/H^+ antiporters in the salt stress response. Plant J, 2002, **30** (5): 529 ~ 539

11 Clough S J, Bent A F. Floral dip: a simplified method for agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J, 1998, **16** (6): 735 ~ 743

12 Venema K, Quintero F J, Pardo J M, *et al*. The *Arabidopsis* Na^+/H^+ exchanger *AtNHX*1 catalyzes low affinity Na^+ and K^+ transport in reconstituted liposomes. J Biol Chem, 2002, **277** (4): 2413 ~ 2418

拟南芥 *AtNHX2* 启动子的克隆及表达模式分析*

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摘要 *AtNHX*2 基因是拟南芥 NHX 基因家族的一员，编码了一种液泡膜中的 Na^+/H^+ 反向运输体并对拟南芥的耐盐能力起着重要的作用. 采用 PCR 扩增的方法克隆了拟南芥 *AtNHX*2 基因启始密码子上游约 2.8 kb 的 DNA 片段，并将其克隆到植物表达载体 pCAMBIA1301-1 中，通过基因枪轰击洋葱表皮瞬时表达的方法，初步检测启动子的活性. 将重组质粒 pCAMBIA1301-1/*AtNHX*2 promoter 转化拟南芥并筛选纯合子. *AtNHX*2 promoter-GUS 分析显示 *AtNHX*2 在所有的组织中均有表达，包括根尖. 在保卫细胞中检测到了强烈的 GUS 表达，这一结果表明，*AtNHX*2 对特殊细胞的 pH 调控和 K^+ 自身稳定方面起着重要的作用. *AtNHX*2 启动子的活性可被 NaCl 抑制，并且抑制的强度和 NaCl 的浓度成正相关. 300 mmol/L KCl 处理可增强启动子的活性，说明 NaCl 和 KCl 是在转录水平上调控 *AtNHX*2 的表达. 在老叶中 GUS 活性比在新叶中 GUS 活性强，这说明了 *AtNHX*2 优先将有毒的离子积累在老叶中，从而有利于植物的正常发育. 在根毛细胞中也观测到了强烈的 GUS 活性，这就暗示了 *AtNHX*2 在扩大的液泡中储存 Na^+.

关键词 *AtNHX*2 启动子，瞬时表达，表达模式分析，盐胁迫

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