

# 杜梨 *PbNHX1* 基因的克隆、表达分析及功能验证

刘 威<sup>1,2,3</sup>, 李 慧<sup>2,3</sup>, 蔺 经<sup>2,3</sup>, 杨青松<sup>2,3</sup>, 常有宏<sup>2,3\*</sup>

(<sup>1</sup>南京农业大学园艺学院, 南京 210095; <sup>2</sup>江苏省农业科学院果树研究所, 南京 210014;

<sup>3</sup>江苏省高效园艺作物遗传改良重点实验室, 南京 210014)

**摘要:**【目的】克隆 1 个杜梨 Na<sup>+</sup>/H<sup>+</sup> 逆向转运蛋白基因 *PbNHX1*, 并对其序列特征、表达特点及功能进行研究。【方法】采用 RT-PCR 和 PCR 克隆 *PbNHX1* 的 cDNA 和 DNA 序列, 利用生物信息学方法进行序列分析, 定量 PCR 检测其在非生物胁迫下转录水平变化, 酵母互补试验检测 *PbNHX1* 基因的离子转运能力。【结果】杜梨 *PbNHX1* 基因 cDNA 编码区长 1 704 bp, 对应基因组 DNA 序列长 3 594 bp, 由 13 个外显子和 12 个内含子组成, 编码蛋白含 567 个氨基酸。系统进化树显示, *PbNHX1* 位于液泡膜型 Na<sup>+</sup>/H<sup>+</sup> 逆向转运蛋白分支上, 与杨树液泡膜型 Na<sup>+</sup>/H<sup>+</sup> 逆向转运蛋白 *PtNHX1.3* 基因亲缘关系较近。正常生长条件下, *PbNHX1* 在杜梨叶片中表达量高于根。施加 200 mmol·L<sup>-1</sup> NaCl、10% (φ) PEG6000 或 100 μmol·L<sup>-1</sup> ABA 后, *PbNHX1* 在叶片中的转录水平持续上升; 其在根中的表达量先升后降, 表达高峰依次出现在处理后 6、3 和 6 h。 *PbNHX1* 的转入可恢复 NaCl、KCl 和潮霉素 B 对 *nhx1* 缺失酵母菌株 AXT3 的生长抑制, 转基因酵母细胞中 Na<sup>+</sup> 和 K<sup>+</sup> 含量显著增加。【结论】*PbNHX1* 具有植物 *NHXs* 基因家族的固有特征, 对盐碱、渗透胁迫和 ABA 处理均存在转录响应, 转入该基因能够提高酵母 *nhx1* 缺失突变体 AXT3 对盐胁迫的耐受能力, 部分恢复其对阳离子的转运功能, 从而促进 Na<sup>+</sup>、K<sup>+</sup> 积累。

**关键词:** 杜梨; *PbNHX1* 基因; 序列特征; 表达特点; 酵母互补

中图分类号: S661.2

文献标志码: A

文章编号: 1009-9980(2018)02-0137-10

## Cloning, expression and functional analysis of *PbNHX1* gene in *Pyrus betulaefolia*

LIU Wei<sup>1,2,3</sup>, LI Hui<sup>2,3</sup>, LIN Jing<sup>2,3</sup>, YANG Qingsong<sup>2,3</sup>, CHANG Youhong<sup>2,3\*</sup>

(<sup>1</sup>College of Horticulture, Nanjing Agricultural University, Nanjing 210095, Jiangsu, China; <sup>2</sup>Institute of Pomology, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, Jiangsu, China; <sup>3</sup>Jiangsu Key Laboratory for Horticultural Crop Genetic Improvement, Nanjing 210014, Jiangsu, China)

**Abstract:** 【Objective】*Pyrus betulaefolia* is one of the main rootstocks for pear *P. betulaefolia* as a rootstock can effectively reduce Na<sup>+</sup> accumulation in the root, inhibit the Na<sup>+</sup> transportation to the scion, and improve the salt tolerance of pear varieties. In order to reveal the molecular mechanism of salt tolerance and provide experimental basis for the further research, we researched the Na<sup>+</sup>/H<sup>+</sup> antiporter protein in *P. betulaefolia*. One *PbNHX1* gene was cloned from this species and its sequence characteristics and expression characteristics were analyzed. 【Methods】The sodium/hydrogen exchanger 1-like gene (XM\_018651314.1) in *Pyrus×bretschneideri* (Chinese white pear) was used as the electronic probe to search the transcriptome database of *P. betulaefolia* NaCl-treated seedlings. Then, one transcript Pbr017120 was obtained for designing gene-specific primers. Indeed, its cDNA and DNA sequences were isolated using RT-PCR and PCR techniques. And the physical and chemical properties of *PbNHX1* protein were analyzed by ProtParam online software. The introns and exons of *PbNHX1* genes were analyzed using Gene Structure Display Server. The signature motifs were analyzed by MEME software and the phylogenetic tree was built by MEGA 6.0. The expression condition of *PbNHX1* under the abiotic stresses, such as 200

收稿日期: 2017-08-21 接受日期: 2017-11-01

基金项目: 国家自然科学基金(31372051)

作者简介: 刘威, 男, 在读硕士研究生, 研究方向为果树逆境生理与分子生物学。Tel: 18801587183, E-mail: lw\_7183@163.com

\*通信作者 Author for correspondence. Tel: 025-84390224, E-mail: cyh@jaas.ac.cn

mmol·L<sup>-1</sup> NaCl, 10% (φ) PEG6000 and 100 μmol·L<sup>-1</sup> ABA, were analyzed by quantitative PCR (qRT-PCR). Finally, yeast complementary experiments with a salt sensitive yeast mutant AXT3 were performed to verify the functions of *PbNHX1* gene. After solid and liquid cultivation, the growth status of transgenic yeast was detected. Their total Na<sup>+</sup> and K<sup>+</sup> contents were also tested by a flame-graphite furnace atomic absorption spectrometer. 【Results】The cDNAs of *PbNHX1* was 1 704 bp, and its DNA was 3 594 bp, which included 13 exons and 12 introns. This gene encoded a protein containing 567 amino acids, its relative molecular weight and isoelectric point (pI) was 62.179 ku and 5.55, respectively. Moreover, the *PbNHX1* elements consisted of C<sub>2 861</sub>H<sub>4 437</sub>N<sub>669</sub>O<sub>805</sub>S<sub>21</sub>. The phylogenetic tree showed that *PbNHX1* was located in the branch of vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter, which was far from the plasma membrane Na<sup>+</sup>/H<sup>+</sup> antiporter gene of *Arabidopsis AtNHX7* or rice *OsNHX8*, and was closely related to poplar vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter gene *PtNHX1.3* or *Arabidopsis AtNHX2*. The expression level of the *PbNHX1* was higher in the leaves than that in its roots under normal growth conditions. After treatment with 200 mmol·L<sup>-1</sup> NaCl, *PbNHX1* transcriptional level obviously increased both in the roots and in the leaves. For example, *PbNHX1* expression level increased firstly and then decreased in the roots once the seedlings were treated with salt. Its expression peak appeared at 6 h. At that time, the amount of *PbNHX1* transcription was 2.4 times higher than that of the control. Then, its expression level began to decrease closely to the original level at 24 h. On the one hand, the expression level of *PbNHX1* kept on rising in the leaves after the treatment of salt. In the case of 10% PEG6000, *PbNHX1* expression level increased firstly and then decreased in the roots. Its expression peak appeared at 3 h, which was 1.2 times higher than that of the control. After that time, its expression level turned to fall down and recovered closely to the original level at 24 h. In the leaves, the expression level of *PbNHX1* continued to rise when the salt existed and it was 22.6 times higher than that of the control at 24 h. After 100 μmol·L<sup>-1</sup> ABA treatment, *PbNHX1* expression level increased firstly and then decreased in the roots. Its expression peak appeared at 6 h, which was 1.6 times higher than that of the control. Then its transcription declined closely to the original level at 24 h. In the leaves, the expression level of *PbNHX1* increased during the treatment period, which was 8.0 times higher than that of the control at 24 h. These results indicated that *PbNHX1* was regulated significantly in the leaves under different abiotic stress. The results of YPD solid culture showed that transform of *PbNHX1* could recover the growth inhibition of NaCl, KCl and hygromycin B to the *nhx1* mutant yeast strain AXT3 when the cells were treated with 20 mg·L<sup>-1</sup> hygromycin B, 20–50 mmol·L<sup>-1</sup> NaCl or 0.5–1.00 mol·L<sup>-1</sup> KCl. Meanwhile, the results of liquid culture showed that transform of *PbNHX1* reduced the growth inhibition of the mutant strain AXT3 to NaCl and KCl when the cells were treated with 50–100 mmol·L<sup>-1</sup> NaCl or 0.5–1.0 mol·L<sup>-1</sup> KCl. Furthermore, the contents of Na<sup>+</sup> and K<sup>+</sup> significantly increased in *PbNHX1* transgenic yeast cells compared with the mutant strain without this gene when 20 mmol·L<sup>-1</sup> NaCl presence. 【Conclusion】Our results have showed that *PbNHX1* gene belong to the *NHX* gene family of *P. betulaefolia*, which has the inherent characteristics of plant *NHXs* family. This gene response to NaCl, osmotic and ABA stresses. Transfer of the *PbNHX1* gene can increase the salt tolerance of the *nhx1* mutant yeast strain AXT3 and partly recover its ion transport capacity and facilitate the accumulation of the Na<sup>+</sup> and K<sup>+</sup> ions.

**Key words:** *Pyrus betulaefolia*; *PbNHX1* gene; Sequence characteristics; Expression feature; Yeast complementation

土壤盐渍化是阻碍农作物正常生长、减少产量的主要逆境因素之一,其对植株的伤害包括离子毒害和渗透胁迫两方面<sup>[1]</sup>。研究表明,高等植物抵御盐

胁迫的有效途径是通过液泡膜质子泵 H<sup>+</sup>-ATPase 和 H<sup>+</sup>-PPase 所建立的跨膜质子梯度驱动膜上的 Na<sup>+</sup>/H<sup>+</sup> 逆向转运蛋白 (vacuolar Na<sup>+</sup>/H<sup>+</sup> exchange or antiport-

er, NHX), 将细胞质中过多的  $\text{Na}^+$  区域化在液泡内, 减轻  $\text{Na}^+$  积累对细胞质代谢酶和膜系统的毒害, 与此同时, 转运到液泡内的  $\text{Na}^+$  作为有益的渗透调节剂, 降低细胞渗透势, 从而抵御高浓度盐分产生的渗透胁迫, 使植物更好地适应盐渍生境<sup>[2]</sup>。

植物  $\text{Na}^+/\text{H}^+$  逆向转运蛋白家族可分为液泡膜型和质膜型两大亚族<sup>[3]</sup>, 液泡膜型  $\text{Na}^+/\text{H}^+$  逆向转运蛋白主要是将进入细胞质中的  $\text{Na}^+$  区隔化到液泡中, 以降低胞质中的  $\text{Na}^+$  浓度; 而质膜型  $\text{Na}^+/\text{H}^+$  逆向转运蛋白主要是将细胞质中的  $\text{Na}^+$  逆向运送到胞外, 从而防止  $\text{Na}^+$  对细胞质中细胞器的危害。第一个从植物中分离得到的液泡膜型  $\text{Na}^+/\text{H}^+$  逆向转运蛋白基因是拟南芥 *AtNHX1*<sup>[4]</sup>。迄今为止, 从不同植物中均已成功克隆其同源基因<sup>[5-12]</sup>, 表明 *NHX1* 普遍存在于高等植物体内, 并在生物进化过程中相对保守<sup>[1]</sup>。通过酵母互补实验已明确 *NHX1* 直接参与  $\text{Na}^+$  转运过程。不同植物来源 *NHX1* 基因的转入, 可恢复 *nhx1* 缺失型酵母菌株的  $\text{Na}^+$  转运功能<sup>[11,13-15]</sup>。但是, 由于不同物种生活习性各异, 因此 *NHX1* 同源基因间的表达模式和具体作用部位存在差异。在盐胁迫下, *NHX1s* 通常表现为转录上调响应, 但主要表达部位不同, 如菊花 *Dgnhx1*、烟草 *DmNHX1*、棉花 *GhNHX1*、盐地碱蓬 *SsNHX1* 和白三叶 *TrNHX1* 主要在植株叶片中表达, 而豇豆 *VuNHX1*、绿豆 *VrNHX1* 和菊苣 *CiNHX1* 在根中的表达显著高于叶中<sup>[5-11]</sup>。因此从已有研究结果来类推 *NHX1* 同源基因的转录特点和功能不太恰当, 从新物种中分离盐胁迫响应 *NHX1* 基因, 解析其表达模式, 进行酵母互补功能验证, 仍有重要意义。

杜梨 (*Pyrus betulaefolia* Bunge) 为梨的主要砧木之一, 是梨属植物重要的抗性种质资源。以杜梨为砧木, 能够改善梨树的耐盐能力<sup>[16-17]</sup>。目前已经分

离获得杜梨 CBL (calcineurin B-like protein)-CIPK (CBL-interacting protein kinase) 信号通路的上游基因家族<sup>[18-19]</sup>, 但该通路所调控的下游功能基因 (如 *NHXs*) 在杜梨耐盐过程中的作用尚不明确。笔者首次克隆了杜梨液泡膜型  $\text{Na}^+/\text{H}^+$  逆向转运蛋白基因 (*PbNHX1*), 分析其序列特点和转录特征, 利用 *nhx1* 缺陷型酵母 (AXT3) 对其功能进行验证, 有助于解析该类基因在杜梨耐盐分子调控机制中的作用, 为梨抗盐碱育种提供候选基因及试验依据。

## 1 材料和方法

### 1.1 植物材料及胁迫处理

以江苏省农业科学院果树研究所保存的杜梨单株实生苗来源的组培苗为试材 (组培苗单株编号 001, 保存: 继代培养基 MS+6-BA 0.2 mg·L<sup>-1</sup> 和生根培养基 1/2MS+IBA 0.5 mg·L<sup>-1</sup>), 生长一致的无菌苗转入生根培养基 30 d 后, 轻轻洗净根部琼脂, 转入 Hoagland 营养液, 培养过程用加氧泵补充氧气, 每 3 d 更换营养液, 培养温度 (25±0.5)°C, 光照周期 14 h/10 h (光照/黑暗)。水培 90 d 后转入含 200 mmol·L<sup>-1</sup> NaCl、10% ( $\varphi$ , 后同) PEG6000 或 100  $\mu\text{mol}\cdot\text{L}^{-1}$  脱落酸 (ABA) 的 Hoagland 营养液, 进行盐、渗透胁迫和 ABA 处理, 并于处理后 0、1、3、6、9、12、24 h 收集植株的根和叶片待用。

### 1.2 方法

1.2.1 *PbNHXs* 基因的分离及序列分析 以中国白梨 (*Pyrus bretschneideri*) 基因组数据库中的 Sodium/hydrogen exchanger 1-like 基因 (XM\_018651314.1) 作为电子探针, 搜索杜梨盐胁迫转录组数据库<sup>[19]</sup>, 获得转录本 Pbr017120 作为候选基因, 设计特异引物 (表 1), 扩增 *PbNHX1* 基因。称取 200 mmol·L<sup>-1</sup> NaCl 处

表 1 杜梨 *PbNHX1* 研究用引物

Table 1 Primers used for the analysis of *PbNHX1* from *Pyrus betulaefolia*

引物名称 Primer name	引物序列 Primer sequences	用途 Usage
<i>PbNHX1</i>	F: 5'-ATGGCAGTTCTTGAAGCCATACAG-3' R: 5'-TCAAGAAGTATGTTTCAGGATGTTTCAG-3'	基因克隆 Gene cloning
<i>PbNHX1</i>	F: 5'-AGATTCCTCATTTTCTCAGCACCA-3' R: 5'-CCAGCAACCAAACCAAGA-3'	荧光定量 PCR Fluorogenic quantitative PCR
<i>PbEF-1<math>\alpha</math></i>	F: 5'-CCGTGGGTATGTGCTTCC-3' R: 5'-GACAGCAATGTGGGAGGTGT-3'	荧光定量 PCR Fluorogenic quantitative PCR
<i>PbNHX1</i>	F: 5'- <u>CGGGATCC</u> ATGGCAGTTCTTGAAGCCATACAGT-3' R: 5'- <u>CGGAATTCTCA</u> AGAAGTATGTTTCAGGATGTTTCAGTATG-3'	酵母表达载体构建 Yeast expression vector construction

注: 下划线表示限制性内切酶切位点。

Note: Restriction endonuclease sites were marked with underlines.



理24 h后的杜梨幼苗根和叶0.3 g,利用RNA提取试剂盒(TaKaRa MiniBEST Plant RNA Extraction Kit, TaKaRa)提取总RNA,进行3次生物学重复,合成cDNA第1链(PrimeScript™ 1st Strand cDNA Synthesis Kit, TaKaRa),并进行RT-PCR扩增(PrimeSTAR® Max DNA Polymerase, TaKaRa)。同时,提取杜梨植株总DNA(TaKaRa MiniBEST Plant Genomic DNA Extraction Kit, TaKaRa)用于扩增*PbNHX1*的基因组序列。所有扩增产物克隆到载体pMD™ 19-T上,转化大肠杆菌测序。

ProtParam 软件(<http://www.expasy.org/tools/prot-param.html>)分析*PbNHX1*编码蛋白理化性质,在线基因结构分析系统(<http://gsds.cbi.pku.edu.cn/index.php>)分析*PbNHX1*内含子和外显子的组成,MEME软件<sup>[20]</sup>(<http://meme-suite.org/tools/meme>)寻找液泡膜型NHX特征基序。利用ClustalW对拟南芥、番茄、杨树、杜梨、水稻、玉米的37个NHX氨基酸序列进行多重比对,利用MEGA 6.0(<http://megasoftware.net>)<sup>[21]</sup>程序采用邻接法(neighbor-joining, NJ)生成NHX1基因的系统进化树,校验参数Bootstrap重复1 000次(分支长度与系统发育距离成正比)。

**1.2.2 *PbNHX1*表达模式研究** 利用SYBR Premix Ex Taq II试剂盒(TaKaRa, RR820)在TaKaRa TP800型实时荧光定量PCR仪上进行qRT-PCR扩增,分析*PbNHX1*的表达特点。25 μL反应体系:12.5 μL SYBR Premix Ex Taq II, 1 μL 10 μmol·L<sup>-1</sup>引物, 2 μL cDNA模板和8.5 μL ddH<sub>2</sub>O。PCR反应程序:95℃变性30 s, 95℃ 5 s和60℃ 30 s, 40个循环。实时荧光定量PCR以*PbEF-1α*为内参基因,不加cDNA为阴性对照,采用2<sup>-ΔΔCt</sup>法计算基因表达量。

**1.2.3 酵母互补试验** 利用带Hind II和Sal I酶切位点的特异引物(表1)进行PCR扩增获得*PbNHX1*基因的全长编码区,连接到酵母表达载体P426(Addgene, Cambridge, USA)上。经过酶切验证和测序确认后,阳性质粒被命名为P426-*PbNHX1*。采用醋酸锂法将P426-*PbNHX1*和空载体P426分别转化到盐敏感酵母突变体AXT3(Δ*ena1-4::HIS3*, Δ*nha1::LEU2*, Δ*nhx1::TRP1*)中<sup>[22]</sup>。它们的单菌落接种到YPD液体培养基,30℃培养至OD<sub>600</sub>为0.5时,取10 μL菌液转入含0、20、50 mmol·L<sup>-1</sup> NaCl或0.5、1.0 mol·L<sup>-1</sup> KCl的100 mL YPD液体培养基过夜培养12 h测定OD<sub>600</sub>;取100 μL菌液分别稀释1、10、100和

1 000倍,各吸取5 μL点到含0、20、50 mmol·L<sup>-1</sup> NaCl或0.5、1.0 mol·L<sup>-1</sup> KCl的YPD固体培养基或20 mg·L<sup>-1</sup>潮霉素B的SD *ura*<sup>-</sup>培养基上,30℃培养3 d。利用Tanon-3500数字凝胶成像系统(上海天能科技有限公司)对细胞生长状态进行拍照。

**1.2.4 Na<sup>+</sup>和K<sup>+</sup>的测定** AXT3、AXT3P426和AXT3PbNHX1的单菌落接种到YPD液体培养基中,30℃培养至OD<sub>600</sub>为0.5时,取10 μL菌液转入含20 mmol·L<sup>-1</sup> NaCl的100 mL YPD液体培养基过夜培养12 h。3 000 g离心10 min收集酵母细胞,在预冷后的10 mmol·L<sup>-1</sup> MgCl<sub>2</sub>、10 mmol·L<sup>-1</sup> CaCl<sub>2</sub>和1 mmol·L<sup>-1</sup> HEPES缓冲液中重复洗涤3次。加入0.4%(φ) HCl使细胞破裂析出胞内离子,95℃反应20 min<sup>[23]</sup>。清除细胞碎片后,用火焰石墨炉原子吸收光谱仪(ZEEnit®700P, Jena, Munich, Germany)测定上清液中总Na<sup>+</sup>和K<sup>+</sup>含量。所用标准品购自Merck公司(Germany)。

**1.2.5 统计分析与作图** 所有试验均设3次生物重复,数据采用SPSS 13.0中的邓肯检测(*P* < 0.05)进行差异显著性检验,用SigmaPlot 10.0作图。

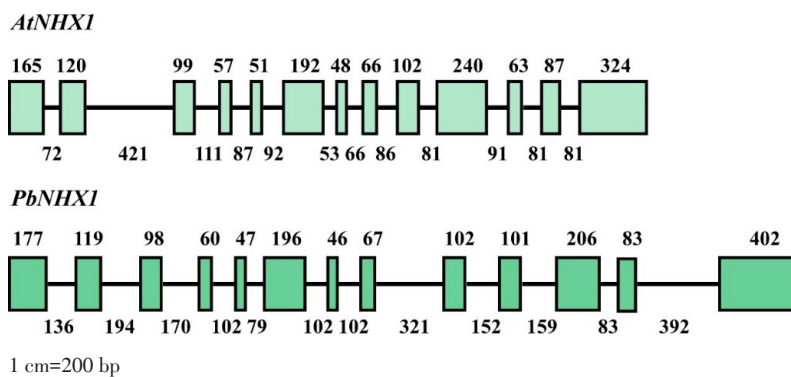
## 2 结果与分析

### 2.1 *PbNHX1*基因的分离

提取200 mmol·L<sup>-1</sup> NaCl处理24 h后的杜梨幼苗总RNA,合成cDNA第1链后,进行RT-PCR扩增,同时以其DNA为模板,扩增*PbNHX1*基因编码区,均获得单一条带,经克隆测序发现*PbNHX1*的cDNA和DNA序列长度分别为1 704和3 594 bp。*PbNHX1* DNA序列由13个外显子和12个内含子组成,与拟南芥*AtNHX1*的外显子和内含子数目相同(图1)。

### 2.2 *PbNHX1*基因的生物信息学分析

用ProtParam在线软件(<http://www.expasy.org/tools/protparam.html>)分析*PbNHX1*编码蛋白的理化性质,预测结果表明*PbNHX1*编码1个含567个氨基酸的蛋白,相对分子质量为62.179 ku,等电点(p)为5.55,元素组成为C<sub>2 861</sub>H<sub>4 437</sub>N<sub>669</sub>O<sub>805</sub>S<sub>21</sub>。MEME软件分析发现,*PbNHX1*所编码蛋白有Na<sup>+</sup>/H<sup>+</sup>逆向转运蛋白的3个特异性基序(84~125、169~197、283~325位氨基酸);并含有真核生物Na<sup>+</sup>/H<sup>+</sup>逆向转运蛋白抑制剂氨氯吡嗪的结合位点(LFFIYLLPPI)(图2)。利用来自不同植物的37个Na<sup>+</sup>/H<sup>+</sup>逆向转运蛋白构建系统进化树,所获得的杜梨*PbNHX1*基因与液泡膜型Na<sup>+</sup>/H<sup>+</sup>



外显子和内含子分别用方框和直线表示,数字为核苷酸的数目。

The boxes represent exons, straight lines indicate introns and numbers mean nucleotide number, respectively.

图1 拟南芥 *AtNHX1* 和杜梨 *PbNHX1* 的基因结构

Fig. 1 The gene structures of *AtNHX1* from *Arabidopsis thaliana* and *PbNHX1* from *P. Betulaefolia*



图2 MEME 软件生成的液泡膜型 NHX1 的特异性基序

Fig. 2 Specific motif of vacuolar type *PbNHX1* generated using MEME

逆向转运蛋白基因如杨树 *PtNHX1.2/PtNHX1.3*、拟南芥 *AtNHX2.3/AtNHX3* 以及玉米 *ZmNHX4* 等亲缘关系较近,与 *PtNHX1.3* 亲缘关系最近;与质膜型  $\text{Na}^+/\text{H}^+$  逆向转运蛋白基因如拟南芥 *AtNHX7*、水稻 *OsNHX8* 等的亲缘关系较远(图3)。上述结果表明,笔者分离的杜梨 *PbNHX1* 基因属于 *NHXs* 家族成员,编码液泡膜型  $\text{Na}^+/\text{H}^+$  逆向转运蛋白。

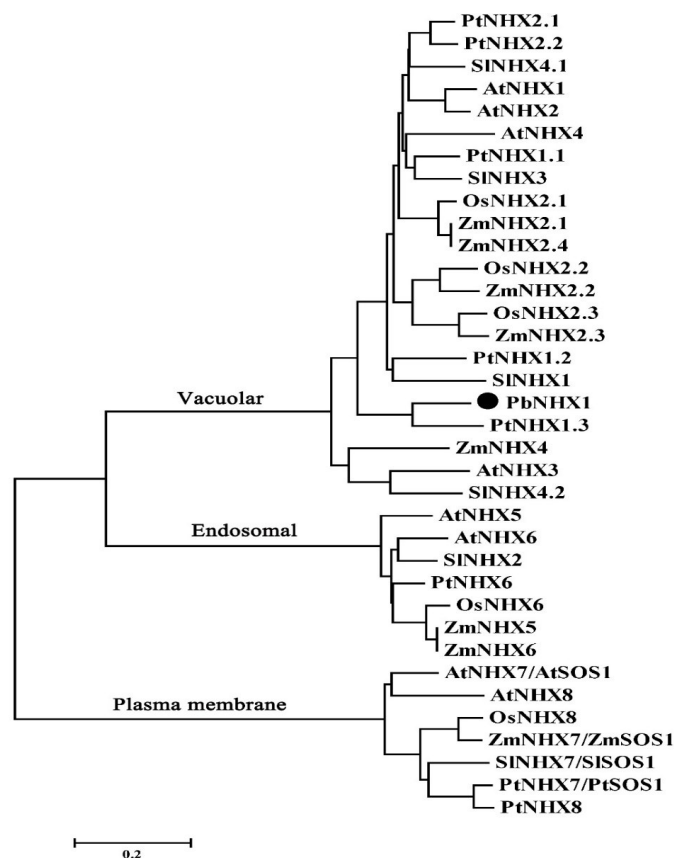
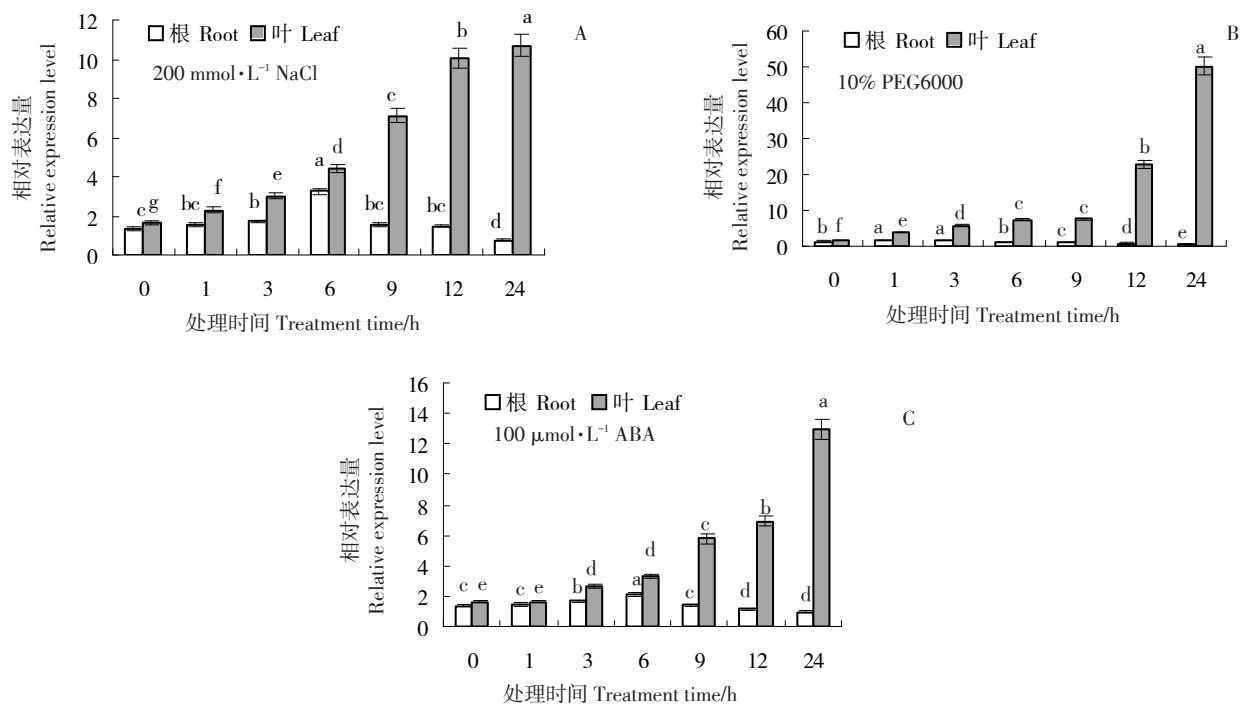
### 2.3 *PbNHX1* 基因的表达特点

正常生长情况下,在杜梨叶和根中都可检测到 *PbNHX1* 基因的表达(图4)。施加  $200 \text{ mmol} \cdot \text{L}^{-1}$   $\text{NaCl}$  后,根和叶中 *PbNHX1* 基因表达量均显著增加,根中表达量在处理6 h时达到峰值(为未处理对照的2.4倍),之后表达量开始下降,至24 h表达量接近

起始水平;叶中表达量持续上升。施加10% PEG6000后,根和叶中 *PbNHX1* 基因表达量均显著增加,根中表达量在处理3 h时达到峰值(为未处理对照的1.2倍),之后表达量开始下降,至24 h表达量接近起始水平;叶中表达量持续上升。施加  $100 \mu\text{mol} \cdot \text{L}^{-1}$  ABA 后,根和叶中 *PbNHX1* 基因表达水平增加,根中表达量在处理6 h时达到峰值(为未处理对照的1.6倍),之后表达量开始下降,至24 h接近起始水平;叶中表达量持续上升。综上所述,不同非生物胁迫下 *PbNHX1* 基因主要在叶片中进行转录调控。

### 2.4 *PbNHX1* 功能分析

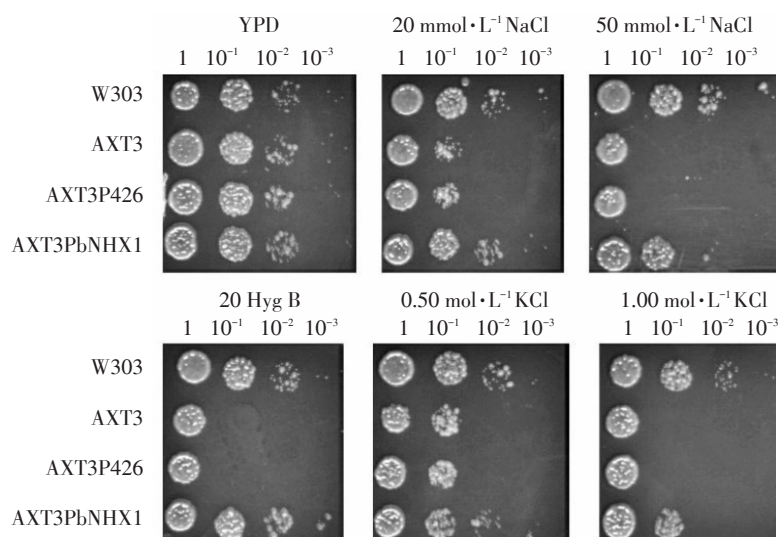
酵母互补试验结果(图5)显示,在 YPD 固体培养基上(对照),野生型酵母菌株 W303、突变株

图 3 植物 *NHXs* 的系统进化分析Fig. 3 Phylogenetic analysis of *NHXs* genes from different plant species

不同小写字母表示在  $P < 0.05$  差异显著。下同。

Different small letters indicate significant difference at  $P < 0.05$ . The same below.

图 4 非生物胁迫下 *PbNHX1* 的表达情况Fig. 4 The expression analysis of *PbNHX1* under different treatments



1、 $10^{-1}$ 、 $10^{-2}$ 、 $10^{-3}$  分别表示菌液稀释 1、10、100 和 1 000 倍。

1,  $10^{-1}$ ,  $10^{-2}$  and  $10^{-3}$  showed bacteria solution diluted 1-, 10-, 100- and 1 000-fold, respectively.

图 5 酵母菌株在不同固体培养基中的生长状况

Fig. 5 The growth situation of different yeast strains in solid medium

AXT3、转入空质粒 P426 的突变体菌株 AXT3P426 和转入 *PbNHX1* 基因的突变体菌株 AXT3PbNHX1 均可正常正长。在含有  $20 \text{ mg} \cdot \text{L}^{-1}$  hygromycin B 的 YPD 培养基上,与对照相比,*PbNHX1* 的表达显著提高了 AXT3PbNHX1 对 hygromycin B 的耐受性。在含有  $20 \text{ mmol} \cdot \text{L}^{-1}$  NaCl 或  $50 \text{ mmol} \cdot \text{L}^{-1}$  NaCl 的 YPD 培养基上, W303 生长正常;由于突变体 AXT3 丧失了  $\text{Na}^+$  转运功能, AXT3 和 AXT3P426 的生长明显受到抑制;而 *PbNHX1* 基因的转入,可以使酵母 AXT3 在培养基的生长得到恢复。在含有  $0.5 \text{ mol} \cdot \text{L}^{-1}$  KCl 或  $1.00 \text{ mol} \cdot \text{L}^{-1}$  KCl 的 YPD 培养基上,各酵母菌株的生长与添加 NaCl 的情况一致(图 5)。*PbNHX1* 转化酵母后可以弥补酵母 *nhx1* 功能的缺失,表明 *PbNHX1* 介导  $\text{Na}^+$  和  $\text{K}^+$  转运。

液体培养结果(表 2)显示,在正常生长情况下(YPD 培养基),4 种菌株的生长无显著差异;加入 50、75 或  $100 \text{ mmol} \cdot \text{L}^{-1}$  NaCl 后, W303 的  $\text{OD}_{600}$  值变化较小,菌株的生长几乎不受影响; AXT3 和 AXT3P426 的  $\text{OD}_{600}$  值随着 NaCl 浓度的增加而显著下降,而 2 者之间无显著差异,说明 NaCl 的加入使突变体菌株的生长受阻,空质粒的转入不影响菌株对 NaCl 的敏感性; AXT3PbNHX1 的  $\text{OD}_{600}$  值逐步减小,但下降幅度较小,说明 *PbNHX1* 的转入可恢复酵母菌株突变体 AXT3 在盐胁迫条件下的生长。这与固

表 2 酵母菌株在不同液体培养基中的生长状况

Table 2 The growth situation of different yeast strains in liquid medium

培养基 Medium	酵母菌株 Yeast strain			
	W303	AXT3	AXT3P426	AXT3P- bNHX1
YPD	$1.84 \pm 0.25 \text{ a}$	$1.72 \pm 0.15 \text{ a}$	$1.72 \pm 0.18 \text{ a}$	$1.80 \pm 0.19 \text{ a}$
$50 \text{ mmol} \cdot \text{L}^{-1}$ NaCl	$1.83 \pm 0.22 \text{ a}$	$1.09 \pm 0.11 \text{ b}$	$1.13 \pm 0.13 \text{ b}$	$1.66 \pm 0.17 \text{ a}$
$75 \text{ mmol} \cdot \text{L}^{-1}$ NaCl	$1.80 \pm 0.31 \text{ a}$	$0.89 \pm 0.10 \text{ c}$	$0.81 \pm 0.07 \text{ c}$	$1.53 \pm 0.16 \text{ b}$
$100 \text{ mmol} \cdot \text{L}^{-1}$ NaCl	$1.79 \pm 0.18 \text{ a}$	$0.65 \pm 0.08 \text{ c}$	$0.62 \pm 0.05 \text{ c}$	$1.43 \pm 0.15 \text{ b}$
$0.50 \text{ mmol} \cdot \text{L}^{-1}$ KCl	$1.83 \pm 0.26 \text{ a}$	$1.23 \pm 0.13 \text{ b}$	$1.33 \pm 0.15 \text{ b}$	$1.60 \pm 0.17 \text{ ab}$
$0.75 \text{ mmol} \cdot \text{L}^{-1}$ KCl	$1.80 \pm 0.33 \text{ a}$	$1.05 \pm 0.09 \text{ b}$	$1.06 \pm 0.09 \text{ b}$	$1.05 \pm 0.11 \text{ b}$
$1.50 \text{ mmol} \cdot \text{L}^{-1}$ KCl	$1.76 \pm 0.18 \text{ a}$	$0.63 \pm 0.07 \text{ c}$	$0.61 \pm 0.06 \text{ c}$	$1.43 \pm 0.14 \text{ b}$

体培养结果类似(图 5)。加入 0.5、0.75 或  $1.0 \text{ mol} \cdot \text{L}^{-1}$  KCl 后,4 种菌株的生长情况与 NaCl 处理基本相同,表明 *PbNHX1* 的转入减弱了 KCl 对突变体菌株 AXT3 的生长抑制。

## 2.5 酵母菌株离子含量

利用火焰石墨炉原子吸收光谱仪对 AXT3、AXT3P426 以及 AXT3PbNHX1 中的  $\text{Na}^+$  和  $\text{K}^+$  含量进行测定(表 3)。正常生长情况下(YPD 液体培养),3 种酵母细胞中  $\text{Na}^+$  含量无显著差异,但是 AXT3PbNHX1 中  $\text{K}^+$  含量显著高于 AXT3 和 AXT3P426。在添加  $20 \text{ mmol} \cdot \text{L}^{-1}$  NaCl 的 YPD 培养基上生长 12 h 后,3 种酵母细胞中  $\text{Na}^+$  和  $\text{K}^+$  含量均明显上升,其中 AXT3 中  $\text{Na}^+$  含量为对照的 3.1 倍,  $\text{K}^+$  含量为对照的 1.4 倍;



表3 不同酵母菌株 Na<sup>+</sup>、K<sup>+</sup>含量Table 3 The contents of Na<sup>+</sup> and K<sup>+</sup> in different yeast strains

酵母菌株 Yeast strain	Na <sup>+</sup> 含量 Na <sup>+</sup> content/(nmol·mg <sup>-1</sup> )		K <sup>+</sup> 含量 K <sup>+</sup> content/(nmol·mg <sup>-1</sup> )	
	对照 Control	20 mmol·L <sup>-1</sup> NaCl	对照 Control	20 mmol·L <sup>-1</sup> NaCl
	AXT3	33.29± 3.15 a	102.33± 10.23 b	75.33± 6.46 b
AXT3P426	32.86± 3.07 a	101.54± 10.26 b	73.52± 5.55 b	108.22± 10.05 b
AXT3PbNHX1	33.82± 3.44 a	275.75± 23.12 a	115.23± 11.02 a	188.23± 15.03 a

AXT3P426中Na<sup>+</sup>含量为对照的3.1倍,K<sup>+</sup>含量为对照的1.5倍。而转*PbNHX1*基因的酵母细胞增加幅度最大,AXT3PbNHX1中Na<sup>+</sup>含量为对照的8.2倍,K<sup>+</sup>含量为对照的1.6倍。上述结果表明,*PbNHX1*可弥补AXT3对Na<sup>+</sup>和K<sup>+</sup>的转运缺陷,促进Na<sup>+</sup>和K<sup>+</sup>在转基因酵母细胞中的积累,且对Na<sup>+</sup>积累的促进更为明显。

### 3 讨论

笔者采用电子探针筛选及RT-PCR方法从杜梨中分离获得了1个液泡膜Na<sup>+</sup>/H<sup>+</sup>逆向转运蛋白基因(*PbNHX1*),经NCBI数据库比对发现*PbNHX1*所编码蛋白与拟南芥液泡膜型Na<sup>+</sup>/H<sup>+</sup>逆向转运蛋白AtNHX1<sup>[4]</sup>具有很高的同源性,且2者基因结构相似(内含子与外显子数目相同,大小类似),*PbNHX1*含有3个液泡膜型NHX1蛋白的特征基序和真核生物Na<sup>+</sup>/H<sup>+</sup>逆向转运蛋白抑制剂氨氯吡嗪的结合位点(LFFIYLLPPI)<sup>[3,5,7,24]</sup>。进化树显示,液泡膜型Na<sup>+</sup>/H<sup>+</sup>逆向转运蛋白又可分为2类,Class I为定位在液泡膜上的,Class II是定位在内膜囊泡上,在动物、真菌内膜上存在相应的同源蛋白<sup>[1-2]</sup>。笔者获得的杜梨*PbNHX1*逆向转运蛋白位于Class I分支上,据此推测该蛋白可能定位于液泡膜上。液泡膜型Na<sup>+</sup>/H<sup>+</sup>逆向转运蛋白基因对于盐胁迫的表达响应具有组织特异性<sup>[5-11,25]</sup>,拟南芥*AtNHX2*在花和根中的表达量相似,但在叶片中的表达量很少,且在花序茎中基本检测不到;而*AtNHX3*和*AtNHX4*只在花和根中表达<sup>[26]</sup>。液泡膜型Na<sup>+</sup>/H<sup>+</sup>逆向转运蛋白基因除了与植物耐盐有关,还与植物干旱胁迫有关,干旱胁迫时,豇豆*VuNHX1*和霸王*ZxNHX1*逆向转运蛋白表达水平显著上升,这说明*VuNHX1*和*ZxNHX1*都受干旱诱导且表达上调<sup>[9,27]</sup>。笔者发现杜梨*PbNHX1*的转录水平受

PEG6000处理而诱导上调,由此可以推测该基因可能参与植株抗旱过程,这还需要通过转基因试验加以证实。

NaCl处理后,杜梨*PbNHX1*在叶中的表达水平较高,而在根中的表达水平较低,这说明*PbNHX1*基因的表达具有组织特异性,表明杜梨*PbNHX1*逆向转运蛋白主要在叶中行使功能。在正常生长的杜梨幼苗中也能检测到*PbNHX1*基因的表达,这说明杜梨*PbNHX1*逆向转运蛋白属于组成型表达,拟南芥*AtNHX1*、甜菜*BvNHX1*和菊芋*HtNHX1*也属于组成型表达<sup>[4,28-29]</sup>。盐胁迫下,杜梨*PbNHX1*与菊花*Dgnhx1*、烟草*DmNHX1*、盐地碱蓬*SsNHX1*和白三叶*TrNHX1*的表达水平上升主要发生在植株叶片组织中,这一现象与Na<sup>+</sup>积累密切相关<sup>[6]</sup>,表明它们可能负责叶片细胞中液泡Na<sup>+</sup>的转运与富集。然而,杜梨*PbNHX1*在盐胁迫过程的具体作用方式仍需进一步的转基因试验证实。ABA处理后,*PbNHX1*的基因表达模式与NaCl处理时的极为相似,以此推测*PbNHX1*的表达可能受ABA的诱导,依赖于ABA途径。这种现象已有报道,棉花*GhNHX1*表达受ABA的诱导表达<sup>[7]</sup>,水稻*OsNHX1*、*OsNHX2*、*OsNHX5*的转录也依赖于ABA<sup>[30]</sup>。这些结果表明ABA在植物对盐胁迫的适应性反应中起着至关重要的作用。此外,PEG处理后,杜梨*PbNHX1*的表达水平在叶中上升量较大,但在根中的表达水平变化较小。这说明杜梨叶片中*PbNHX1*逆向转运蛋白对渗透胁迫较为敏感,且在胁迫中叶片首先进行响应表达。*NHX1*基因的转录水平也响应PEG处理而上升(如苹果*MdNHX1*和拟南芥*AtNHX1*)<sup>[4,12]</sup>,表明它们在植物抵抗渗透胁迫的过程中亦起着重要作用。

啤酒酵母和植物Na<sup>+</sup>/H<sup>+</sup>转运蛋白的潜在结构和功能相似,使其成为研究离子转运蛋白运输特点和生理功能的良好模型<sup>[13]</sup>,为验证植物*NHXs*基因功能提供了一种简便有效的方法<sup>[31]</sup>。不同植物来源*NHX1*基因的表达可以减弱酵母*nhx1*突变体对盐胁迫的敏感性<sup>[6-8,11-15,24,32-35]</sup>。杜梨*PbNHX1*的转入可以使突变体酵母菌株AXT3在含20 mmol·L<sup>-1</sup> NaCl或50 mmol·L<sup>-1</sup> NaCl的YPD培养基上恢复正常生长,显著减弱了其对NaCl、KCl胁迫的敏感性,这与前人研究结果类似<sup>[11,13-15,32-35]</sup>,说明*NHX1s*基因的转入能够部分弥补突变体酵母菌株AXT3中*NHX1*的功能。分别将*AtNHX1*和*ScNHX1*转入ATX3突变型酵母



中,在含有 NaCl 的 AP 培养基 2 者长势相似且明显优于对照组,也验证了 *NHX1* 基因可以提高酵母耐盐能力<sup>[13]</sup>。在添加 20 mmol·L<sup>-1</sup> NaCl 条件下,转 *PbNHX1* 基因 AXT3 细胞中 Na<sup>+</sup> 和 K<sup>+</sup> 含量显著增加。拟南芥 *AtNHX1* 的转入同样可促进酵母细胞中 Na<sup>+</sup>、K<sup>+</sup> 积累,且对 Na<sup>+</sup> 积累的促进作用尤为明显<sup>[35]</sup>,而 *AtNHX3* 具有 K<sup>+</sup> 离子转运活性<sup>[36]</sup>,水稻 *OsNHX1*、*OsNHX2*、*OsNHX3* 和 *OsNHX5* 具有转运 Na<sup>+</sup> 和 K<sup>+</sup> 的活性<sup>[31]</sup>。这些结果表明,NHXs 家族既可转运单一的 Na<sup>+</sup>/K<sup>+</sup>,也可同时转运 Na<sup>+</sup>/K<sup>+</sup>。综上所述,杜梨 *PbNHX1* 与其他 *NHX1* 基因功能相同,在盐胁迫过程中具有转运阳离子的功能。

#### 参考文献 References:

- [1] BASSIL E, BLUMWALD E. The ins and outs of intracellular ion homeostasis: NHX-type cation/H<sup>+</sup> transporters[J]. Current Opinion in Plant Biology, 2014, 22(22): 1–6.
- [2] RODRIGUEZ- ROSALES M P, GALVEZ F J, HUERTAS R, ARANDA M N, BAGHOUR M, CAGNAC O, VENEMA K. Plant NHX cation/proton antiporters[J]. Plant Signaling & Behavior, 2009, 4(4): 265–276.
- [3] HAMADA A, SHONO M, XIA T, OHTA M, HAYASHI Y, TANAKA A, HAYAKAWA T. Isolation and characterization of a Na<sup>+</sup>/H<sup>+</sup> antiporter gene from the halophyte *Atriplex gmelini*[J]. Plant Molecular Biology, 2001, 46(1): 35–42.
- [4] SHI H Z, ZHU J K. Regulation of expression of the vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter gene *AtNHX1* by salt stress and abscisic acid[J]. Plant Molecular Biology, 2002, 50(3): 543–550.
- [5] LIU Q L, XU K D, ZHONG M, PAN Y Z, JIANG B B, LIU G L, JIA Y. Cloning and characterization of a novel vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter gene (*Dgnhx1*) from chrysanthemum[J]. Plos One, 2013, 8(12): e83702.
- [6] ZHANG H, LIU Y X, CHAPMAN S, LOVE A J, XU Y, XIA T. A newly isolated Na<sup>+</sup>/H<sup>+</sup> antiporter gene, *DmNHX1*, confers salt tolerance when expressed transiently in *Nicotiana benthamiana* or stably in *Arabidopsis thaliana*[J]. Plant Cell Tissue and Organ Culture, 2012, 110(2): 189–200.
- [7] WU C G, YANG G D, MENG Q W, ZHENG C C. The cotton *GhNHX1* gene encoding a novel putative tonoplast Na<sup>+</sup>/H<sup>+</sup> antiporter plays an important role in salt stress[J]. Plant Cell Physiology, 2004, 45(5): 600–607.
- [8] TANG R, LI C, XU K, DU Y H, XIA T. Isolation, functional characterization, and expression pattern of a vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter gene *TrNHX1* from *Trifolium repens* L.[J]. Plant Molecular Biology Reporter, 2010, 28(1): 102–111.
- [9] MISHRA S, ALAVILLI H, LEE B H, PANDA S K, SAHOO L. Cloning and characterization of a novel vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter gene (*VuNHX1*) from drought hardy legume, cowpea for salt tolerance[J]. Plant Cell Tissue and Organ Culture, 2015, 120(1): 19–33.
- [10] MISHRA S, BEHURA R, AWASTHI J P, DEY M, SAHOO D, BHOWMIK S S D, PANDA S K, SAHOO L. Ectopic overexpression of a mungbean vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter gene (*VrNHX1*) leads to increased salinity stress tolerance in transgenic *Vigna unguiculata* L. walp[J]. Molecular Breeding, 2014, 34(3): 1345–1359.
- [11] LIANG M X, LIN M M, LIN Z Y, ZHAO L, ZHAO G M, LI Q, YIN X Z. Identification, functional characterization, and expression pattern of a NaCl-inducible vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter in chicory (*Cichorium intybus* L.) [J]. Plant Growth Regulation, 2015, 75(3): 605–614.
- [12] SUN M H, MA Q J, LIU X, ZHU X P, HU D P, HAO Y J. Molecular cloning and functional characterization of *MdNHX1* reveals its involvement in salt tolerance in apple calli and *Arabidopsis*[J]. Scientia Horticulturae, 2017, 215: 126–133.
- [13] QUINTERO F J, BLATT M R, PARDO J M. Functional conservation between yeast and plant endosomal Na<sup>+</sup>/H<sup>+</sup> antiporters I[J]. Febs Letters, 2000, 471(2/3): 224–228.
- [14] GOUIAA S, KHOUDI H, LEIDI E O, PARDO J M, MASMOUDI K. Expression of wheat Na<sup>+</sup>/H<sup>+</sup> antiporter *TNHS1* and H<sup>+</sup>-pyrophosphatase *TPP1* genes in tobacco from a bicistronic transcriptional unit improves salt tolerance[J]. Plant Molecular Biology, 2012, 79(1/2): 137–155.
- [15] WU C X, GAO X H, KONG X Q, ZHAO Y X, ZHANG H. Molecular cloning and functional analysis of a Na<sup>+</sup>/H<sup>+</sup> antiporter gene *ThNHX1* from a halophytic plant *Thellungiella halophila*[J]. Plant Molecular Biology Reporter, 2009, 27(1): 1–12.
- [16] MATSUMOTO K, TAUMURA F, CHUN J P, IKEDA T, IMANISHI K, TANABE K. Enhancement in salt tolerance of Japanese pear by using *Pyrus betulaefolia* rootstock[J]. Horticulture Research, 2007, 6(1): 47–52.
- [17] OKUBO M, FURUKAWA Y, SAKURATANI T. Growth, flowering and leaf properties of pear cultivars grafted on two Asian pear rootstock seedlings under NaCl irrigation[J]. Scientia Horticulturae, 2000, 85(1/2): 91–101.
- [18] 许园园, 蔺经, 李晓刚, 李慧, 常有宏. 杜梨 *PbCBL10* 基因表达与启动子功能分析[J]. 果树学报, 2014, 31(6): 1024–1031. XU Yuanyuan, LIN Jin, LI Xiaogang, LI Hui, CHANG Youhong. Expression of the *PbCBL10* gene and functional analysis of its promoter in pear plants (*Pyrus betulaefolia*) [J]. Journal of Fruit Science, 2014, 31(6): 1024–1031.
- [19] LI H, LIN J, YANG Q S, LI X G, CHANG Y H. Comprehensive analysis of differentially expressed genes under salt stress in pear (*Pyrus betulaefolia*) using RNA-Seq[J]. Plant Growth Regulation, 2017, 82(10): 409–420.
- [20] BAILEY T, ELKAN C. Fitting a mixture model by expectation

- maximization to discover motifs in biopolymers[C]//Seattle, USA: Proceedings of the Second International Conference on Intelligent Systems for Molecular Biology (ISMB-94), 1994.
- [21] TAMURA K, PETERSON D, PETERSON N, STECHER G, NEI M, KUMAR S. Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods[J]. *Molecular Biology and Evolution*, 2011, 28: 2731-2739.
- [22] GIETZ D, JEAN A S T, WOODS R A, SCHIESTL R H. Improved method for high efficiency transformation of intact yeast cells[J]. *Nucleic Acids Research*, 1992, 20(6): 1425.
- [23] PEREZ-VALLE J, JENKINS H, MERCHAN S, MONTIEL V, RAMOS J, SHARMA S, SERRANO R, YENUSH L. Key role for intracellular K<sup>+</sup> and protein kinases Sat4/Hal4 and Hal5 in the plasma membrane stabilization of yeast nutrient transporters[J]. *Molecular and Cellular Biology*, 2007, 27(16): 5725.
- [24] MA X L, ZHANG Q, SHI H Z, ZHU J K, ZHAO Y X, MA C L, ZHANG H. Molecular cloning and different expression of a vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter gene in *Suaeda salsa* under salt stress[J]. *Biologia Plantarum*, 2004, 48(2): 219-225.
- [25] ZÖRB C, NOLL A, KARL S, LEIB K, YAN F, SCHUBERT S. Molecular characterization of Na<sup>+</sup>/H<sup>+</sup> antiporters (*ZmNHX*) of maize (*Zea mays* L.) and their expression under salt stress[J]. *Journal of Plant Physiology*, 2005, 162(1): 55-66.
- [26] AHARON G S, APSE M P, DUAN S L, HUA X J, BLUMWALD E. Characterization of a family of vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporters in *Arabidopsis thaliana*[J]. *Plant and Soil*, 2003, 253(1): 245-256.
- [27] WU G Q, XI J J, WANG Q, BAO A K, MA Q, ZHANG J L, WANG S M. The *ZxNHX* gene encoding tonoplast Na<sup>+</sup>/H<sup>+</sup> antiporter from the xerophyte *Zygophyllum xanthoxylum* plays important roles in response to salt and drought[J]. *Journal of Plant Physiology*, 2011, 168(8): 758-767.
- [28] XIA T, APSE M P, AHARON G S, BLUMWALD E. Identification and characterization of a NaCl-inducible vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter in *Beta vulgaris*[J]. *Physiologia Plantarum*, 2002, 116(2): 206-212.
- [29] 严一诺, 孙淑斌, 徐国华, 刘兆普. 菊芋 Na<sup>+</sup>/H<sup>+</sup> 逆向转运蛋白基因的克隆与表达分析[J]. *西北植物学报*, 2007, 27(7): 1291-1298.
- YAN Yinuo, SUN Shubin, XU Guohua, LIU Zhaopu. Cloning and analysis of a Na<sup>+</sup>/H<sup>+</sup> antiporter gene in *Helianthus tuberosus* L.[J]. *Acta Botanica Boreali-Occidentalia Sinica*, 2007, 27(7): 1291-1298.
- [30] FUKUDA A, NAKAMURA A, HARA N, TOKI S, TANAKA Y. Molecular and functional analyses of rice NHX-type Na<sup>+</sup>/H<sup>+</sup> antiporter genes[J]. *Planta*, 2011, 233(1): 175-188.
- [31] WEI Q, GUO Y J, CAO H Z, KUAI B K. Cloning and characterization of an *AtNHX2*-like Na<sup>+</sup>/H<sup>+</sup> antiporter gene from *Ammopiptanthus mongolicus* (Leguminosae) and its ectopic expression enhanced drought and salt tolerance in *Arabidopsis thaliana*[J]. *Plant Cell Tissue and Organ Culture*, 2011, 105(3): 309-316.
- [32] WU G X, WANG G, JI J, LI Y, GAO H L, WU J, GUAN W Z. A chimeric vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter gene evolved by DNA family shuffling confers increased salt tolerance in yeast[J]. *Journal Biotechnology*, 2015, 203: 1-8.
- [33] YOKOI S, QUINTERO F J, CUBERO B, RUIZ M T, BRESSAN R A, HASEGAWA P M, PARDO J M. Differential expression and function of *Arabidopsis thaliana* NHX Na<sup>+</sup>/H<sup>+</sup> antiporters in the salt stress response[J]. *Plant Journal*, 2002, 30(5): 529-539.
- [34] BARRAGAN V, LEIDI E O, ANDRES Z, RUBIOL, LUCA A D, FERNANDEZ J A, CUBERO B, PARDO J M. Ion exchangers *NHX1* and *NHX2* mediate active potassium uptake into vacuoles to regulate cell turgor and stomatal function in *Arabidopsis*[J]. *Plant Cell*, 2012, 24(3): 1127-1142.
- [35] XU K, ZHANG H, BLUMWALD E, XIA T. A novel plant vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter gene evolved by DNA shuffling confers improved salt tolerance in yeast[J]. *Journal of Biological Chemistry*, 2010, 285(30): 22999-23006.
- [36] LIU H, TANG R J, ZHANG Y, WANG C T, LÜ Q D, GAO X S, LI W B, ZHANG H X. *AtNHX3* is a vacuolar K<sup>+</sup>/H<sup>+</sup> antiporter required for low-potassium tolerance in *Arabidopsis thaliana*[J]. *Plant, Cell and Environment*, 2010, 33(11): 1989-1999.