Transformation and expression of Na^+/H^+antiporter vacuolar (*AtNHX1) gene* in tobacco plants under salt stress

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ABSTRACT

Saline soil is a serious problem worldwide, and it is necessary to improve the salt tolerance of plants to avoid the progressive deterioration of saline soil. Agrobacterium based system was employed to transfer a vacuolar Na^+/H^+ antiporter (AtNHX1) gene into tobacco leaf discs. Hundred thirty putative transgenic tobacco plants were obtained. The leaf painting assay was applied to select the transformants containing the bar gene (basta herbicide resistance). In addition, the stable integration of the AtNHX1 gene was confirmed by PCR analysis. The expression of encoding sequence AtNHX1 gene was detected by the accumulation of salt in the transgenic tobacco plants under the salt stress conditions. Transgenic tobacco plants expressing the AtNHX1gene were able to grow in the presence of 150 to 300 mM NaCl and accumulated significantly at high concentrations of sodium ions in the leaves. However, the wild type plants could not tolerate a concentration up to 150 mM NaCl. This remarkable high salt tolerance in transgenic tobacco plants can be helpful in the future for applying important phytoremdiation plants in high saline lands.

Key words: *Transformation, transgenic tobacco, AtNHX1 gene, salt–tolerance, Phytoremdiation.*

INTRODUCTION

Salinity is one of the major environmental factors limiting plant growth and its productivity worldwide. In response to various environmental stresses, plants have developed different physiological and biochemical strategies to adapt or tolerate stress conditions (Moghaieb *et al.*, 2000). In Egypt, there is a remarkable and continuous growth in population and land scarcity as well. Therefore, the challenge for agricultural researchers is to produce high-yielding crop varieties. The effort exerted in this context is interrupted by two major limiting factors, i.e. drought and salinity. They are considered as major constraints and have adverse impacts on crop production, food security and socio-economic aspects in the Middle East. However, about 40% of all arable lands cannot be used because of the potential salinity problems. High salinity causes ion imbalance due to elevated toxic levels of the cytoplasmic sodium and drought stress (Town and Mahamed., 2008). Na+/H+antiporters are ubiquitous membrane proteins that play major roles in cellular pH and Na+ homeostasis throughout the biological kingdom (Shi and Zhu., 2002).However, *NHX1*gene is found in *Sacharomyces cerevisiae* and localized to a late endosomal /prevacuolar compartment, where it mediates intracellular sequestration of

Na+ in a pH-dependent manner (Yan *et al.*, 2005).This finding indicates a significant role for intracellularly localized Na+/H+ antiporters intermediating NaCl tolerance through prevacuolar compartmentation of Na+ (Shi and Zhu., 2002).

Over-expression of the *Arabidopsis tonoplast* membrane–bound Na+/H+antiporter, *AtNHX1*gene, under a strong constitutive promoter resulted in salt-tolerant *Arabidopsis* as reported by (Apse *et al.*, 1999), *Brassica napus* (Zhang *et al.*, 2001), Tomato (Zhang and Blumwald, 2001), wheat (Brini *et al.*, 2007) and *Tall fescue (*Lming *et al.*, 2006). *AgNHX1, an AtNHX1* homologues genes from the halophytic plant *Atriplex gmelini* (Hamada *et al.*, 2001), over expression in rice (*Oryza sativa*) plants and improved salt tolerance of the transgenic rice (Ohta *et al.*, 2002). The *AtNHX1*gene homologues from many plant species have been isolated; mostly based on their sequence homology to the *Arabidopsis* gene. Thus, the *NHX1* gene seems to be highly conserved among many different plant species. The genetic manipulation of this system in crop species are likely to result in improving salt tolerance up to 300 mM NaCl (Zhang *et al.*, 2004).

The transgenic tomato plants expressing the antiporter gene *AtNHX1* exhibits the ability to grow and produce fruit using even irrigation water with 300 mM NaCl (Zhang and Blumwald, 2001). In addition, it was reported that the transgenic *Brassica* plants expressing the *AtNHX1* gene grown in high salinity conditions accumulated sodium up to 6% of total dry weight (Zhang *et al* .,2001). The synthesis of stress induced proteins, accumulation of non-toxic osmolytes (such as proline) biochemical and physiological make-up of certain cell lines provide an evolutionary value to the cell survival under adverse conditions (Al-Nagger *et al* ., 2008). Taking into consideration that a mature *Brassica* plant in the field can weight 2 kg fresh weight or 300 grams dry weight could accumulate 18 grams of sodium when grown in the presence of 200 mM NaCl. This significant amount of sodium taken up by transgenic plants would suggest that, (in addition to value as an agronomic crop) these transgenic plants expressing *AtNHX1* gene could be used as one component needed to reclaim saline soils.

The present investigation was carried out to achieve the following objectives: (1) to investigate the regeneration capacity of tobacco plants using modified regeneration medium; (2) to introduce the *AtNHX1* gene into tobacco leaf disc explants using *Agrobacterium*-mediated transformation ; (3) to select the putative transgenic tobacco plants by phenotypic characterization criterion (basta test); (4) to confirm the integration of *AtNHX1* gene in the genomic DNA of T0 plants by PCR; (5) to evaluate the transgenic tobacco plants for salt stress tolerance using several analytical methods.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of tobacco plants *(Nicotiana tabacum*) used for transformation were kindly provided by the Genetic Engineering Center, Faculty of Agriculture, and Cairo University. Tobacco seeds *(Nicotiana tabacum L. cv. Wisconsin*) were surface sterilized in 70% (v/v) ethanol and 10% (v/v) sodium hypochloride and then washed thoroughly in sterilized water. The seeds were germinated and subcultured on half-strength MS medium (Murashige and Skoog., 1962) supplemented with 15 g sucrose and 7 g agar (pH 5.8). All reagents were obtained from Sigma-Aldrich (St. Louis, Mo.) unless stated otherwise. Approximately 1-2cm sections of aseptic leaves of one month-old plants were used for regeneration and transformation experiments.

Bacterium strain and plant expression vector

The *Agrobaterium tumefacien* strain LBA4404 containing the binary vector 35S At-NHX1 is used in this study , the vacuolar Na+/H+ antiport target gene under the control of 35S promoter and terminator, *bar* gene as a reporter gene and the selective kanmycin resistance gene, was used for transforming tobacco explants Fig. (1).

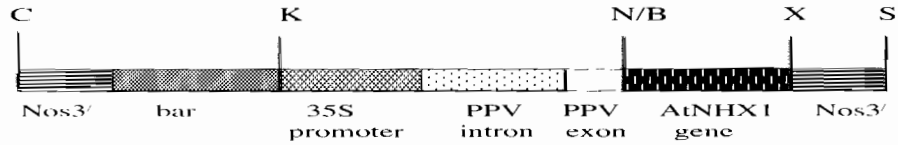

Fig. (1): Plasmid map of the transformation vector 35S ATNHX1. C: ClaI, K: KpnI, N: NcoI, B: BamHI, X: XbaI and S: ScaI.

Tobacco regeneration and transformation

Introduction of the *AtNHX1* gene to tobacco leaf disc explants was done using Agrobacterium-mediated transformation system. The leaf disc explants were dipped in an Agrobacterium soluble culture for 10 min and then blotted dry before culturing on co-cultivation medium. The explants were rinsed several times with a sterile water, to remove excess of bacteria and then blotted dry. The infected leaf explants were placed on co-cultivation MS1 medium (MS + 6-BA 0.5mg/l + NAA 0.01 mg/l) at 25°C in the dark for 2 days. The shoots were regenerated on a selection MS2 medium (MS1 + 500 mg l–1 cefotaxime) to inhibit bacterial growth. The well-grown shoots (2–3 cm in length) were excised carefully and then transferred onto rooting MS3 medium (MS + NAA 1mg/ l +GA0.02 mg/ l +250 mg l–1 cefotaxime). All media were supplemented with 3% sucrose and 2×6 g l–1 phytagel (Sigma, St. Louis, MO, USA) ; the pH was adjusted to 5.8. The conditions for shooting and rooting were the same as those for germination. The rooted shoots were multiplied by nodal bud cultures for maintaining clones under *in vitro* conditions. Some of these clones were transferred to pots for hardening and then , moved to a growth room for acclimatization.

Selection of putative transgenic tobacco plants by basta herbicide assay (*bar* gene)

The leaf painting assay for detection of the *bar* gene (herbicide resistance) was used. The bar gene encoding the enzyme phosphinothricin acetyl transferase (DeBlock *et al.*, 1987) which inactivates phosphinothricin was considered as a selectable marker. Transformation construct of the *AtNHX1* gene contains the *bar* gene and the transformed plants were tested by the leaf painting assay for detection of *bar* gene (Delaney *et al.*, 1989). Non- transformed tobacco leaves were also treated to serve as a control. The young plants (around 4-6 weeks old after acclimatization) were painted by 2g /l of basta solution (dilution at concentration of 2g / l glufosinate) ; the resistant leaflets were scored after 2 weeks.

Genomic DNA isolation and PCR analysis

Genomic DNA was extracted from tobacco leaves using the cetyltrimethyl ammonium bromide (CTAB) method (Gaoge *et al.*, 2005). Two sets of primers were used for the detection of the *bar* and *ATNHX1* genes by PCR. The sequence of the specific primers for the *AtNHX1* gene was F 5/ TTT TGG CTT AAA TTC ATA TTC AA 3/ and R 5/ GGC TTA AAG TGT CCA TG 3/. While,

the sequence of the specific primers for the *bar* gene is F 5/ GAG GAG TGG ACG GAC GAC 3/ and R 5/ GAA GTC CAG CTG CCA GAA AC 3/. The PCR reactions were carried out in a 20 µl volume containing 40 ng DNA template, 20 pmole /1ml primers, 200 µl of dNTps mix, 2 µl 10x amplification buffer, 0.2 mM MgCl2, and one unit Taq DNA polymerase . The volume was completed up to 20 µl with sterilized distilled water. The PCR temperature profile used for the amplification consists of initial denaturation cycle 94°C/ 5 min followed by 94°C/1min 53°C/1min, 72°C/1min and terminal extension cycle at 72°C /7min for *AtNHX1* gene. The PCR program for *bar* gene is similar to that of *AtNHX1* gene except the annealing temperature which was 53°C. Finally, the PCR products were electrophoresed on 1% agarose gel.

Salt tolerance assays of transgenic plants

To test whether *AtNHX1*gene expressed in transformed tobacco plants, a number of 60 T0 transformed plants were analyzed for their salt tolerance in growth room conditions. The control plants (non- transformed) of similar age and height were also analyzed. Six seeds from each *AtNHX1*-expressed transformed tobacco plants (T1) and non-transformed plants (C) were germinated in pots containing a mixture of peatmoss and sandy soil (1:1/v: v). The salt treatments were conducted in an incremental manner for two weeks each period i.e., starting with 50 mM, followed by 100 mM, 150 mM, 200 mM, 250 mM, and finally 300 mM NaCl according to ((Lming *et al.*, 2006). Leaves from the growing plants were excised carefully to determine the concentration of Na+ and Cl^+ content. In addition, the dry weight was measured. The Na+ and the Cl^+ contents were extracted with 1 N HCl solution at 60°C for 1 hr. The supernatants were analyzed by atomic absorption using a flame photometer (Eiko Instruments Inc., and Tokyo 2008).

RESULTS AND DISCUSSION

Tobacco regeneration and transformation

Regeneration in tobacco was done *via* direct organogenesis according to (Tang *et al.*, 2005). In this study, a new enhanced system for regeneration and transformation of tobacco plants was developed. It was found that the concentration of BA 0.1 mg / l was efficient in decreasing the growth stage period. In addition, 0.02 mg / l GA was used in shooting medium to improve shoot formation (Table 1). The transformation of tobacco plants was achieved using Agrobacterium -mediated transformation using the plasmid 35S AtNHX1 harboring the *AtNHX1* gene, and the *bar* genes. In this study, the leaf disc explants were co-cultivated with Agrobacterium solution and the infected leaf explants were transferred to a shooting medium .One week later, treated explants grew larger and thicker. Then shoots started to appear from the edges of these explants and reached 2–3 cm in length after 3-4 weeks. The shoots rooted easily on a rooting medium after 2 weeks and then the rooted plantlets (5-6 cm in length) were ready for acclimatization in the soil (Fig.2 A, B, C,and D). About 130 putative transgenic plants were obtained after Agrobacterium co-cultivation and these plants were used for molecular analysis and functional test.

Table (1): Concentrations of growth regulators to improve shoot formation.

Concentration (mg/l)		No. plants	Survival		shoots	Growth (weeks)
BA	GA		No.	%	%	
0.3	0.04	20	15	75	67	5-6
0.2	0.03	20	16	80	79	4-5
0.1	**0.02**	20	**18**	**90**	**83**	**3-4**
0.01	0.01	20	10	50	60	6-7

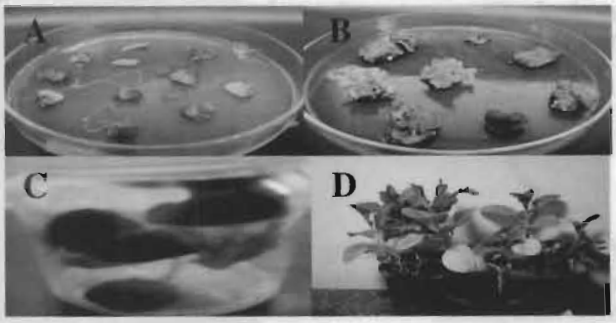

Fig. (2): Tobacco transformation: (A) leaf discs after infection. (B, C) shoot formation; and (D) fertile regenerated tobacco plants.

Evaluation of Transformed Plants

Basta herbicide resistance test

The most reliable criterion to assess the production of transformants, containing the herbicide-resistant *bar* gene, is leaf painting with the herbicide bastausing 0.2 % as a recommended dose (Spencer *et al.,* 1990). Herbicide resistance of putative transformed tobacco plants was done at a biocontainment stage by painting the middle green parts of the tobacco leaf plants from both sides with 1g/l basta.The data of basta treatment are presented in Table (2) .The results in Fig. (3) show that the leaves of the herbicide-resistant tobacco plants retained the green color while in the others, either non-resistant to the herbicide or control (non- transformed); the green color turned yellow (necrotic) within 7 days. Moreover, results in Fig. (4) show the survival of transgenic tobacco plants after treatment with the herbicide using the spraying method and the death of the control in non-transformed tobacco plants. The present results show successful use of leaf painting to identify the transgenic tobacco plants. Similar results were reported by (Moemen et *al.*, 2005) who selected putative transgenic faba bean. Also, (Chaewan, *et al.,* 2007) reached to the same conclusion in Chinese cabbage transformation.

As presented in Table (2), out of 300 explants, 178 and 130 regenerated plants were produced from non-transformed and transformed plants, respectively. When basta herbicide was applied to the regenerated plants, all non -transformed plants showed susceptibility to basta, while the 81 transformed plants were basta tolerant. The mean of transformation frequency was (62.30) under basta herbicide treatment.

Table (2): Expression frequency of the bar gene in transformed plants.

Treatment	No.of cultured explants	No.of regenerated plants	Basta Tolerant plants	Trans- formation %
Non-transformed plants	300	178	0	—
Transformed plants(T0)	300	130	81	62.30

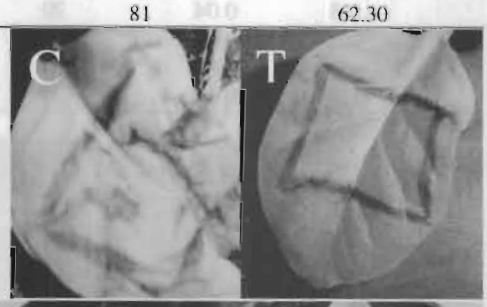

Fig. (3): Application of the herbicide basta using the painting method: (C) non- transformed plants; and (T) transformed plants

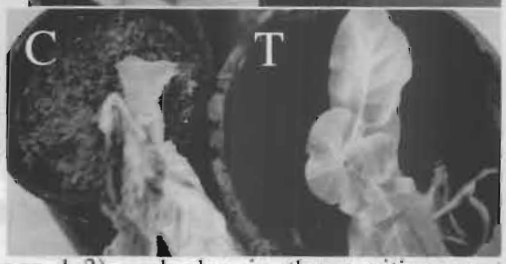

Fig.(4): Application of the herbicide basta using the spraying method:(C) non-transformed plants; (T) transformed plants.

PCR analysis

Polymerase chain reaction (PCR) is a reliable technique to determine the presence or absence of a specific DNA fragment in plant genomic DNA (Roger and Bendich, 1985). Genomic DNA was subjected to PCR analysis using two specific primers for the *bar* gene. In this respect, the screening for the *bar* gene in both *Agrobacterium tumefacien* strain LBA4404 containing the 35S promoter At-NHX1 plasmid and the transformed tobacco plants was carried out. The expected amplification product of 350 bp for the *bar* gene was detected as shown in Fig. (5). However, this amplified fragment for the *bar* gene had manifested in the transformed plants (lanes 1-3) and also in the positive control (lane p). Moreover, the negative control resulted from non-transformed tobacco plants shows no amplified band as clarified in lane C. Therefore, it is evident that the *bar* gene was present only in the genomic DNA background of the transformed plants.

PCR analysis also confirmed the presence of the *AtNHX1* gene in the transformed plants as shown in Fig. (6) Which gave the expected amplification product of 500bp. However, the amplified fragment for *AtNHX1* gene representing the negative control disappeared (lane C), while lanes (P, 1, 2, 3,4 and 5) showed the expected amplified band of molecular weight 500 bp .

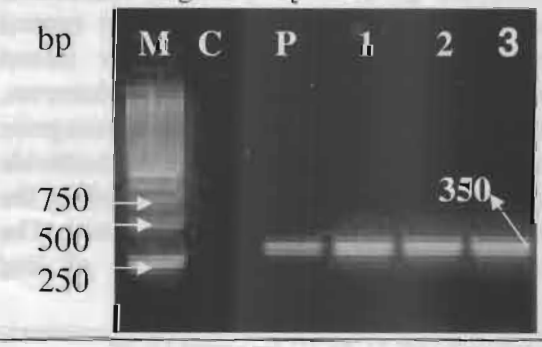

Fig. (5): The PCR amplification of the bar gene fragment in putative transgenic tobacco plants; M: 1Kb DNA ladder; C: non transgenic plant P: Positive control; 1, 2, 3 positive tested plant samples.

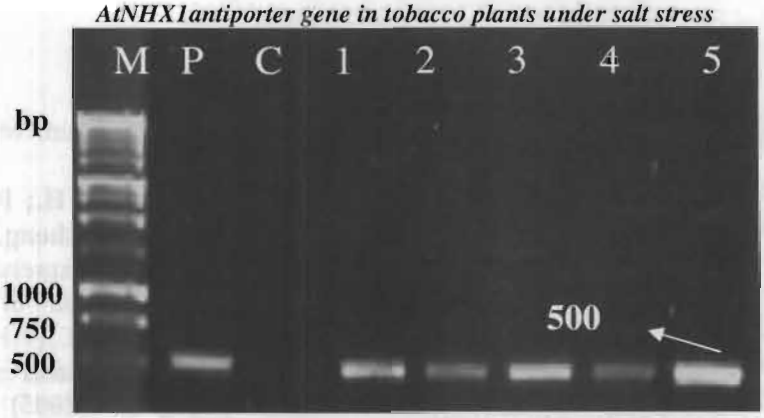

Fig. (6): The PCR amplification of the AtNHX1 gene fragment in putative transgenic tobacco plants; M: 1Kb DNA ladder, C: non transgenic plant P: Positive control; 1,2,3,4 and 5 positive plant samples.

Maximum expression of *AtNHX1* gene in transgenic tobacco plants

A number of 60 T0 transgenic plants were tested for the expression of *AtNHX1* gene by salt stress tolerance treatment in a growth room. The salt treatments were conducted in an incremental manner for two weeks (each period) as described in materials and methods. It started with 50 mM, followed by 100 mM, 150 mM, 200 mM, 250 mM, and finally 300 mM NaCl. As seen in Fig. (7). growth of non - transformed plants was severely inhibited by 150 mM NaCl treatment. However, the growth of *AtNHX1*-expressed transformed tobacco plants was more tolerant to the same concentration of NaCl and to the other remaining tested concentrations as well. After 2 weeks, the transformed plants under all other tested NaCl concentrations were significantly vigorous than those of the non- transformed plants. To test the maximum tolerance to NaCl by tobacco transformed plants, the NaCl concentration was increased up to 600 mM. The results indicated that the transgenic T0 plants could not grow up at more than 400 mM NaCl.Similar results were reported by Apse *et al.* (1999) in *Arabidopsis* plants. They reported that the transgenic plants showed over expression of *AtNHX1gene* in soil irrigated with 200 mM NaCl .The same results were also reported by Zhang and Blumwald (2001) in *Brassica napus* and by Hong and Zhanguo (2005) in cotton plants. The transgenic plants were able to grow, flower, and produce seeds in the presence of 200 mM NaCl without any obvious changes in quantity and quality of the products. However, it was found that non - transgenic plants could only grow in soil containing NaCl up to 150 mM which is considered the minimum level of *AtNHX1* gene expression under natural condition

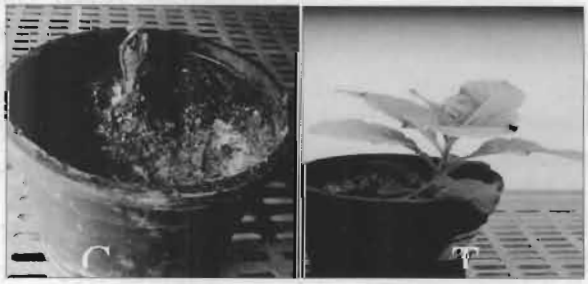

Fig. (7): Evaluation of salt-tolerance in transgenic tobacco plants over- expressingAtNHX1 gene, irrigated with 300 mM NaCl. (C) non-transgenic plants;(T) transgenic plants.

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الملخص العربي

التحول الوراثى والتعبير الجينى لجين تبادل الصوديوم والهيدروجين (AtNHX1) فى نباتات الدخان تحت ظروف الملوحة العالية

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من المعروف ان التربة الملحية هي مشكلة خطيرة في جميع أنحاء العالم ، لذلك من الضروري تحسين مقاومة النباتات للملوحة لتفادي مشاكل التربة الملحية .ولتحقيق ذلك تم استخدام التحول الوراثى بواسطة الاجروبكتريم لنقل جين *AtNHX1* لنباتات الدخان . وقد تم الحصول على 130 نبات محول وراثيا من الدخان. وقد تم اختيار النباتات المحولة وراثيا المحتوية على جين *Bar* وبالإضافة إلى ذلك معرفة اندماج جين *AtNHX1* فى جينوم النباتات عن طريق استخدام تفاعل البلمرة المتسلسل (PCR). ولمعرفة تعبير الجين *AtNHX1* من خلال تراكم الملح تم اختيار النبات المحولة وراثيا تحت ظروف الملوحة العالية. وقد وجد أن النباتات المحورة وراثيا المحتوية على جين *AtNHX1* قادرة على النمو في وجود 150 الى300 ملى مولر من كلوريد صوديوم ، والتي تراكمت بشكل كبير فى تركيزات عالية من أيونات الصوديوم في أوراق نباتات الدخان المحورة وراثيا ، وإن كانت النباتات البرية يمكن أن تقاوم تركيز يصل إلى 150 ملى مولر كلوريد الصوديوم. إن مقاومة الملوحة الملحوظة في نباتات الدخان المحورة وراثيا ربما يكون مفيدا في المستقبل لتطبيق ظاهرة ال phytoremdiation فى تقليل نسبة الملوحة والاستفادة من زراعة الأراضي الملحية بمحاصيل ذات قيمة اقتصادية .