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ORIGINAL ARTICLE

Molecular cloning and expression of a vacuolar Na^+/H^+ antiporter gene (*AgNHX1*) in fig (*Ficus carica* L.) under salt stress

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Abstract Soil salinity can be a major limiting factor for productivity in agriculture and forestry and in order to fully utilize saline lands productively in plantation forestry for fig production, the genetic modification of tree species for salt tolerance may be required. Na^+/H^+ antiporters have been suggested to play important roles in salt tolerance in plants. Here, we isolated *AgNHX1* a vacuolar Na^+/H^+ antiporter from a halophytic species *Atriplex gmelini* and introduced it into fig (*Ficus carica* L.) cv. Black Mission via *Agrobacterium*-mediated transformation. Leaf discs explants of fig were co-cultivated for 2 days with *Agrobacterium tumefaciens* strain LBA 4404 harboring the binary vector pBI121 containing the *AgNHX1* gene and the *hpt* selectable marker gene which encodes hygromycin phosphotransferase. Explants were cultured on MS medium containing 30 mg L^{-1} hygromycin, 3 % sucrose, 0.2 mg L^{-1} kinetin and 2.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid solidified with 2.5 g L^{-1} phytagel in darkness for callus formation. The calli were cultured on MS medium containing 2.0 mg L^{-1} zeatin riboside in combination with 0.4 mg L^{-1} indole acetic acid in the light for plant regeneration. Putative regenerated transformant shoots were confirmed by polymerase chain reaction (PCR) and Southern hybridization for the *AgNHX1* gene. Reverse transcriptase polymerase chain reaction analysis indicated that the gene was highly expressed in transgenic plants, but the degree of this expression varied among transformants. Overexpression of the *AgNHX1* gene conferred high tolerance to salt stress and transgenic fig plants overexpressing *AgNHX1* developed normally under salinity conditions compared to those of non-transgenic plants. Salt treated transgenic plants contained high proline and K^+ but less Na^+ compared to non-transgenic control plants.

Keywords *Agrobacterium tumefaciens* · *AgNHX1 antiporter gene* · Fig (*Ficus carica* L.) · RT-PCR · Southern blot hybridization · Transformation

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
2ip	*N*-6-(Δ^2-isopentenyl) adenine
BAP	Benzylaminopurine
CTAB	Cetyltrimethylammonium bromide
Hpt	Hygromycin phosphotransferase
IAA	Indole acetic acid
IBA	Indole-3-butaric Acid
LB	Luria broth medium
NAA	α-Naphthaleneacetic acid
OD	Optical density
ORF	Opening reading frame
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase polymerase chain reaction
TDZ	Thiadiazuron (*N*-phenyl-*N*-1,2,3,-thiadiasol-5-ylurea)
ZR	Zeatin riboside

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Introduction

The common fig (*Ficus carica* L.) of the family *Moraceae*, has been cultivated for its fruit and saleable produce commercially for many years. Fig is native to western Asia and has subsequently been transplanted and cultivated throughout Mediterranean region and in other similar climatic zones around the world. The main producers of edible figs include Turkey, Egypt, Morocco, Spain, Greece, California, Italy, Brazil and elsewhere in regions with mild winters that are usually dry and with summers that are hot often requiring irrigation (Küden 1996). As a perennial plantation crop the use of continuous groundwater irrigation is commonplace but can lead to build-up mineral salts in the rooting zone reducing the production potential (Tóth et al. 2008). Plant breeding of fig is laborious and time consuming often with variable results and successful genetic transformation of commercial fig cultivars provides a promising tool for the introduction of desirable genes to improve current fig cultivars (Flaishman et al. 2008a; Akdemir et al. 2012). Genetic and transformation methodologies offer the opportunity for the production of transgenic cultivars with improved agronomic characteristics, such as disease resistance, fruit storability, and enhanced fruit quality and flavour (Azafadi 2012; Meneses and Orellana 2013; Bakhsh and Hussain 2015). In addition, transformation of figs could provide the means for the production of desirable proteins in the edible parts of fig leading to enhanced nutritional and/or pharmaceutical composition (Yancheva et al. 2005; Flaishman et al. 2008b). The cultivar "Black Mission" is the most renowned fig in agricultural production and it produces two crops a year, with big and sweet fruits with distinctive flavour figs that can eaten fresh or dried.

Plants having the genetic ability to grow on salty land are classified as salt tolerant (glycophytes) or salt resistant (halophytes) on the basis of coping with excess Na^+ in the cytocel. Halophytes have evolved the ability to grow normally under high salinity (Flowers and Yeo 1986; Khan and Duke 2001; Roohi et al. 2011) but glycophytes have the ability to develop a metabolic steady state to growth under salt stress and often restrict the intake of Na^+ into the cytoplasm (Niu et al. 1993; Zhu 2007; Flowers and Colmer 2008). Some glycophytes can reduce Na^+ ions out of the cytosol by transporting them into the vacuolar lumen or out of the cell using Na^+/H^+ exchangers localized in the vacuolar and plasma membranes (Apse and Blumwald 2007). Bassil et al. (2012) confirmed that Na^+/H^+ antiporters play a vital part in the homeostatic mechanism and are found in the plasma membrane, where they assist in moving Na^+ from cells or sequester it into the vacuole. Given these hypothesis and results, Borsani et al. (2003) and Khan (2011) indicated that to enhance the salt tolerance in a salt sensitive plant, genetic engineering with sodium and hydrogen (Na^+/H^+) antiporters could lead to an improvement in salt tolerance.

Hamada et al. (2001) have successfully isolated an assumed Na^+/H^+ antiporter gene (*AgNHX1*) from *Atriplex gmelini* and made this available for transformation. The isolated cDNA is 2607 bp in length and contains one open reading frame, which comprises 555 amino acid residues with a predicted molecular mass of 61.9 kDa. The amino acid sequence of the *AgNHX1* gene showed more than 75 % identity with those of the previously isolated *NHX1* genes from glycophytes, *Arabidopsis thaliana* and *Oryza sativa*. There is evidence that overexpression of *AtNHX1* in wild type *Arabidopsis thaliana* plants conferred higher salt tolerance (Apse et al. 1999). The transformed gene for a putative plant vacuolar Na^+/H^+ antiporter (*AtNHX1*) was isolated from *Arabidopsis* and the activity of Na^+/H^+ antiporter increased upon treatment with NaCl and showed more increase plant tolerance to NaCl in transformants plants compared to wild types (Yu et al. 2007; Dorani-Uliaie et al. 2012). Also, overexpression of *AtNHX1* in tomato resulted in transgenic plants that were able to grow, flower and set fruits at higher salt concentrations (Zhang and Blumwald 2001; Razzaque et al. 2014).

This work described here concerns the in vitro regeneration and transformation of fig (*Ficus carica* L.) cv. Black Mission based on the infection of leaf sections with *Agrobacterium tumefaciens* harboring the *AgNHX1* gene and to determine whether overexpression of *AgNHX1* under 300 mM NaCl improve the mRNA and salt tolerance. These results are the first recorded that test the efficiency of cloning *AgNHX1* in the fig (*Ficus carica* L.) genome.

Materials and methods

In vitro regeneration of fig (*Ficus carica* L.)

The leaves of *Ficus carica* L. cv. Black Mission were taken from in vitro formed plants produced from shoot tips and the in vitro leaves were used as explants. Explants were cut into two pieces longitudinally and cultured on MS medium containing 100 mg L^{-1} myo-inositol, 30 g L^{-1} sucrose, 0.2 mg L^{-1} kinetin and 2.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) for 4 weeks to induce calli. The explants with induced calli were transferred to MS medium supplemented with 100 mg L^{-1} myo-inositol, 30 g L^{-1} sucrose, 1.0–3.0 mg L^{-1} zeatin riboside combined with 0.1–0.5 mg L^{-1} indole acetic acid (IAA) and 1–5 mg L^{-1} thiadiazuron (TDZ) combined with 0.2–0.5 mg L^{-1} naphthaleneacetic acid (NAA) at 4 weeks for shoot induction.

The small new shoots were transferred to MS medium supplemented with 100 mg L^{-1} myo-inositol, 30 g L^{-1} sucrose and 1 mg L^{-1} *N*-6-(Δ^2-isopentenyl adenine (2ip) for shoot elongation. The developed shoots were transferred to MS medium with 2.0 mg L^{-1} benzyl amino purine (BAP) at 4 weeks for shoot multiplication. The elongated shoots were transferred to MS medium supplemented with 30 g L^{-1} sucrose, 1.0 mg L^{-1} indole-3-butyric acid (IBA), 0.5 mg L^{-1} NAA and 15 mg L^{-1} hygromycin in the presence of 2.0 mg L^{-1} activated charcoal for rooting. The rooted plantlets were transferred to soil and grown on in a growth chamber for 4 weeks under a photoperiod of 16/8 h (light/dark) and finally transferred to the greenhouse. The applied treatments were taken from the literature of *Ficus carica* as described by Soliman et al. (2010). Ten Petri dishes, each containing five explants were used per treatment. The sterilized explants were cultured on the media under complete aseptic conditions in a laminar Air Flow Hood. Petri dishes and jars were then placed in an incubation room at 25 $\pm$ 2 °C under 16 h photoperiod of 35 µmol m^{-2} s^{-1} supplied with cool white fluorescent lamps.

Plasmid construction and *Agrobacterium* preparation

Total RNA was isolated from the halophyte *Atriplex gmelini*. First strand cDNA was synthesized with a SMART RACE cDNA Amplification Kit (Clontech). Primers for 3́-RACE and 5′-RACE were 5′-CAT CAG TGT CAA TTC GAG AAA CAA CAG-3′ and 5′-CTA TGT TCT GTC TAC CAA ATT GTT GTT GCT-3′. After obtaining the full length of *AgNHX1*, we amplified its coding region with primers 5′-CTA ACG TCA ACG AGG CAC CGG TAG AAG-3′and 5′-CGC TAC TCT CTA GGC TAC AGC ACC TAC-3′, which contain BamH1 and Sac1 sites for cloning, respectively. The vector for plant transformation was constructed by replacing *AgNHX1*of the pBI-121 vector (Fig. 1). This binary plasmid contains *hpt* selectable marker gene which encodes hygromycin phosphotransferase within the TDNA region, under the nos promoter, and the CaMV 35S promoter. The pBI-121-*AgNHX1* plasmid transferred to *Agrobacterium tumefaciens* strain LBA4404 by electroporation and was used for transformation. The bacteria was grown overnight at 28 °C in 5 mL Lauria broth (LB) liquid medium supplemented with 50 mg L^{-1} rifampicin and 30 mg L^{-1} hygromycin on a shaker at 150 rpm to mid-log phase (OD600 = 0.8–1). Then, the bacterial cells were collected by centrifugation at 2500×*g* for 5 min and re-suspended in liquid Luria broth (LB) medium to a final OD600 of 0.2 for use in transfection.

Hygromycin sensitivity test

In order to test the transformed explants, a reliable selection method is required; this was achieved by using the hygromycin resistance gene. To determine the least lethal dose of hygromycin it was added to MS medium in different concentrations (0, 5, 10, 15, 20, 25, 30, 35, 40 mg L^{-1} hygromycin) and ten leaf explants for each concentration was assessed. The hygromycin was sterilized by filtration through disposable micropore filters (0.22 µm) and incorporated into precooled (45–50 °C) autoclaved medium. The percentages of explant survival (hygromycin resistant) were recorded after 4 weeks from culturing.

Agrobacterium-mediated transformation of (*Ficus carica* L.)

Leaf explants were then inoculated with *A. tumefaciens* strain LBA4404 harboring the plasmid pBI121 contains the *AgNHX1* and *hpt* genes. Explants were co-cultivated with *Agrobacterium* for 2 days on MS medium-free hormones. Following co-cultivation, the explants were transferred to the callus medium but supplemented with 300 mg L^{-1} carbinicillin and 30 mg L^{-1} hygromycin to select for transformed cells. After 4 weeks of incubation, callus was transferred to regeneration medium containing the selection antibiotics. The cultures were reincubated under the same conditions. At least five Petri dishes, each containing four explants were used per treatment and all experiments were repeated at least three times.

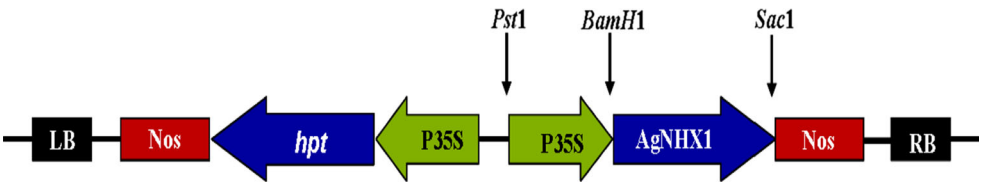

Fig. 1 Diagram of T-DNA of the recombinant binary vector pBI121 carrying the *AgNHX1* gene driven by CaMV 35S promoter. *LB* left border, nopaline synthase gene terminator, *hpt* selectable marker gene, 35S cauliflower mosaic virus 35S promoter, *AgNHX1* fig Na^+ antiporter gene, nopaline synthase gene terminator, *RB* right border

Detection of transgenic plants

Polymerase chain reaction (PCR) analysis

Genomic DNA was extracted from leaf tissue of putatively transformed and non-transformed control tissues using the acetyl tri-methyl-ammonium bromide (CTAB) method. The presence of the *AgNHX1* transgene in putative transgenic fig plants was detected by polymerase chain reaction (PCR) using Sigma RED Extract-N-Amp Tissue PCR Kit following the manufacturers' instructions. Primers were forward primer, 5′-TCC CGT GTA CTT GGG AAT GC-3′; reverse primer, 5′-CGC CCA CAA TAC CAA ACA CC-3′, which amplified a 2600 bp segment. The PCR reactions were preheated to 95 °C for 2 min, followed by 35 cycles with denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 1 min, and a final extension step at 72 °C for 10 min.

Southern blot hybridization

Genomic DNA was isolated from 5 weeks-old in vitro propagated plants from all transformed and untransformed control plants. It was then digested with HindIII. RT-PCR-amplified fragment (448 bp) of *AgNHX1* was labelled with [α-^{32}P] and used as a probe. Hybridization was conducted in the hybridization buffer (6 × SSC, 0.5 % SDS, 0.2 mg mL^{-1} salmon sperm DNA) at 65 °C for 24 h. The membrane was washed once at room temperature for 15 min in 0.2 SSC, 0.5 % SDS and twice at 55 °C for 20 min in 0.1 SSC, 0.5 % SDS. The DNA probe was then used in Southern blot hybridization as described in Sambrook et al. (1989). The DNA samples which were blotted onto the membrane were the PCR products of *Ficus carica* L. genomic DNA amplified using *AgNHX1* conserved region primer pair. Hybridization was conducted in the hybridization buffer (6 × SSC, 0.5 % SDS, 0.2 mg mL^{-1} salmon sperm DNA) at 65 °C for 24 h. The membrane was washed once at room temperature for 15 min in 0.2 SSC, 0.5 % SDS and twice at 55 °C for 20 min in 0.1 SSC, 0.5 % SDS. The DNA probe was then used in Southern blot hybridization as described in Sambrook et al. (1989). The DNA samples which were blotted onto the membrane were the PCR products of *Ficus carica* L. genomic DNA amplified using *AgNHX1* conserved region primer pair.

RT-PCR analysis of AgNHX1 expression in transgenic fig plants

Total RNA was extracted using RNA-plus solution as described previously (Fukuda et al. 1991). Northern blot analysis was performed according to the method described by Hayakawa et al. (1992). RT-PCR was performed using total RNA pre-treated with primers forward (′5-CTT TAT GTT GGT ATT GAT GCC TTG G-3′) and reverse (5′-ATT GAT TTT GGA GTG GTT GGT TCT G-3′) and cDNA was synthesized under conditions of 94 °C for 5 min; then 33 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s; with a final step of 72 °C for 7 min. DNA probes were made by a multiprime labelling system (Amersham Pharmacia Biotech, Cleveland, OH) with a portion of the cloned cDNA fragment and 32P-dCTP. For assessing the relative quantities, the loaded RNAs were stained with ethidium bromide after electrophoresis.

Measurement of fresh, dry weight and Na^+, K^+contents

Transgenic and non-transgenic fig plants were grown in greenhouse for 7 months and then treated with 100–300 mM L^{-1} NaCl. Ten individual plants of each treatment for each condition were used. After 3 months under NaCl treatment, plants were assessed by measuring fresh weight, dry weight and Na^+, K^+ content. Dry weight was measured after 48 h incubation at 80 °C. Also, dried samples were ground using a pestle and mortar for determination of mineral composition according to Abdel-Wanis et al. (2012). Cation contents were determined by atomic absorption spectrophotometry according to Jackson (1985).

Determination of proline content

Proline content was measured as described by Bates et al. (1973). 100 mg of frozen plant material was homogenized in 10 mL of 3 % sulphosalicylic acid and the residue was removed by centrifugation. 200 µl of supernatant was reacted with 200 µl glacial acetic acid and 200 µl acid ninhydrin (1.25 g ninhydrin warmed in 30 mL glacial acetic acid and 20 mL 6 M phosphoric acid until dissolved) for 30 min at 100 °C. The reaction was then terminated in an ice bath. The reaction mixture was extracted with 600 µl toluene. The chromophore containing toluene was warmed to room temperature and its optical density was measured at 520 nm.

Statistical analysis

Data was analysed using ANOVA and the differences among means for all treatments were tested for significance at 5 % level using Duncan (1955) new multiple range test as described by Snedecor and Cochran (1967). Means followed by the same letter are not significantly different at $p \leq 0.05$.

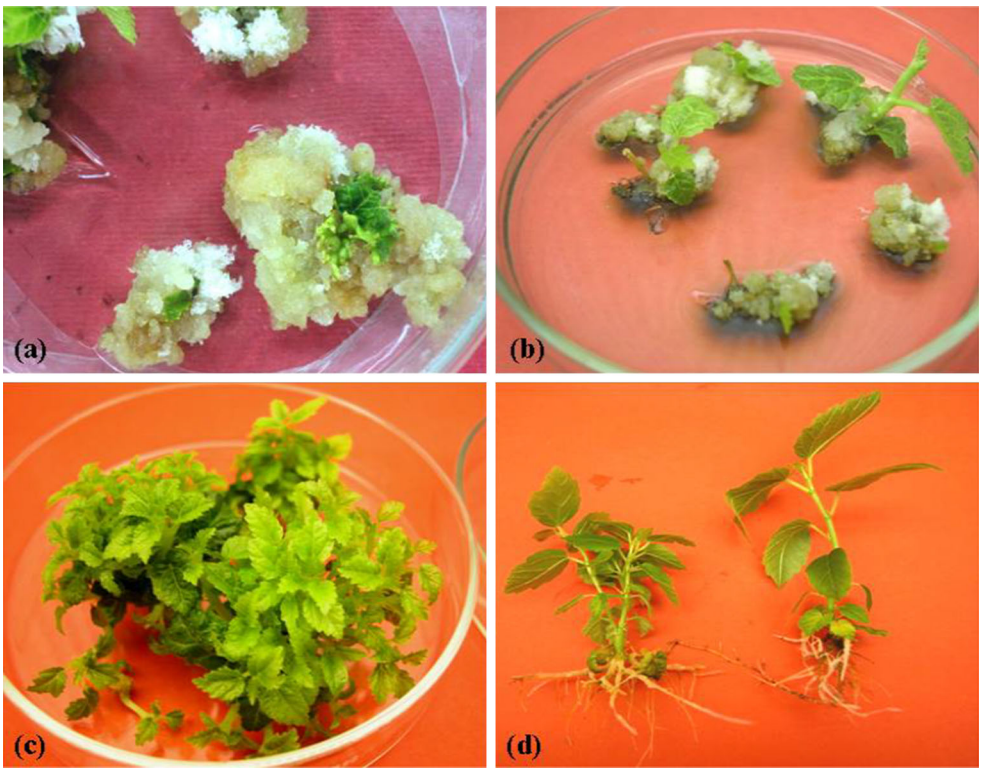

Fig. 2 Regeneration of transgenic plants of *Ficus carica* L. cv. Black Mission via indirect somatic embryogensis from leaf explants. **a** Callus induction in transformed tissue; **b** transgenic shoot regeneration on MS medium supplemented with 2.0 mg L^{-1} zeatin riboside and 0.2 mg L^{-1} IAA in presence of 20 mg L^{-1} hygromycin; **c** shoot multiplication of transgenic plants on MS medium supplemented with 2.0 mg L^{-1} BAP; and **d** rooting of transgenic plants on MS medium supplemented with 1.0 mg L^{-1} IBA and 0.5 mg L^{-1} NAA

Results and discussion

In vitro regeneration of fig (*Ficus carica* L.)

Leaf explants were excised from in vitro plants, and cultured on MS medium supplemented with 100 mg L^{-1} myo-inositol, 30 g L^{-1} sucrose, 0.2 mg L^{-1} kinetin and 2.0 mg L^{-1} 2, 4-D solidified with 2.5 g L^{-1} phytagel (Fig. 2a). Then the calli were cultured on MS medium containing different concentrations and combinations of zeatin riboside in combination with IAA or TDZ in combination with NAA to establish the best condition for plant regeneration.

The results showing shoot regeneration from the range of concentrations of growth regulators tested are presented in Table 1. Various ranges of regeneration efficiencies (46–70 %), mean number of shoots per explant (1.58–4.85) and mean length of shoots formed per explant (1.00–1.72 cm.) were obtained when leaf explants were grown in media containing different concentrations of growth regulators. The best results of the shoot formation percentage (78 %) and mean number of shoots per explant (5.25) was obtained on MS medium supplemented with 2.0 mg L^{-1} zeatin riboside in combination with 0.4 mg L^{-1} IAA compared to the other treatments. While, the best results of mean length of shoots formed per explant were produced on 4.0 mg L^{-1} TDZ and 0.4 mg L^{-1} NAA; reaching 1.75 cm compared to the other treatments (Fig. 2b). After 4–6 weeks, the shoots were transferred to MS medium with 2.0 mg L^{-1} BAP for shoot multiplication (Fig. 2c). The elongated shoots were transferred to MS medium supplemented with 30 g L^{-1} sucrose, 1.0 mg L^{-1} IBA, 0.5 mg L^{-1} NAA and 2.0 mg L^{-1} activated charcoal for rooting (Fig. 2d). The rooted plantlets were transferred to soil and grown under a photoperiod of 16/8 h (light/dark) in a plant growth chamber for 4 weeks and finally transferred to greenhouse. These results agree with those obtained by Soliman et al. (2010) who reported a method for callus induction and regeneration from leaf explants of *Ficus carica* L. cv. Sultani on MS medium supplemented with 2.0 mg L^{-1} 2,4-D and 0.2 kinetin for callus induction and MS medium supplemented with 2.0 mg L^{-1} TDZ and 4 mg L^{-1} 2iP for shoot formation. Wounded leaf explants cultured on MS medium supplemented with TDZ in combination with IBA produced more multiple shoots than did other combinations of auxin and cytokinin (Kim et al. 2007). Yakushiji et al. (2003) and Dhage et al. (2012) also reported a method for the induction of organogenesis from leaf

Table 1 The best concentrations of growth regulators to produce shoots of *Ficus carica* cv. Black Mission using leaf segment explants after 5 weeks

Concentration (mg L^{-1})		% of explant forming shoots	Mean number of shoots/explant	Mean length of shoots (cm)
ZR	IAA			
1.0	0.1	55	1.98^{e}	1.44^{c}
1.5	0.2	63	2.46^{d}	1.54^{bc}
2.0	0.4	78	5.25^{a}	1.45^{c}
2.5	0.5	70	3.28^{c}	1.00^{e}
3.0	0.5	60	1.89^{e}	1.25^{d}
TDZ	IAA			
1.0	0.2	46	1.58^{e}	1.72^{ab}
2.0	0.2	58	2.00^{d}	1.65^{b}
3.0	0.2	66	3.75^{c}	1.35^{d}
4.0	0.4	69	4.85^{b}	1.75^{a}
5.0	0.5	55	1.62^{e}	1.65^{b}

Means followed by the same superscript letters are not significantly different at $p \leq 0.05$

explants of *F. carica* on MS medium supplemented with different combinations of 2,4-D, TDZ and phloroglucinol (PG), and the frequency of adventitious bud differentiation from leaf fragments was relatively low (22 %), and no adventitious buds were observed without PG.

Transformation of fig (*Ficus carica* L.) cv. Black Mission

Hygromycin sensitivity

Hygromycin is necessary to study the sensitivity of growth and differentiation of fig tissues and it is important selection of transformed shoots. To determine the optimum concentration of hygromycin for the selection of transformed fig shoots, a kill curve experiment was carried out using cultured non-transformed fig explants. The selective media were prepared by adding filter sterilized with different concentrations 5, 10, 15, 20, 25, 30, 35, 40 mg L^{-1} hygromycin. Results showed that increasing the hygromycin concentrations was accompanied by a decrease in the percentage of the survival of explants with the same conditions for regeneration stage. The lethal dose of hygromycin was estimated at 30 mg L^{-1} (Fig. 3). Therefore, this concentration was chosen as a selection threshold for transformed fig tissues. In agreement with our results, Lifang et al. (2001) demonstrated that the concentrations of the hygromycin used in the regeneration and subculture media for wheat plants was 20 and 30 mg L^{-1}, respectively. Sjahril et al. (2006) reported that 25 mg L^{-1} hygromycin was used in the regeneration medium for selection of putative transgenic *Phalaenopsis* orchid plantlets. The results are also consistent with those obtained by Angenon et al. (1994) and Cosson et al. (2015) as

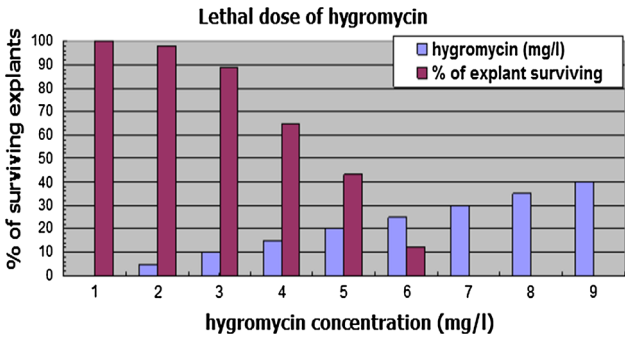

Fig. 3 Effect of hygromycin concentrations on leaf segments of *Ficus carica* L. cv. Black Mission

who reported that the selection agent should fully inhibit growth of untransformed plant cells, therefore the lowest concentration of the selection agent that suppresses growth of untransformed cells is generally used. They also demonstrated that the sensitivity of plant cells to the selection agent depends upon the genotype, the explant type and the developmental stage. Most of studies have reported that hygromycin can be used as a more efficient selectable marker, compared to kanamycin, and that it is an effective agent in plant transformation, very efficient in terms of regeneration, does not inhibit regeneration and does not affect the subsequent fertility of transgenic plants (Reynaerts et al. 1988; Twyman et al. 2002; Htwe et al. 2014).

Transformation via Agrobacterium

Leaves of *F. carica* were used as explants, and incubated with *A. tumefaciens* strain LBA4404 harboring the plasmid pBI121

contains the *AgNHX1* and *hpt* genes for 20 min before to co-cultivation media. Then, explants were co-cultivated with *Agrobacterium* for 48 h on the MS medium-free hormones. Following co-cultivation, the explants were cultured on MS medium supplemented with 100 mg L^{-1} myo-inositol, 30 g L^{-1} sucrose, 0.2 mg L^{-1} kinetin and 2.0 mg L^{-1} 2, 4-D solidified with 2.5 g L^{-1} phytagel for 4 weeks. After that, the explants were transferred to the regeneration medium containing 2 mg L^{-1} zeatin riboside in combination with 0.4 mg L^{-1} IAA with 300 mg L^{-1} carbinicillin and 30 mg L^{-1} hygromycin to select for transformed cells for 4 weeks. Adventitious shoots were excised when approximately 2–3 cm in length, and transferred to MS medium containing 2 mg L^{-1} BAP for shoot multiplication, then hygromycin resistant shoots were transferred to rooting medium containing 1 mg L^{-1} IBA, 0.5 mg L^{-1} NAA and 2 mg L^{-1} activated charcoal (Fig. 2d).

Molecular analysis of transgenic fig plants

PCR detection of transformed plants

Successful introduction of the transgenes (*Ag*NHX1 gene) was confirmed by PCR using specific primers for each of the transgenes. A fragment of 2600 bp (Fig. 4) was amplified from the DNA of the hygromycin resistant fig plants by using the *Ag*NHX1 specific primers. These bands were not amplified with non-transformed plants. It was observed that PCR-positive plants (38 %) for *Ag* NHX1 gene, indicating that the gene was successfully transferred to the explants by using the *Agrobacterium*-mediated transformation method. The polymerase chain reaction is a very powerful technique that is now used in many areas of biology. A simple and obvious use of PCR in plant transformation studies is to use it to confirm the presence of a foreign gene in transgenic plant tissues (Lassner et al. 1989; Yi et al. 2013; Singh et al. 2014). Polymerase chain reaction as indicator for the presence of *gus intron gene* into transformed fig plants cv. Sultani grown on selective media by obtaining the expected product size was detected by Soliman et al. (2010).

Southern blot analysis

PCR-positive plants were further analyzed by Southern blot analysis to study confirm the presence of the fig transgenic plants and to study the integration patterns using *Ag NHX1* as a probe. Genomic DNA was digested with *Hin*dIII and data revealed that only one band was obtained but was the integration of *AgNHX1* gene into the genomic DNA of the fig transformed plants (Fig. 5).

RT-PCR detection of transgenic fig plants

Transgenic fig positive in their PCR identification were selected for expression analysis of the *AgNHX1* gene. RT-PCR and primers specific for their cDNAs were used, based on relatively high salt tolerances in those plants and the expression of *AgNHX1* corresponding to be measured after 200, 250 and 300 mM NaCl treatments. An *AgNHX1*-specific band with expected size of 448-bp was amplified from DNase-treated RNA prepared from the leaves of transgenic fig plants (Fig. 6). Relative amounts of the mRNA in the leaves increased up to 4 and 7 times in response to treatment with 200 and 300 mM NaCl, respectively. In examining the accumulation of mRNA for the introduced *AgNHX1*, transgenic plants were detected by RNA gel blot analysis (Fig. 6).

Salt tolerance of transgenic plants and analysis of proline content

The survival rates of control fig plants decreased with increasing salt concentration from 100 to 250 mM and at the

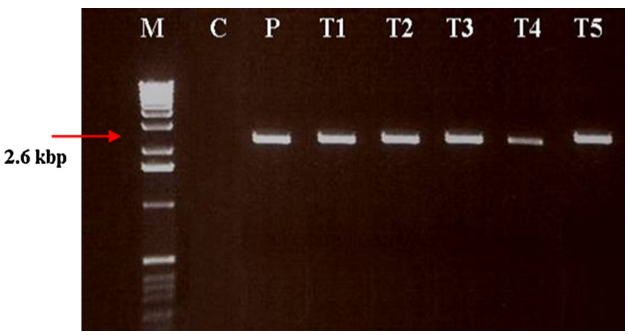

Fig. 4 PCR analysis of DNA amplified with *AgNHX1*. Lanes T1–T5 DNA from the transgenic plants, lane C DNA from non-transformed plants (negative control). Lane P DNA from the plasmid *AgNHX1* (positive control); M: DNA marker (1 Kb plus DNA ladder)

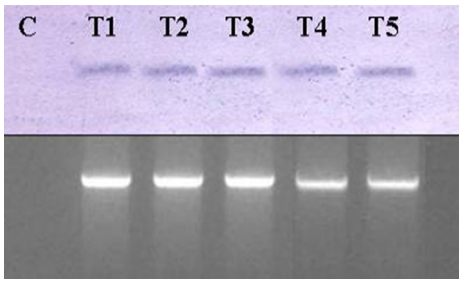

Fig. 5 Southern blot analysis of transgenic fig shoots. Ten micrograms of the plant genomic DNA was digested with *Hin*dIII. Filters were hybridized with the α-32P labeled, PCR-generated *AgNHX1* fragment; Lane C non-transformed plant and Lanes T1, T2, T3, T4 and T5 fig transformed plants

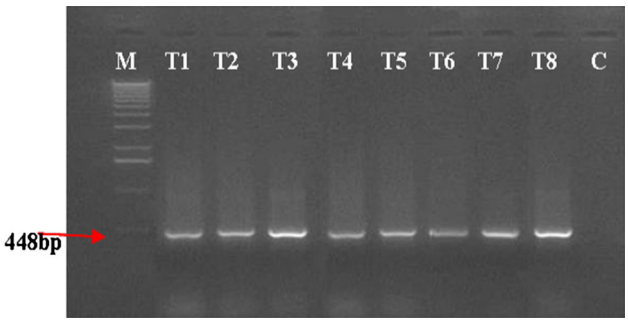

Fig. 6 RT-PCR demonstrating *AgNHX1* and expression in fig (*Ficus carica* L.) cv. Black Mission. PCR products are electrophoresed on 1 % agarose gel and stained with ethidium bromide. Lanes T1, T2, T3, T4, T5, T6, T7 and T8 transformed lines, Lane C; non-transformed plant. M: DNA marker (1 kb plus DNA ladder)

high salt concentrations (300 mM) all control plants died. Transgenic fig plants however had a high survival rate when exposed to 300 mM NaCl. The data showed that the Na^+ content was significantly lower in the transgenic *AgNHX1* plants than in non-transformed plants, and the K^+ content of transgenic plants was higher that in the non-transformed plants (Table 2). Also, the results indicated that the proline content in transgenic leaves was higher than in non-transformed leaves. Transgenic plants stressed with 300 mM NaCl displayed a four fold increase in proline content compared with non-transgenic plants (Fig. 7). The accumulation of solutes such as glycine and proline has been linked to water stress, salinity and other abiotic plant stresses (Ashraf and Harris 2004; Munns and Tester 2008; Lu et al. 2009; Bhauso et al. 2014), indicating an essential role for these solutes in tolerance to these stresses. Proline accumulates under salt stress and acts as a reserve source of carbon, nitrogen and energy during recovery from stress (Watanabe et al. 2000; Chen et al. 2007; Kumar et al. 2010; Anjum et al. 2011; Lum et al. 2014; Jariteh et al. 2015).

In the present study, it was shown that the salt tolerance of fig (*Ficus carica* L.) cv. Black Mission was improved by the introduction of the *AgNHX1* gene. The transgenic fig plants expressing *AgNHX1* exhibited improved shoot

Table 2 Effects of different levels of NaCl on growth, sodium, potassium and proline content of in vitro grown *Ficus carica* L. cultivar Black Mission of *AgNHX1* expressing transgenic plants and non-transgenic control

NaCl (mM)	Fresh weight (gm)		Dry weight (gm)		K^+ (mg L^{-1})		Na^+ (mg L^{-1})		Proline content (μg g^{-1} Fw)	
	C	T	C	T	C	T	C	T	C	T
200	12.65^{f}	38.8^{d}	2.25^{h}	4.35^{g}	20.3^{e}	38.5^{d}	95.8^{a}	40.6^{c}	19.3^{e}	87.65^{b}
250	11.02^{f}	37.5^{d}	1.82^{h}	4.24^{g}	21.8^{e}	42.7^{c}	97.2^{a}	42.4^{c}	20.2^{e}	92.38^{a}
300	00.00^{i}	37.4^{d}	00.00^{i}	4.02^{g}	00.00	42.8^{c}	00.00^{i}	42.9^{c}	00.00^{i}	92.45^{a}

C non-transgenic plant (control), *T* transgenic plant

Means followed by the same superscript letters are not significantly different at $p \leq 0.05$

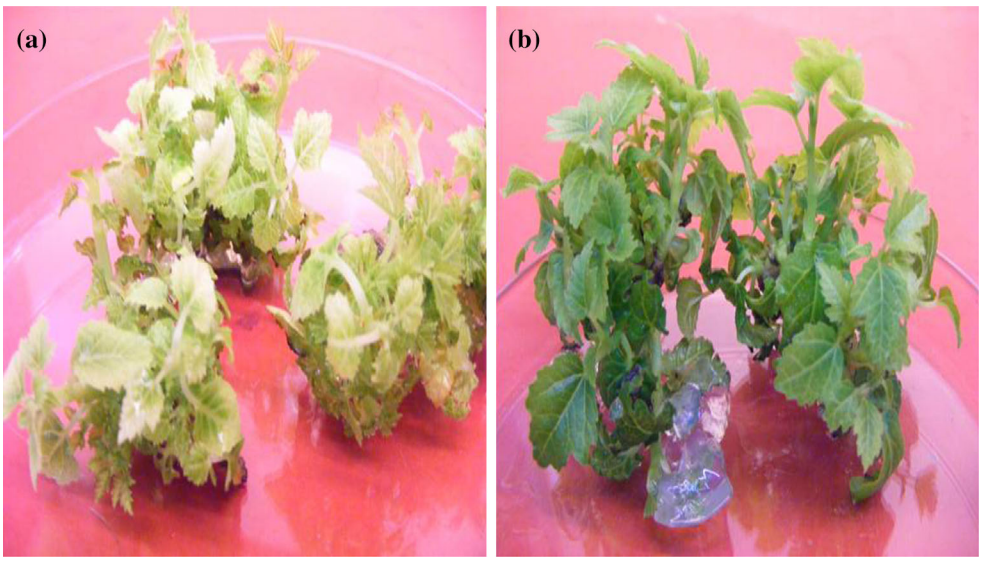

Fig. 7 In vitro shoots of *Ficus carica* L. cultivar Black Mission after 4 weeks from culturing on medium supplemented with 300 mM NaCl **a** control; **b** transgenic fig plants carrying *AgNHX1* gene

Fig. 8 Transgenic fig plants carrying *AgNHX1* gene grown in the presence of 300 mM NaCl solution

growth under the salt condition of 300 mM NaCl in the greenhouse (Fig. 8). A significant difference in shoot growth was observed between the *AgNHX1* transgenic plants and non-transgenic plants. A comparative analysis on Na^+ accumulation revealed that transgenic plants accumulated significantly less Na^+ in leaves than the non-transgenic control after 35 days of salt treatment. Similar results have been reported for vacuolar antiporter overexpressing plants such as tomato (Gisbert et al. 2000), wheat (Xuea et al. 2004), fescue (Zhao et al. 2007), Arabidopsis (Li et al. 2007), sugar beet (Liu et al. 2008) and apple rootstock (Li et al. 2010). Hamada et al. (2001) successfully isolated a Na^+/H^+ antiporter gene from a halophytic plant, *Atriplex gmelini*, and named it *AgNHX1*. The isolated cDNA is 2607 bp in length and contains one open reading frame, which comprises 555 amino acid residues with a predicted molecular mass of 61.9 kDa. The amino acid sequence of the *AgNHX1* gene showed more than 75 % identity with those of the previously isolated *NHX1* genes from glycophytes, *Arabidopsis thaliana* and *Oryza sativa*. The sensitivity to salt of cytosolic enzymes is similar in both glycophytes and halophytes, indicating that the maintenance of a high cytosolic K^+/Na^+ concentration ratio is a key requirement for plant growth in high salt (Greenway and Munns 1980; Maris and Eduardo 2002; Rajabi and Vazan 2013). The compartmentation of Na^+ ions into vacuoles provides an efficient mechanism to avert the toxic effects of Na in the cytosol (Li et al. 2010). The transport of Na into the vacuoles is mediated by a Na^+/H^+ antiporter that is driven by the electrochemical gradient of protons generated by the vacuolar H^+-translocating enzymes, the H^+-ATPase and the H^+-PPase (Blumwald 1987; Yamaguchi et al. 2003; Bassil et al. 2011; Hui et al. 2012; Bakhsh and Hussain 2015).

Conclusion

Adverse abiotic stress environmental affects yield and quality of most crops more than biotic stress. Thus, the major goal of scientists in the field of plant breeding, genetics and biotechnology is to alleviate abiotic stress on the plant and maintain high productivity and quality under abiotic stress. Plant biotechnology has the potential to address various problems in agriculture such as salt stress. The overexpression of a vacuolar Na^+/H^+ antiporter gene (*AgNHX1*) appears to hold great promise in improving agricultural productivity under abiotic salt stress. Thus, regeneration and transformation systems have to optimize as a necessary requirement for the introduction of the desired gene into plants. Given this observation, the hypothesis of this research was to enhance salt tolerance in fig (*Ficus carica* L.) by the transfer of Na^+/H^+ antiporter gene (*AgNHX1*) using *Agrobacterium tumefaciens*. To achieve this goal, we succeeded first to establish a useful protocol for production of a sufficient number of regenerated in vitro plants, which then allowed routine introduction of *AgNHX1* gene into the fig genome by by a co-cultivation technique. Second, we demonstrated that expression of *AgNHX1* gene in fig putative plantlets conferred tolerance to salt stress in the transgenic plantlets. The physiological mechanisms responsible for enhanced tolerance to salt stress can be accounted for by enhanced accumulation of proline, inhibition of Na^+ accumulation and retention of K^+ via up-regulating of the Na^+/H^+ antiporter gene (*AgNHX1*). The results are the first recorded to assess the effectiveness of transformation method of cloning Na^+/H^+ antiporter gene (*AgNHX1*) using *Agrobacterium tumefaciens* and to enhance the salt tolerance in fig (*Ficus carica* L.).

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