ISOLATION AND SEQUENCE ANALYSIS OF THE Na^+/H^+ ANTIPORTER cDNA FROM *Atriplex halimus* AND *Suaeda pruinosa*

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The desert plant species are adapted to tolerate multiple stresses including drought, high temperature, high solar radiation, high speed wind, and salinity (Batanouny, 2001). Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids (Cowen, 1999). Most of the secondary metabolites of herbs and spices are commercially important and used in a number of pharmaceutical compounds. Flavonoids and phenolic acids are the most important groups of secondary metabolites and bioactive compounds in plants (Kim *et al*., 2003).

In general mRNA is degraded rapidly by ribonucleases (RNases) and, therefore it, must be extracted quickly and efficiently (Sambrook *et al*., 1989). This is not easy to accomplish when working with aromatic plants that contain large amounts of polysaccharides and polyphenolic compounds (Gehrig *et al*., 2000; Kiefer *et al*., 2000). Phenolic compounds are readily oxidized to form covalently linked quinines and interact irreversibly with nucleic acids leading to their oxidation and degradation (Salzman *et al*., 1999).

Various protocols have been used for RNA isolation from plant species rich in polyphenolics or polysaccharides compounds (Dong and Dunstan, 1996; Geuna *et al*., 1998; Gehrig *et al*., 2000; Wang *et al*., 2005; Provost *et al*., 2007). Isolation of RNA from plants rich in secondary metabolites using commercial kits often results in contaminated preparations which are not suitable for downstream applications (Kalinowska *et al*., 2012).

In plants, the Na^+/H^+ antiporter, which is located in the plasma and vacuolar membrane can actively exclude excessive Na^+ from the cytosol or compartmentalize it into tonoplast to remove Na^+ toxicity (Apse *et al*., 1999; Hasegawa *et al*., 2000; Shi *et al*., 2002). In particular, the vacuolar Na^+/H^+ antiporter had been demonstrated to play a key role in salt

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tolerance of plants (Blumwald *et al*., 2000). The vacuolar Na^+/H^+ antiporter is a transmembrane protein which can catalyze the exchange of Na^+ and H^+ across the vacuolar membrane and maintains cellular pH and Na^+ homeostasis in plants (Apse *et al*., 1999; Shi and Zhu, 2002).

Recently many genes encoding vacuolar Na^+/H^+ antiporters (NHX1) have been characterized and isolated from several plant species, including glycophytic species such as *Arabidopsis thaliana* (Apse *el at*., 1999), *Triticum aestivum* (Brini *et al*., 2007) and *Zea mays* (Zorb *et al*., 2005), wheat (Xue *et al*., 2004), tomato (Zhang and Blumwald, 2001), *Gossypium hirsutum* (Wu *et al*., 2004) and *Medicago sativa* (Yang *et al*., 2005), halophytic species, *Atriplex gmelini* (Hamada *et al*., 2001), *Atriplex dimorphostegia* (Li *et al*., 2003), *Thellungiella halophila* (Wu *et al*., 2009), *Brassica napus* (Wang *et al*., 2003), *Beta vulgaris* (Xia *et al*., 2002), and *Suaeda salsa* (Ma *et al*., 2004). Nucleotide and amino acid analysis of these genes showed that they are highly homologous with similar structural features and contain conserved domains.

In the present study, two modified protocols for total RNA isolation are described here as a simple, fast, convenient and does not require DEPC (diethylene pyrocarbonate) treatment. Reverse transcription of the RNA followed by PCR amplification was used to confirm that the RNA produced is able to generate cDNA. Touchdown PCR program is used to amplify cDNA of vacuolar Na^+/H^+ antiporters (NHX1). The full length vacuolar Na^+/H^+ antiporters (NHX1) from *Atriplex halimus* L. and *Suaeda pruinosa* were isolated, sequenced and analyzed.

MATERIALS AND METHODS

Plant materials

One hundred milligram of collected frozen tissue samples from three plant species, *Atriplex halimus* and *Suaeda pruinosa* were placed in sterile 2 ml microfuge and immediately dipped in liquid nitrogen, are crush into fine powder using tissue Lyser II machine for homogenization to avoid browning and degradation during RNA extraction. All reagents and materials including UltraPure™ DNase/RNase-Free distilled water were autoclaved, without treating with DEPC (Diethyl pyrocarbonate).

Bioinformatic analysis

Nucleotide sequences of NHX genes were searched in NCBI database, The National Center for Biotechnology Information GenBank Database, (http://www.ncbi.nlm.nih.gov), while the European Molecular biology laboratory (EMBL), (www .2ebi.ac.uk) were used for sequence analyses. The ClC workbench 6.9 software was used to analyze coding sequences of ORFs of NHX gene sequences (http://www.clcbio.com). Protein sequences was predicted from the obtaining Open Reading Frame (ORF) NHX nucleotide sequence using the web: www.Expasy.org/translate. Multiple sequence alignment (for both amino acid

and nucleic acid) was used for ClustalW program (Thompson *et al*., 1994) (http://www2.ebiac.uk/clustalw). The European Bioinformatics institute software was used to design several primers for NHX gene. The oligonucleotide primers were synthesized and sequences analysis at *GATC* Biotech Company**,** Germany.

Total RNA extraction and purification

For establishing the suitable method for isolating total RNA from plants under this study, two different methods were evaluated TRIzol® Reagent and RNeasy Plant Mini Kit. For TRIzol® method, total RNA was extracted using 100 mg tissue/1 mL TRIzol® Reagent according to the manufacturer's instruction (Invitrogen, Cat no. 15596-026). Isolated RNA was cleaned *vis* RNeasy Plant Mini Kit. For RNeasy Plant Mini Kit method, 100 mg of plant tissue was used for each reaction. Polyethylene Glycol (PEG- MW 6000) was add to RLC or RLT buffer provided with kits breach of a concentration of 30 mg /1 ml and was incubated at 60°C for 3 hours and keep worm before use. Fine powder of samples was subjected to RNA extraction following the manufacturer's procedure according (RNasy mini plant Kit Cat No: 74904). RNA were suspended in 30-50 µl in RNase free water and stored in -80°C for further analysis.

Purified RNA samples were measured using NanoDrop spectrophotometer (NanoDrop, Technologies Inc.). The integrity of total RNA was verified using 1.2% non-denaturing agarose gel electrophoresis.

RNA analysis by one step RT-PCR

Positive RNA (2 X 105 copies/ µL), provided by TaKaRa one step RNA PCR Kit was applied as control according to the manufacturer's instruction (TaKaRa Bio INC. Cat. No. RR024A). Two micro liter of total RNA extraction from plant species were used as a template. Primers ubiquitous 18s rRNA universal primer sequences were used as positive control for one step RT-PCR to amplify 1 kb of the 18S rRNA, (18S-Fwd: 5`- CAG TAG TCA TAT GCT TGT CTC AAA-3`/ 18S-Rev: 5`- GAC TAG GAC GGT ATC TGA TCG T-3`). (Brunner *et al*., 2004; Ashoub *et al*., 2006). Sample amplified products were analyzed using 1.2% agarose gel electrophoreses staining with ethidium bromide.

Amplification of cDNA Na^+/H^+ antiporter gene by RT-PCR Two Steps

Two steps were performed to amplify the cDNA for the NHX gene. For preparing the first strand cDNA synthesis of total RNA 10 pg - 500 ng, oligo $(dT)_{20}$ and M-MLV RT (SuperScript III Reverse Transcriptase) were mixed and used according to the manufacturer's instructions (Invitrogen, Cat No. 18080-085). The cDNA synthesis reaction was stored at -20°C to be used for second step PCR.

Oligonucleotide primers designing

All primers were designed from Na^+/H^+ antiporter genes at the conserved nucleotide sequences region which were determined based on the multiple se-

quence alignment of other Na^+/H^+ antiporter genes families sequences from selected plant species in the universal database.

Primers were designed to amplify the full NHX genes. Alignment between related species to plants under this study showed a homology at the 5` end of the gene (ORF), while the 3` end sequences were species specific. Therefore, one primer at the 5' end (NHX- F1) designed for two species, while two different primers were designed for the 3` end from each species (NHX-R1A and NHXR1S, for *A. halimus* and *S. pruinosa*, respectively). Two primers were designed at the conservative region of the most related species to amplify the core region of the gene (NHXF2 and NHXR2) representing together the full NHX gene (Table 1 and Fig. 1).

PCR amplification cDNA of NHX1 gene

The High-Fidelity DNA polymerase, Phusion® *Taq* (Thermo Scientific, Product codes: F-530L, 500 Unit) with the ability to perform proof reading was used to amplify the cDNA. It generates blunt ends in the amplification products. Reaction was done in a 50 µl total volume. Reaction contained 4 µl cDNA, 10 µl 5X Phusion HF Buffer, 1 µl 10mM dNTP mix, 2.5 µl primer 1 (10 µM), 2.5 µl primer 2 (10 µM), 0.5 µl Phusion DNA polymerase, 29.5 µl DEPC H_2O and spin for 15 Sec. Touchdown PCR program was used to amplification for Na^+/H^+ antiporter cDNA gene. One cycles 30 sec of preheated at 98°C, 10 cycles 30 sec. for denaturation at 98°C, 30 sec for annealing at 58-52°C was decreased (2°C/Cycles) and 30 cycles; 30 sec. of denaturation at 98°C, 30 sec. of annealing 52°C, 1 min of extension at 72°C and followed by final extension at 72°C for 10 min. (Korbie and Mattick, 2008; Hecker and Roux, 1996).

A volume of 10 µl of each sample were analyzed using 1.2% agarose gel electrophoreses and stained with ethidium bromide (Eth-Br). The PCR fragments of each sample were excised and purified from the agarose gel with a clean, sharp scalpel. The gel slice was weighed in a colorless tube and the QIAquick® Gel Extraction Kit (Qiagen, cat. no. 28706) was used to elute the DNA from the gel. For PCR product, the sample was centrifuged according to the manufacturer's procedure in the QIAquick PCR Purification Kit (Qiagen, Cat. No.28106).

Nucleotide sequencing and analyses

DNA sequences of amplified NHX genes were determined by *GATC*, Biotech AG-Germany. The nucleotide and amino acid sequences were then compared with those in GenBank database. Homology comparison and phylogenetic relationship were analyzed using the CLUSTALW multiple sequence alignment algorithm (Thompson *et al*., 1994). Evolutional distances were calculated using the neighbor-joining method (Saitou and Nei, 1987). Sequence analysis at BLAST search and ExPASY-translate tool data analysis were conducted on the NCBI platform.

RESULTS AND DISCUSSION

There are several previous publications on using guanidine isothiocyanate as a single step method for its efficiency in isolating RNA from plants (Chomczynski and Sacchi, 1987) such as from Rice and Arabidopsis. However, this method proved to be unsuitable for isolating RNA with high quality from plants rich in secondary metabolites (Ghangal *et al*., 2009), but there were several trials for RNA isolations from such plants (Dong and Dunstan, 1996; Geuna *et al.*, 1998; Gehrig *et al.*, 2000; Wang *et al.*, 2005; Provost *et al.*, 2007).

This study was conducted in an attempt to investigate a simple, fast and convenient protocol for isolating RNA from plants rich on phenolic compounds and polysaccharides.

Both non Kit based method for isolating RNA using the commercial reagent TRIzol® Reagent (Invitrogen) and followed by purification step with a Qiagen spin-column and RNeasy plant mini Kit (Qiagen) purified with PEG for RNA extraction showed high efficiency for isolating high-quality and quantity RNA suitable for using in sensitive downstream applications.

Two Egyptian native (non-domesticated) plant species were collected from North West of Marsa Matrouh, belong to family Amaranthaceae, *Atriplex halimus* L., and *Suaeda pruinosa* (Fig. 2) and used in total RNA extracted.

Results showed good quality of the isolated RNA provided by NanoDrop spectrophotometric measurements, as it gave A_{260}/A_{280} absorbance ratio between 1.9-2.0; indicating that RNA was relatively free of DNA and protein contamination (Fig. 3).

Also, when isolated total RNA were run on 1.2% agarose gel electrophoresis, both 28S rRNA and 18S rRNA showed clearly RNA integrity with no RNA degradation, indicating that RNA is also relatively free of RNase (Fig. 4). Similar results were also reported by Kiefer *et al*. (2000), Tattersall *et al*. (2005) and Portillo *et al*. (2006).

The RNA quality was tested by one step RT-PCR for TaKaRa kit using universal oligonucleotide primers, which were designed based on the conserved 18S rRNA. Reaction products and 1 Kbp DNA leader plus, was calculate, were separated on 1.2% agarose TAE of gel electrophoresis and visualized under UV light following Et-Br staining (Fig. 5).

Results showed that the expected 1 kb DNA fragment of the 18S rRNA was amplified. This result is in agreement with those observed by Brunner *et al*. (2004) and Ashoub *et al*. (2006). This protocol allowed RNA isolation with high purity from plant species under this study. The method may be suitable for other plant

species from the same family rich in polyphenols and polysaccharides.

Amplification of NHX1 cDNA by touchdown PCR program

Amplification of full length and partial cDNA fragments of AhNHX1 and SpNHX1 gene were carried out using Touchdown (TD)-PCR program (Hecker and Roux, 1996; Korbie and Mattick, 2008).

For preparing cDNA, 1000 ng RNA was used to synthesize single-strand cDNA using reverse-transcribtase with oligo $dT_{20.}$ The synthesized cDNA was used as a template for amplifying the full and partial *NHX1* gene.

One pair of specific primer (NHX-F1 and NHX-R1A) was used to amplify the full length of *NHX1* gene which gave fragment size of 1668 bp with *A. halimus*. For the amplification of the NHX cDNA from *S. pruinosa*, two PCR reactions were carried out. One pair of specific primer (NHX-F1 and NHX-R2) were used to amplification the 5' end fragment 1 (790 bp) and another pair of specific primer (NHX-F2 and NHX-R1S) for amplifying the 3' fragment 2 (872 bp). Both fragments represent the full length cDNA of the gene with 1662 bp (Fig. 6).

Nested PCR was also carried out using the primers NHX-d1 and NHXd2 which gave fragments of about 600 bp for both *A. halimus* and *S. pruinosa* (Fig. 7) to confirm the nucleotide sequence of the core region of the two genes for AhNHX1 and SpNHX1, respectively.

Phylogenetic relationships based on sequence analyses

Sequences of NHX1 genes from different species were obtained from the GenBank. Phylogenetic analysis showed the relationship between some plant origin Na^+/H^+ antiporters and species, *Atriplex* spp., and *Suaeda* spp. (Fig. 8).

All purified of PCR products were sequenced. The assembly of contiguous sequences showed sequence identity with Na^+/H^+ antiporter sequence genes from data. Results of sequences data analysis of purified PCR fragment of Na^+/H^+ antiporter genes were submitted by dried submission of sequence data to NCBI GenBank as BankIt online with accession number KJ452334.1 and KJ452335.1 for AhNHX1, and SpNHX1 cDNA, respectively.

The sequence analysis of the cDNA for vacuolar Na^+/H^+ antiporter gene AhNHX1 revealed that it has 1668 bp for the full length of ORF, encoding a protein of 555 amino acids with a calculated molecular mass of 61.582 KDa, while the full length of the SpNHX1 ORF was 1662 bp encoding a protein of 553 amino acids with a calculated molecular mass of 61.579 KDa.

Highly conserved regions of amino acid sequences of plant vacuolar Na^+/H^+ antiporter, including amiloride-binding domain (^{85}LFFIYLPPI^{94}), NHE (Na^+/H^+

exchange) domain, and 10 and 9 transmembrane segments (TM), were found in the deduced amino acid sequence of AhNHX1 and SpNHX1, respectively.

As shown in Fig. (9), the third transmembrane domain (TM3) of AhNHX1 contains a putative amiloride-binding motif (85-FFIYLLPPI-94) that was common to vacuolar Na^+/H^+ antiporter.

Multiple alignments of vacuolar Na^+/H^+ antiporters was carried out with corresponding gene from other related species (Table 2). The results showed that AhNHX1 and SpNHX1 sequences shared high identity with other plant vacuolar Na^+/H^+ antiporters.

Phylogenetic relationship analysis indicated that AhNHX1 and SpNHX1 were clustered into the vacuolar Na^+/H^+ antiporter group (Fig. 10). Taken together, these results suggest that AhNHX1 and SpNHX1 are new members of the vacuolar Na^+/H^+ antiporter families.

The hydropathy plot generated by the SOSU1 program (http://harrier.nagahama-i-bio.ac.jp/sosui/cgi-bin/adv_sosui.cgi) indicated that the AhNHX1 had ten putative hydrophobic regions and SpNHX had nine putative hydrophobic region (Hirokawa, *et al*., 1998).

The hydrophobicicity values were calculated by the program TMpred available at http: www. Ch.embnet.org/ software /TMPRED-form.html (Fig. 11&12). According to the preliminary topological model. There are three potential N-glycosylation sites in the AhNHX1 and SpNHX1, located at Asn-36-49-292. These sites correspond to the position of the consensus N-glycosylation sites in human NHE1 (Counillon *et al*., 1994), indicating that the AhNHX1 and SpNHX1 protein is glycosylated.

SUMMARY

Two Egyptian native (non-domesticated) plant species, *Atriplex halimus* L., and *Suaeda pruinosa* from family Amaranthacae were collected from North West Coast of Marsa Matrouh governorate, Egypt under abiotic stress. For cDNA synthesizing and analysis, it is essential to isolate RNA in good quality and quantity to be used for RT-PCR amplification. Two protocols; TRIzol® Reagent with RNeasy Plant Mini Kit for RNA cleanup and RNeasy Plant Mini Kits plus polyethylene glycol (PEG-6000) were evaluated for their efficiency in RNA isolation. Both methods proved to be suitable for isolating RNA from plants rich in secondary metabolites and bioactive compounds as they produced pure and with reasonable amount of RNA, without contamination with DNA. The ultimate goal of this study was to identify the vacuolar antiporter NHX1 candidate genes from Egyptian *Atriplex halimus* and *Suaeda pruinosa*. Multiple sequence alignments were conducted to *NHX1* genes from plants related to *Atriplex* and *Suaeda* to identify the conserved regions and to de-

sign primers for cloning those genes. According to NHX1 family conserved region, several primers were synthesis either to amplify the full genes or core regions of the genes. Phusion *Taq* DNA Polymerase for RT-PCR amplification for cDNAs took place. Only touchdown PCR program (TD-PCR) succeeded in amplifying the cDNAs of the Na^+/H^+ antiporter genes when using with Phusion *Taq*. This technique increased specificity, sensitivity and yield to amplify cDNA. The obtained cDNA vacuolar Na^+/H^+ antiporter gene *AhNHX1* from both *Atriplex halimus* and *Suaeda pruinosa* were sequenced and submitted to GenBank under accession numbers KJ452334.1 and KJ452335.1, respectively. Alignment of the amino acid sequences revealed highly conserved regions representing vacuolar Na^+/H^+ antiporter. Computer analysis for the prediction of amino acid sequence of AhNHX1 and SpNHX1 recognized different domains including amiloride-binding domain (^{85}LFFIYLPPI^{94}), NHE (Na^+/H^+ exchange) domain, and 10 and 9 transmembrane segments (TM). In addition, phylogenetic relationship analysis indicated that AhNHX1 and SpNHX1 were clustered into the vacuolar Na^+/H^+ antiporter group. The obtained results indicated that AhNHX1 and SpNHX1 genes are new members of the vacuolar Na^+/H^+ antiporter families.

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Table (1): list of specific and degenerate pair primer sequences.

Primer Name	Nucleotide sequences of Na^+/H^+ antiporter
NHX-F1 for all species	5` ATG TGG TCA CAG TTA AGC TCT TT
NHX-F2 for all species	5` ATG ATG CTT ATG GCT TAT CTA TC
NHX-R2 for all species	5` GAT AGA TAA GCC ATA AGC ATC AT
NHX-R1A for *Atriplex* spp.	5`CTA TGT TCT GTC TAC CAA ATT GTT
NHX-R1S for *saueda* spp.	5` TCTGCACCAACTGCCTCAATTATCG
NHX-d1 for all species	5`GGSTTTCARGTRAARAAGAAGCAR 3`
NHX-d2 for all species	5` RTYACATTGTGCCAKGTRTAATGRGAC3`

Note: S= (G/C), R= (A/G), Y=(C/T), and K= (G/T).

Table (2): Sequences homology of plant vacuolar Na^+/H^+ antiporter genes related to AhNHX1 KJ452341.1/AHY19032.1) and SpNHX1 (KJ452342.1/AHY19033.1) localization in tonoplast.

Name of plant species	Accession no. of nucleotide sequences	Accession no. of amino acid	Identity and similarity to vacuolar Na^+/H^+ antiporter (%), bp/(aa)	
			KJ452341.1/ AHY19032.1	KJ452342.1/ AHY19033.1
Atriplex gemelini	AB038492.1	BAB11940.1	97/(99)	87/(90)
Atriplex dimorphostegia	AY211397.1	AAO48271.1	97/(98)	87/(89)
chenopodium glaucum	AY371319.1	AAQ72785.1	93/(94)	88/(89)
Halostachys caspica	GU188850.1	ADK62565.1	88/(90)	92/(93)
Kalidium foliatum	AY825250.1	AAV73803.1	87/(89)	90/(93)
Salsola komarovi	AB531436.1	BAJ06110.1	86/(89)	88/(92)
Salsola soda	EU073422.1	ABU49649.1	85/(87)	88/(90)
Suaeda maritime subsp Salsa	AY261806.1	AAP15178.1	84/(86)	90/(92)
Mesembryanthemum crystallinum	AM746985.1	CAN99589.1	83/(87)	84/(88)
Suaeda corniculata	DQ512716.1	ABF68604.1	84/(87)	90/(92)
Suaeda japonica	AB198178.1	BAE95195.1	85/(88)	90/(93)

Comparison of sequences from AhNHX1 (full length) and SpNHX1 (full length) were applied. Homology values of nucleotide are bold. Values of amino acid are in brackets. *Atriplex gemelini* (AB038492.1/BAB11940.1), *Atriplex dimorphostegia* (AY211397.1/AAO48271.1), *chenopodium glaucum* (AY371319.1/AAQ72785.1), *Halostachys caspica* (GU188850.1/ADK62565.1), *Kalidium foliatum* (AY825250.1/AAV73803.1), *Salsola komarovi* (AB531436.1/BAJ06110.1), *Salsola soda* (EU073422.1/ABU49649.1), *Suaeda maritime subsp. Salsa* (AY261806.1/AAP15178.1), *Mesembryanthemum crystallinum* (AM746985.1/CAN99589.1), *Suaeda corniculata* (DQ512716/ABF68604.1) and *Suaeda japonica*(AB198178.1/BAE95195.1).

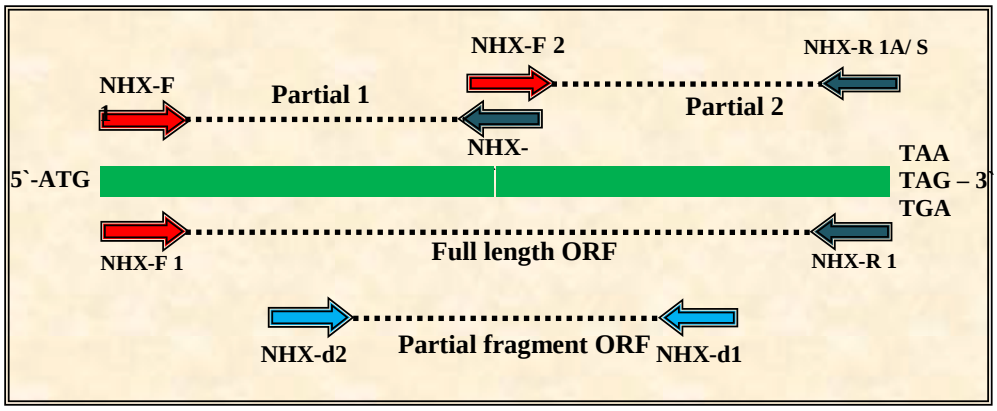


Fig. (1): Diagram showing primer pair designs of cDNA Na^+/H^+ antiporter gene:
Specific primer NHX-F1 and NHX-R2 to amplify partial 1 ORF
Specific primer NHX-F 2 and NHX-R1S to amplify partial 2 ORF
Specific primer NHX-F1 and NHX-R1A to amplify full length ORF
Degenerate primer NHX-d1 and NHX-d2 to amplify nested PCR

Fig. (2): Plant Species: *Atriplex halimus* L. and *Suaeda pruinosa* of family Amaranthacae collected from North West Coast of Marsa Matrouh.

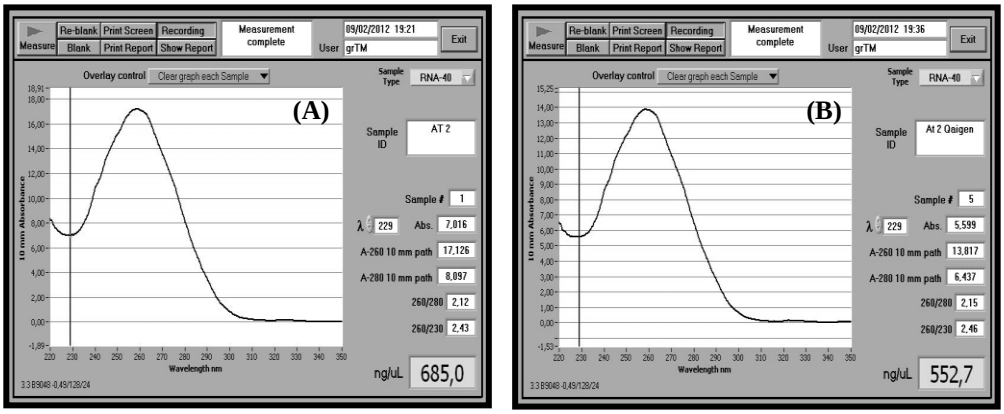


Fig. (3): Measurement of extracted RNA using nanodrop spectrophotometry showing high quality of total RNA free appreciable levels of organic contaminants by two modify methods using (A) TRIzol® reagent and (B) RNeasy plant mini kit.

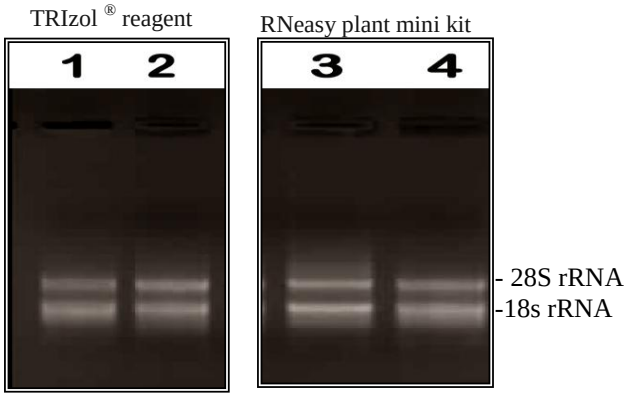


Fig. (4): RNA extracts from *Atriplex halimus* (Lanes 1 and 3) and *Suaeda pruinosa* (Lanes 2 and 4) using TRIzol ® reagent and RNeasy plant mini kit, respectively.

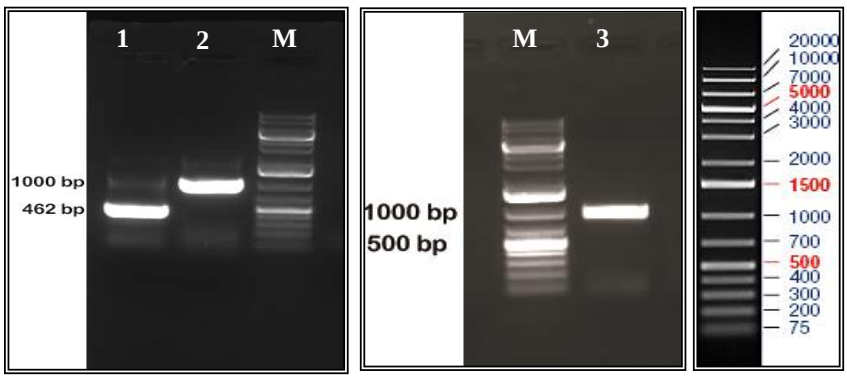


Fig. (5): Amplification of positive control rRNA 462 bp provide by TaKaRa Kit (lane 1) 1 K bp 18S ribosomal RNA of two plant species: *A. halimus* and *S. pruinosa* (lanes 2 & 3, respectively), using TaKaRa one step RT-PCR Kit. M: GeneRuler 1 Kb plus DNA Ladder.

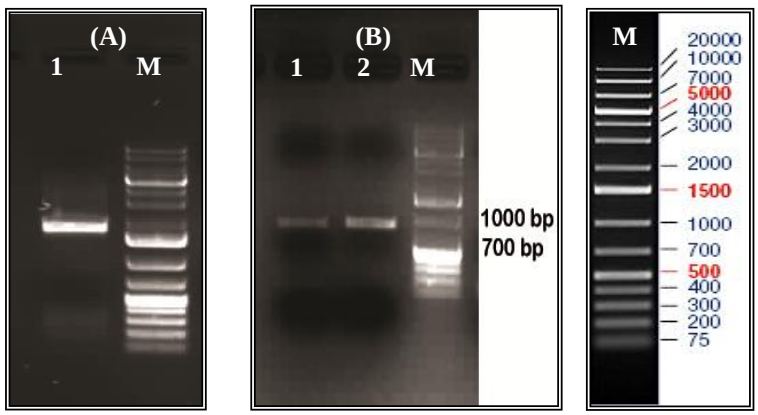
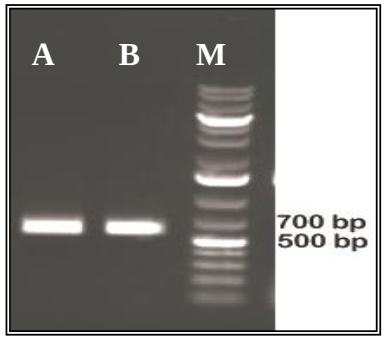


Fig. (6): RT-PCR product of cDNA using specific primer pair to amplify (A): Lane 1 full length of of Na^+/H^+ antiporter ORF for *Atriplex halimus*, (B): Lanes (1 and 2) fragment and 2 length of *Suaeda pruinosa* ORF, M: DNA size markers GeneRuler 1 Kb plus DNA Ladder.

Fig. (7): RT-PCR product of cDNA of core region for Na^+/H^+ antiporter ORF (600 bp) using degenerate primer pair for plant species: A. *Atriplex halimus*, and B. *Suaeda pruinosa*. M: GeneRuler 1 Kb plus DNA Ladder.



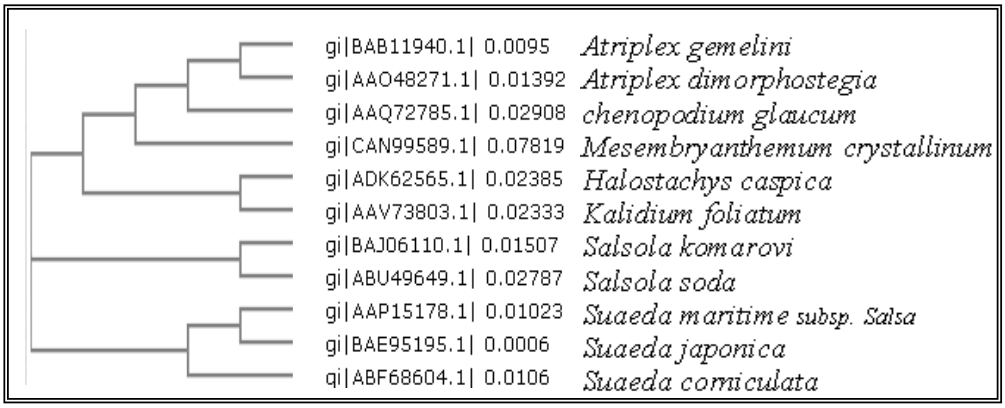


Fig. (8): Phylogenetic analysis based on full length Na^+/H^+ antiporter genes from related plant species to *Atriplex* and *Suaeda spp*, phylogenetic relationship were analyzed with CLUSTALW program and tree view.

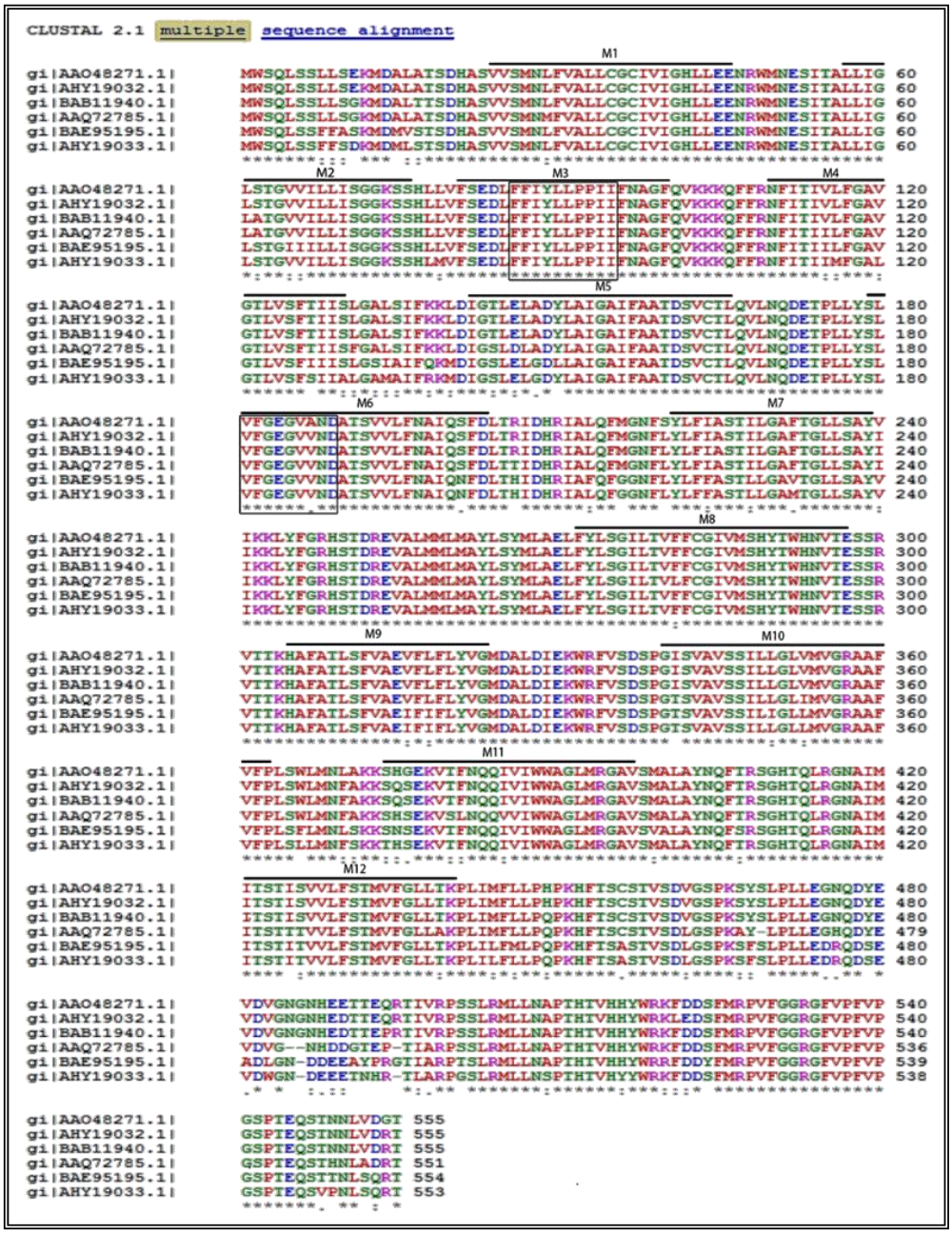


Fig. (9): Multiple sequence alignment of *AhNHX1* (AHY19032.1) and *SpNHX1* (AHY19033.1) with other putative Na+/H+ exchanger proteins. Putative membrane spanning domains of NHX1 (M1~M12) are indicated by overlines. Amino acid sequences were aligned using the Clustal X program. The accession numbers of protein sequences of the Na^+/H^+ antiporters are as follows: AAO48271.1, (*Atriplex dimorphostegia*), BAB11940.1, (*Atriplex gmelini*), and AAQ72785.1 (*Oxybasis glauca*), and BAE95195.1 (*Suaeda japonica*). The amiloride binding sites are enclosed with a box.

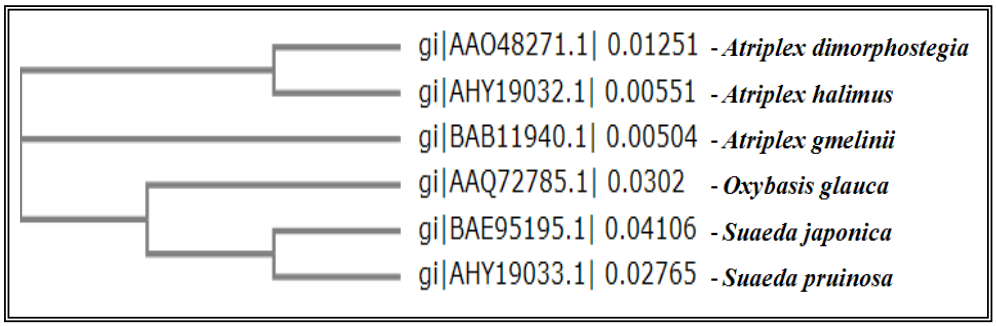


Fig. (10): Phylogenic tree produced from multiple sequence alignment of *AhNHX1* (AHY19032.1) and *SpNHX1* (AHY19033.1) with other putative Na+/H+ exchanger proteins. Software packages, ClustalW2 and generate phylogenetic tree.

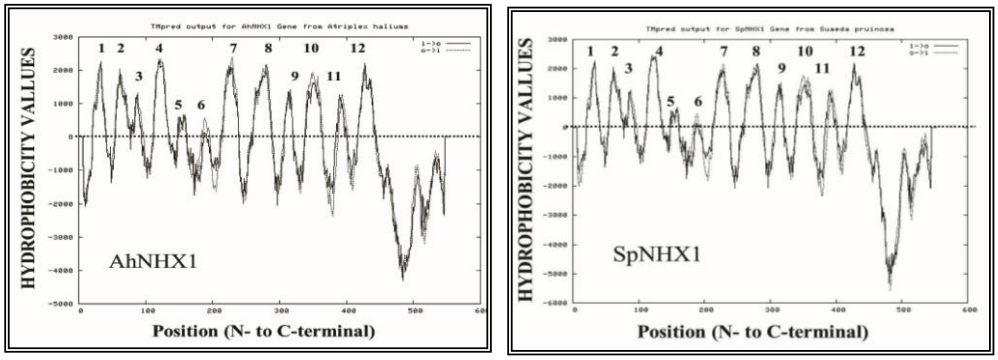


Fig. (11): Hydrophobicity plot of AhNHX1 and SpNHX1 gene product. The hydrophobicity values were calculated by program TMpred available at (http://www.ch.embnet.org/cgi-bin/TMPRED_form_parser).

Query title : AhNHX1 Gene from Atriplex haliums

Total length : 555 A. A.

Average of hydrophobicity : 0.496216

This amino acid sequence is of a MEMBRANE PROTEIN which have 10 transmembrane helices.

No.	N terminal	transmembrane region	C terminal	type	length
1	24	VVSMNLFVALLCGCIVIGHLLEE	46	PRIMARY	23
2	51	NESITALLIGLSTGVVILLISG	72	PRIMARY	22
3	79	LVFSEDLFFIYLLPPIIFNAGFQ	101	SECONDARY	23
4	111	FITIVLFGAVGTLVSFTIISLGA	133	PRIMARY	23
5	146	ELADYLAIGAIFAATDSVCTLQV	168	SECONDARY	23
6	220	LYLFIASTILGAFTGLLSAYII	241	PRIMARY	22
7	261	MAYLSYMLAELFYLSGILTVFFC	283	PRIMARY	23
8	305	HAFATLSFVAEVFLFLYVGMDAL	327	SECONDARY	23
9	342	SVAVSSILLGLVMVGRAAFVFPL	364	PRIMARY	23
10	421	ITSTISVVLFSTMVFGLLTKPLI	443	SECONDARY	23

Query title : SpNHX1 Gene from Suaeda pruinosa

Total length : 553 A. A.

Average of hydrophobicity : 0.452983

This amino acid sequence is of a MEMBRANE PROTEIN which have 9 transmembrane helices.

No.	N terminal	transmembrane region	C terminal	type	length
1	24	VVSMNLFVALLCGCIVIGHLLEE	46	PRIMARY	23
2	51	NESITALLIGLSTGVVILLISG	72	PRIMARY	22
3	79	MVFSEDLFFIYLLPPIIFNAGFQ	101	SECONDARY	23
4	111	FITIIMFGALGTLVSFSIIALGA	133	PRIMARY	23
5	219	FLYLFFASTLLGAMTGLLSAYVI	241	SECONDARY	23
6	261	MAYLSYMLAELFYLSGILTVFFC	283	PRIMARY	23
7	305	HAFATLSFVAEIFIFLYVGMDAL	327	SECONDARY	23
8	344	AVSSILLGLLMVGRAAFVFPLSL	366	SECONDARY	23
9	422	TSTITVVLFSTMVFGLLTKPLIL	444	SECONDARY	23

Fig. (12): A hydropathy plot generated by the SOSU1 program for *Atriplex haliums* which have 10 TM (transmembrane helices) and for *Suaeda pruinosa* which have 9 TM (transmembrane helices), (http://harrier.nagahama-i-bio.ac.jp/sosui/cgi-bin/adv_sosui.cgi).