Scientific Journal for Damietta Faculty of Science 3 (1) 2014, 1-9

ISSN Print 2314-8594
ISSN Online 2314-8616

Role of Na^+ manipulating genes in *Phragmites australis* adaptation to different habitats

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Received: 14 September 2013 / Accepted: 08 December 2013

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Abstract

Phragmites australis adapts to different habitats and tolerate drought and/or salt stress. In the present study, *P. australis* was collected from mesophytic, brackish water, sand dune, fresh water and salt marsh habitats. The leaf fresh weight was optimum in mesophytic habitats and it was negatively affected by stress found in other habitats. Water content followed the same trend for the fresh weight. The decrease in water content could be a tolerance strategy. Na^+ content was higher in roots than in leaves for all habitats. In contrast, K^+ content was higher in leaves than in roots. K^+/Na^+ ratio was higher than unity in leaves for all habitats even within roots collected from salt marsh. Except for leaves collected from mesophytic habitats, K^+/Na^+ ratio was less than one (1.1). *NHX1* expression level was higher in roots than that in leaves suggesting that this plant may accumulate Na^+ inside root vacuoles to restrict its movement to shoot. However, the transcript level of *SOS1* was not detected in any root collected from different habitats. Therefore, this reed may have other mechanisms for Na^+ extrusion. However, *SOS1* transcript level was detected in leaves collected from different habitats suggesting that *SOS1* could play different roles in *P. australis* tolerance.

*Keywords***:** brackish water; fresh water; mesophytes; reeds; salt marsh; sand dune; *NHX1*; *SOS1*

Introduction

Abiotic stress becomes major problems that threaten crop productivity. Up to 20% of irrigated lands are salt affected [1]. Many researches have been focused on studying the salt tolerance mechanism in glycophytes, which are naturally salt sensitive species. Therefore the attention should be shed towards the naturally adapted species to these harsh environments Detailed understanding of the tolerance mechanisms that play roles in species acclimation, thrive and maintain growth in stress environments can lead to develop a proper breeding programs for crop tolerance [2].

Both salt and drought stresses decrease water potential and hence limit the available water to plants causing osmotic stress. In addition to osmotic stress, plants expose to salinity suffer from ionic stress due to salt accumulation [2-4].

Salt tolerance is a complicated mechanism and many genes regulate this process. Sodium compartmentalization and extrusion are two main mechanisms of salt tolerance mechanisms [3,5,6].

The vacuolar Na^+/H^+ antiporter (NHX1) has been reported to regulate the sequestration of sodium into vacuole to prevent the toxicity of cytosol and hence sodium will play as a cheap osmolytes [7,8]. Proton generated from the vacuolar H^+-PPase and H^+-ATPase achieved the movement of Na^+ cross the tonoplast [9, 10]. Plasma membrane Na^+/H^+ antiporter (SOS1) controls the extrusion of sodium cross the plasma membrane back to the media [11]. Also this mechanism is energized by proton generated from plasma membrane H^+-ATPase [12].

It is well known that dicotyledonous halophytes overcome the external, excess of salts by accumulating salts inside their vacuoles. Contrarily, monocotyledonous halophytes tend to exclude sodium to maintain high (more than unity) K^+/Na^+ ratio that seems to be crucial for salt tolerance [13, 14].

Phragmites austrails (Cav.) Trin, ex Steud. (Poaceae) is an emergent grass and hydrophytic species whose typical habitats are fresh and brackish water of swamps, riversides, estuaries and coasts. Moreover, the plant can adapt to different habitats such as drought and salinity [15-17]. This study aims to investigate the contribution of sodium manipulating genes in the adaptation of *P. autralis* to different habitats.

Materials and Methods

Plant materials

P. australis was collected from fresh and brackish water, sand dune, mesophytic and salt marsh habitats in the north eastern section of the Nile Delta. Plant samples were collected on during July, 2013 at the midday to keep the unity of sampling. Samples were collected when *P. australis* reaches the maximum growth rate. To measure the growth parameters, samples were collected in plastic bags to prevent the loss of water. Others were collected in liquid nitrogen and then were kept at -80^{o}C for the biochemical analyses. Soil and water samples were collected from the rhizosphere around the roots of the plants.

Soil and water analyses

Salinity was measured in terms of electric conductivity using YSI Model 33 S-C-T Meter. Soil extract (1:5) was prepared to measure Na^+ and K^+ by flam photometer (PFP7, Jenway). Na^+ and K^+ concentration were determined from the standard curve in the range of 20-100 ppm for each ion. Three separate replicates were made.

Leaf fresh weight and water content

Fresh weight of leaves was measured and then leaves were dried on the oven at 60^{o}C to calculate the water content. Water content was calculated as a percentage of the differences between the fresh and dry weights on fresh weight basis.

Leaf Na^+ and K^+ contents

Na^+ and K^+ have been extracted as described by [18]. About 100 mg of dried leaf or root tissue was firstly homogenized in 2 ml of boiled water. The mixture was kept in water bath at 100^{o}C for 1 h. The residue was removed by centrifugation at 14000 rpm for 20 min. The diluted supernatant (1:10 with H_2O) was used to measure Na^+ and K^+ ions by flame photometry. The concentrations of Na^+ and K^+ ions were measured by using standard curves in the range of 20-100 ppm for Na^+ and K^+. Three replicates were used for each treatment.

Quantification of gene expression by semi-quantitative RT-PCR

Total RNA was extracted from about 50 mg of frozen leaves using TRI-reagent (Biovision, Egypt) according to the manufacture's protocol. To prevent DNA contamination, the extracted RNA was treated with DNA-free kit (Thermo scientific, UK) for 30 min at 37^{o}C. Then, poly A tail mRNA was isolated by reacting 10 µl of RNA with 2 µl of oligo $dT_{(18)}$ and 3 µl free RNase and DNase H_2O for 5 min at 70^{o}C and then the reaction was terminated onto ice for at least 2 min. The reverse transcription was conducted by using MMLuV-reverse transcription kit according to the supplier's recommendations (Thermo scientific, UK).

The primers for each gene were designed according to the sequence of *P. australis* found in the gene bank NCBI using primers 3 program. The primers used in amplifying *NHX1, SOS1* and *18S rRNA* and the number of cycles are listed in table 1. PCR conditions were adjusted as follows initial denaturation at 94^{o}C for 3 min, followed by 35-40 cycles according to each gene of denaturation at 94^{o}C for 30 sec, annealing for 52^{o}C for 30 sec and extension at 72^{o}C for 50 sec. the PCRs were adjusted for the number of cycles

to ensure the intensity of bands within the linear phase of the amplification.

For each gene, three replicates were used from three different isolations of RNA; one of them is shown here. PCR products were resolved by electrophoresis in 1% agarose gels, stained with ethidium bromide and visualized by UV. The intensity of bands was estimated by using Image Studio "V 3.1" program. *18S rRNA* was used as a reference in normalization.

Table 1. Primers used in quantification of genes by semi-quantitative RT-PCR. Number of cycles inside the linear phase is shown for each gene. N=No. of cycles.

Gene name	Forward primer	Reverse primer	N
NHX1	GAAGGTGTTGTTAATGATGC	CAATGTCCAATGCATCCATC	35
SOS1	TCCTGTTCGAAAGCTCGTTT	AAACAATTCCAAACGCAAGG	40
18S rRNA	CCACCCATAGAATCAAGAAAGAG	GCAAATTACCCAATCCTGAC	30

Statistical analysis

All statistical methods were performed according to [19], using SPSS, version 13.0. We used one-way ANOVA followed by LSD analyses.

Results

Salinity, Na+ and K+ of different habitats

The highest salinity was recorded for salt marsh habitats (33.66 mS cm^{-1}) followed by slightly saline water habitat as shown in table 2.

Leaf fresh weight and water content

Leaves collected from mesophytic habitats recorded the highest significant fresh weight followed by that collected from salt marsh habitats (Fig. 1A). Water content was in parallel relationship with the different habitats in terms of salinity. The highest water content was recorded in leaves collected from mesophytic habitat followed by fresh water, sand dune, brackish water and finally that of leaves collected from salt marsh habitats (Fig. 1B).

Leaf and root Na^+ content

The highest accumulation of Na^+ was recorded within leaves collected from salt marsh habitats (1046.719 ± 39 µmol g^{-1} DWt) followed by brackish water (933 ± 115 µmol g^{-1} DWt). The lowest accumulation was in leaves collected from mesophytic habitats (554 ± 49 µmol g^{-1} DWt) (Fig. 2A). The same trend was found in roots (Fig. 2B), where the most significant accumulation was found within roots collected from salt marsh (3946 ± 189 µmol g^{-1} DWt). Generally, the accumulation in roots was significantly higher than that detected in leaves for all habitats (Fig 2 A and B).

Leaf and root K^+ content

In contrast to Na^+, the highest accumulation of K^+ was found in leaves collected from mesophytic habitats where the low salt concentration (1782±20 µmol g^{-1} DWt) (Fig. 3A).

Table 2. Soil chemical characteristics of *P. australis* growing in the different habitats of the NE part of the Nile Delta. Data is the mean of triplicates samples ± SE. Data labelled with different letter are significantly different at $p \leq 0.05$ according to LSD test.

Parameters	Habitats				
	Fresh water	Mesophytic	Salt marsh	Sand dune	brackish water
Salinity (mS cm^{-1})	0.75 ± 0.1^{c}	0.50 ± 0.0^{c}	33.66 ± 2.3^{a}	0.37 ± 0.07^{c}	1.67 ± 0.1^{b}
K^+ (µmol g^{-1} Dwt)	3.29 ± 0.1^{e}	6.39 ± 0.56^{d}	215.16 ± 12.6^{a}	20.25 ± 1.26^{c}	94.92 ± 6.3^{b}
Na^+(µmol g^{-1} Dwt)	49.70 ± 0.8^{e}	93.09 ± 1.57^{d}	7100.68 ± 0^{a}	157.79 ± 0^{c}	2998.06 ± 157.0^{b}

While the least significant accumulation was within leaves collected from salt marsh habitats (1264±9 µmol g^{-1} DWt). The same trend was recorded in roots, while the highest significant accumulation was in roots collected from mesophytic habitats (1129 ± 29 µmol g^{-1} DWt) and the lowest one was in roots from salt marsh habitats (877 ± 12 µmol g^{-1} DWt) (Fig. 3B). Generally, the accumulation of K^+ in roots was significantly lower than that in leaves (Fig. 3 A and B).

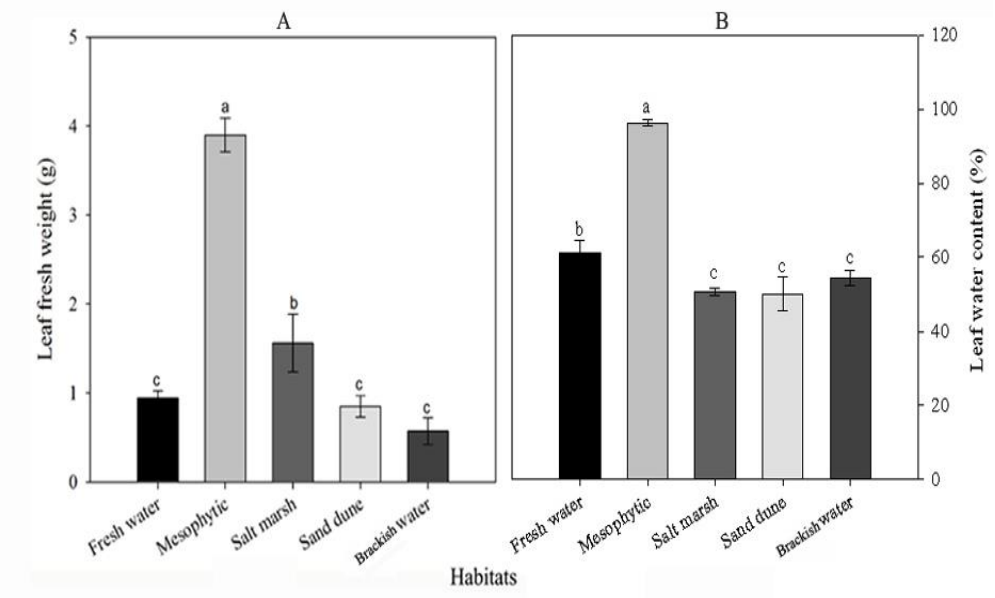

Fig. 1 Leaf fresh weight (A) and leaf water content (B) collected from different habitats. Data is the mean of triplicates samples ± SE. Bars labelled with different letters are significantly different at p≤0.05.

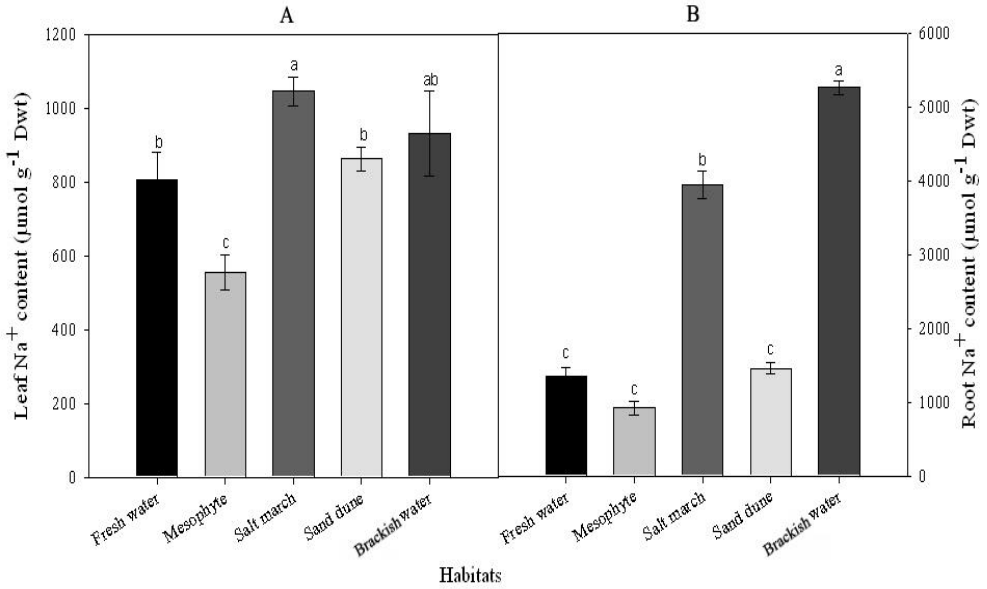

Fig. 2 Leaf (A) and root (B) Na^+ content collected from different habitats. Data is the mean of triplicates samples ± SE. Bars labelled with different letters are significantly different at p≤0.05.

K^+/Na^+ ratio

For all habitats, $K^{+/}Na^+$ ratio of leaves was higher than unity. The highest ratio was about 3.6 for mesophytic habitat and the lowest one was about 1.2 for salt marsh habitat (Fig. 4A). In contrast to leaves, K^+/Na^+ was less than one in roots except for roots collected from mesophytic habitats (1.1) (Fig. 4B).

Expression of NHX1

NHX1 transcript level was higher in roots than in leaves. In brackish water habitat, NHX1 mRNA was significantly higher in roots by about 40%. Also in sand dune habitat, the transcript level showed significant increase by about 75% in roots compared to that recorded in leaves. NHX1 mRNA was not detected in the roots of salt marsh, mesophytic or fresh water habitats. The highest significant transcript level detected in leaves was in the brackish water habitats (Fig. 5 A and B).

Expression of SOS1

No detectable bands were recorded in any root for all habitats. The highest significant transcript level was found in brackish water habitat followed by sand dune and fresh water habitats (Fig. 6A and B).

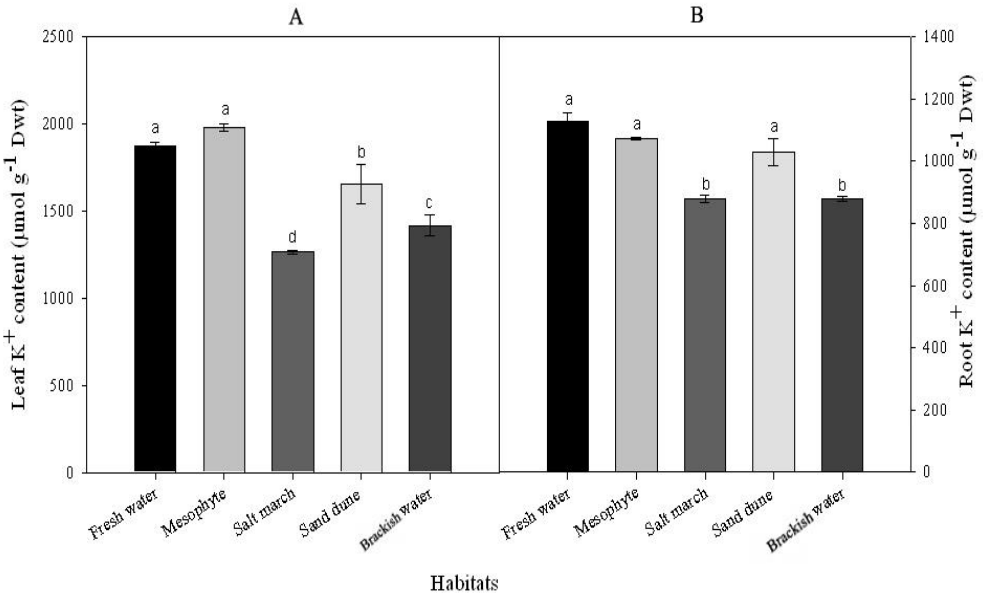

Fig. 3 Leaf (A) and root K^+ content (B) collected from different habitats. Data is the mean of triplicates samples ± SE. Bars labelled with different letters are significantly different at $p \leq 0.05$.

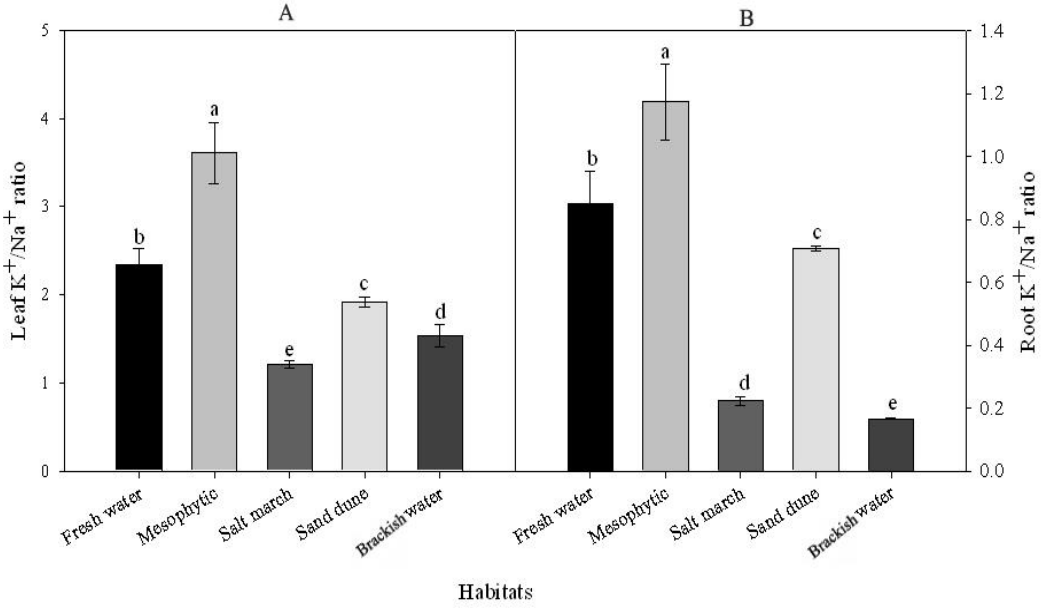

Fig. 4 Leaf (A) and root (B) K^+/Na^+ collected from different habitats. Data is the mean ± SE. Bars labelled with different letters are significantly different at $p \leq 0.05$.

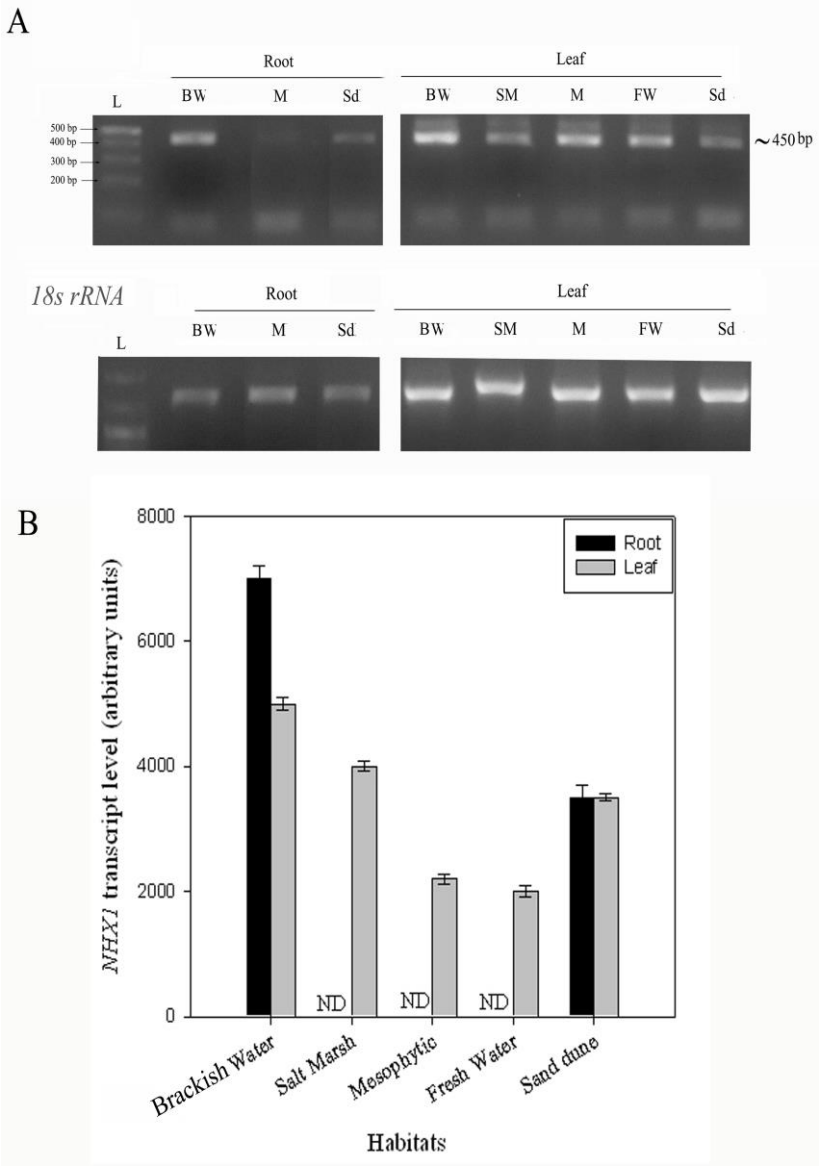

Fig. 5 Semi-quantitative RT-PCR of *NHX1* in leaves collected from different habitats. A: Ethidium bromide-stained gels after different cycles showing the growth of the bands before reaching the plateau (saturated cycles) and B: quantification of expression in terms of band volumes. BW: brackish water, FW: fresh water, M: mesophytic, Sd: sand dune and SM: salt marsh.

Discussion

P. australis has an ability to adapt to different habitats including saline, dry and hydrophytic ones. Its acclimation to these different habitats is accompanied by changing in morphological, structural, and physiological and could be genetic features [20]. *P. australis* not only adapted to saline habitat but also it has an ability to maintain growth and production contrasting the glycophytes species especially monocotyledonous ones that fail to survive under salt condition. The main reason has been attributed to the disability of monocot glycophytes to maintain higher K^+/Na^+ ratio (see introduction).

In the present study, the optimum growth could be found in mesophytic habitats whose salt concentration was low (Table 2). Leaf fresh weight was negatively affected by the concentration of external salt (Fig. 1A). Leaf fresh weight was higher in salt marsh than that in fresh water habitat, which could be attributed to the area and the thickness of leaf. Also,

photosynthetic parameters may play a role in that growth variation (Unpublished data).

Furthermore, the decrease in leaf fresh weight could be attributed to decrease in water content rather than dry weights.

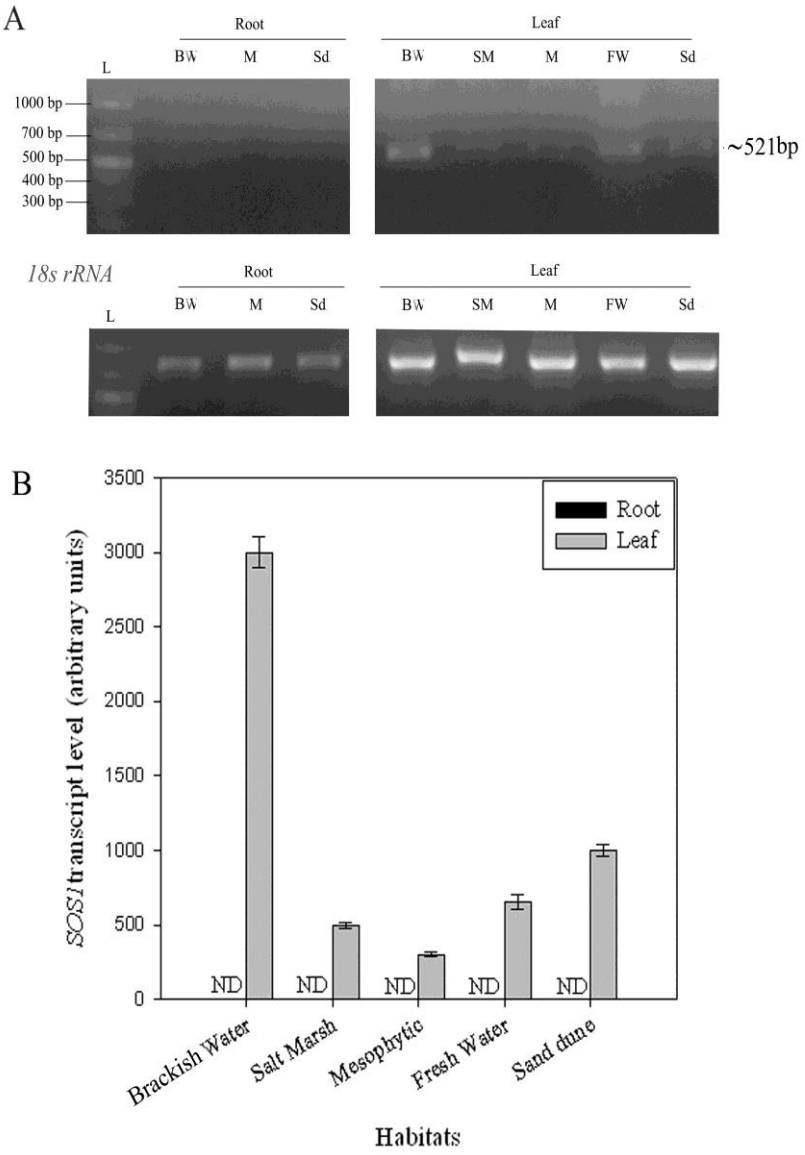

Fig. 6 Semi-quantitative RT-PCR of *SOS1* in leaves collected from different habitats. A: Ethidium bromide-stained gels after different cycles showing the growth of the bands before reaching the plateau (saturated cycles) and B: quantification of expression in terms of band volumes. BW: brackish water, FW: fresh water, M: mesophytic, Sd: sand dune and SM: salt marsh

Water content reflected the adaptation of *P. australis* to different habitats. Where, water content significantly decreased by increasing the magnitude of outer stress condition (Fig. 1B). The decrease in water content during the exposure to harsh conditions seems to be a tolerance mechanism for many plants including halophytes [21,22].

The highest Na^+ content was recorded in leaves collected from salt marshes and the lowest one was in leaves collected from mesophytic habitat. However, the highest Na^+ content was found in roots collected from brackish water habitat. Generally, root Na^+ content was significantly higher than that of leaves in all habitats (Fig. 2 A and B). Contrarily to Na^+ content, the highest K^+ content was detected in

leaves collected from mesophytic habitats and the lowest one was within leaves in salt marsh habitats. The same trend was recorded for roots. Generally, K^+ content was significantly higher in leaves than that in roots for all habitats (Fig. 3).

Perusal data showed that *P. australis* could prevent the accumulation of Na^+ inside its leaves and accumulated them inside the roots as a strategy for tolerance. K^+/Na^+ ratio (Fig. 4 A and B) could confirm this hypothesis, where *P. australis* could maintain this ratio more than unity inside its leaves in all habitats even in salt marsh. However, this ratio was less than one in its roots that could be attributed to the direct contact of roots to the salt in the soil.

NHX1 and *SOS1* expressions were measured in leaves and roots collected from different habitats to evaluate their roles in maintaining K^+/Na^+ ratio higher than one especially for leaves. *NHX1* expression was higher in roots compared to that in leaves. The highest significant expression was detected within roots and leaves collected from brackish water followed by those collected from salt marsh habitats (Fig. 5).

SOS1 transcript level was not recorded in any root collected from different habitats. Meanwhile, the highest expression value was detected in leaves collected from brackish water habitats (Fig. 6).

From these expression data, *NHX1* may be more effective than SOS1 in plant roots and that could be a trial to accumulate Na^+ inside root parts to restrict the transport of ions to leaves. *P. australis* use this strategy even with mesophytic habitats. However, the expression of *SOS1* in leaves could be to remove the excess of Na^+ by re-circulating them via the phloem or other pathways to prevent the toxicity of cytosol. *SOS1* could play role in K^+ homeostasis [23]. Additionally, the long cytoplasmic tail of *SOS1* may have a vital role in oxidative stress response via the interaction between this tail and the regulator of oxidative stress response (RCD) [24].

Understanding the mechanism by which *P. australis* could maintain its productivity under these harsh environments especially under saline one could help in enhancing the crop tolerance especially monocots to survive under stress conditions. Further studies would be effective to investigate other genes that control the ion channels inside the roots and the leaves of this reed.

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الملخص العربى

دور الجينات المتحكمة بعنصر الصوديوم فى تأقلم نبات البوص للبيئات المختلفة

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لنبات البوص قدرة على التكيف مع البيئات المختلفة وذلك بتحمل الجفاف و زيادة نسبة الملوحة. و في دراستنا الحالية تم تجميع نبات البوص من خمس بيئات مختلفة (البيئة الوسطية، المياه متوسطة الملوحة، الكثبان الرملية، المياه العذبة، والأراضي الملحية) بدلتا النيل. سجل الوزن الطري للورقة أعلى قيمة في البيئة الوسطية وتأثر سلبيا بالإجهاد الموجود بالبيئات الأخرى. وتبين ان التغير فى المحتوى المائي والوزن الطرى يتبعا مسلك متشابه . ولوحظ أن هذا النقص فى المحتوى المائي للورقة إستراتيجية للتكيف مع البيئات المختلفة. أظهرت الدراسة ارتفاع محتوى الصوديوم في الجذور عن الأوراق في كل البيئات و بالعكس فإن محتوى البوتاسيوم كان أعلى في الأوراق عن الجذور. وكانت نسبة البوتاسيوم على الصوديوم أعلى من الواحد الصحيح في أوراق كل البيئات حتى في الأوراق المجمعة من البيئات شديدة الملوحة بينما نسبة البوتاسيوم على الصوديوم كانت أقل من الواحد الصحيح في كل الجذور من جميع البيئات ما عدا البيئة الوسطية. إن الجين المسئول عن تجميع الصوديوم داخل الفجوة كان أعلى في الجذور من الأوراق و ذلك قد يكون نتيجة أن النبات يراكم الصوديوم داخل فجوة الجذور ليحد من حركة الصوديوم إلى الساق. وعلى الرغم من ذلك فإن الجين المسئول عن طرد الصوديوم خارج الجدار الخلوي لا يمكن تحديده في أى من الجذور المجمعة. و لذلك من الممكن وجود أليات للنبات غير المتعارف عليها لطرد الصوديوم. و على الرغم من ذلك فإن هذا الجين كان معبرا في جميع الأوراق المجمعة، وربما يدل ذلك على ان هذا الجين قد يلعب أدوارا أخري في قدرة نبات البوص على التكيف.