A constitutively active form of a durum wheat Na⁺/H⁺ antiporter SOS1 confers high salt tolerance to transgenic *Arabidopsis*

Kaouthar Feki¹, Francisco J. Quintero², Habib Khoudi¹, Eduardo O. Leidi², Khaled Masmoudi^{1,3}, Jose M. Pardo^{2*} and Faïçal Brini^{1*}

¹Plant Protection and Improvement Laboratory, Centre of Biotechnology of Sfax (CBS)/ University of Sfax, Route Sidi Mansour Km 6, B.P “1177” 3018 Sfax, Tunisia.

²Instituto de Recursos Naturales y Agrobiología, Consejo Superior de Investigaciones Científicas, 41012, Sevilla, Spain.

³International Center for Biosaline Agriculture (ICBA), United Arab Emirates.

*Corresponding authors

Faïçal Brini, Plant Protection and Improvement Laboratory, Centre of Biotechnology of Sfax (CBS), Route Sidi Mansour Km 6, B.P’1177’, 3018, Sfax, Tunisia. Tel: +216 74 872 091; Fax: +216 74 875 818; Email: faical.brini@cbs.rnrt.tn

Jose M. Pardo, Instituto de Recursos Naturales y Agrobiología, Consejo Superior de Investigaciones Científicas, 41012, Sevilla, Spain. Tel.: +34 954624711; Fax: +34 954624002; Email: pardo@irnase.csic.es

Abstract

Key message Expression of a truncated form of *TdSOS1* in *Arabidopsis* exhibited an improved salt tolerance. This finding provides new hints about this form that can be considered as a salt tolerance determinant.

The SOS signaling pathway has emerged as a key mechanism in preserving the homeostasis of Na^+ and K^+ under saline conditions. We have recently identified and functionally characterized, by complementation studies in yeast, the gene encoding the durum wheat plasma membrane Na^+/H^+ antiporter (*TdSOS1*). To extend these functional studies to the whole plant level, we complemented *Arabidopsis sos1-1* mutant with wild-type *TdSOS1* or with the hyperactive form *TdSOS1 Δ 972* and compared them to the *Arabidopsis AtSOS1* protein. The *Arabidopsis sos1-1* mutant is hypersensitive to both Na^+ and Li^+ ions. Compared with *sos1-1* mutant transformed with the empty binary vector, seeds from *TdSOS1* or *TdSOS1 Δ 972* transgenic plants had better germination under salt stress, and more robust seedling growth in agar plates as well as in nutritive solution containing Na^+ or Li^+ salts. The root elongation of *TdSOS1 Δ 972* transgenic lines was higher than *Arabidopsis sos1-1* mutant transformed with *TdSOS1* or with the endogenous *AtSOS1* gene. Under salt stress, *TdSOS1 Δ 972* transgenic lines showed greater water retention capacity, and retained low Na^+ and high K^+ in their shoots and roots. Our data showed that the hyperactive form *TdSOS1 Δ 972* conferred a significant ionic stress tolerance to *Arabidopsis* plants and suggest that selection of hyperactive alleles of the SOS1 transport protein may pave the way for obtaining salt-tolerant crops.

Keywords: Durum wheat, *Arabidopsis*, Na^+/H^+ antiporter SOS1, hyperactive form, ionic stress tolerance

1 Introduction

2 Salinity is one of the most severe environmental stresses affecting plant productivity
3 worldwide. High concentration of sodium (Na^+) in the soil solution impairs cell metabolism
4 and photosynthesis by imposing an osmotic stress on cell water relations and by the toxicity
5 of Na^+ in the cytoplasm. Plants prevent the excessive Na^+ accumulation in the symplast
6 through three mechanisms that function in a cooperative manner i.e, restriction of Na^+ influx,
7 active Na^+ extrusion at the root-soil interface, and vacuolar sequestration of Na^+ (Niu et al.
8 1995; Tester and Davenport 2003). Moreover, transport systems that control net Na^+ uptake
9 into the root xylem vessels and regulate Na^+ movement to shoots are salt tolerance
10 determinants (Shi et al. 2002; Olías et al. 2009; Munns et al. 2012).

11 In *Arabidopsis thaliana*, three SOS genes (*SOS1*, *SOS2* and *SOS3*) have been found to
12 function in a common pathway (Wu et al. 1996; Zhu 2000). The SOS signal transduction
13 pathway is important for ion homeostasis and salt tolerance in plants (Zhu 2003; Pardo et al.
14 2006). The biochemical and physiological functions of *SOS1* have been demonstrated first in
15 *Arabidopsis thaliana*, and later in other plant species by genetic criteria (Shi et al. 2000, 2002;
16 Oh et al. 2009; Olías et al. 2009). *SOS1* encodes for a plasma membrane Na^+/H^+ antiporter
17 involved in removing Na^+ ions from cells (Shi et al. 2000; 2002, Pardo 2010). *SOS1* is
18 preferentially expressed in the epidermis of the root tip region and in xylem parenchyma cells
19 (Shi et al. 2002). In salt stress conditions, *SOS1* protein controls long-distance Na^+ transport
20 since this ion is transported from the root to the shoot via the xylem (Shi et al. 2002). This
21 critical function was demonstrated also in the halophytic *Arabidopsis* relative *Thellungiella*
22 *salsuginea* (Oh et al. 2009) and in tomato (Olías et al. 2009). In *Arabidopsis*, *SOS1* likely
23 works in concert with *HKT1* at the plasma membrane of xylem parenchyma cells to finely
24 regulate the amount of Na^+ being delivered to the shoot (Sunarpi et al. 2005; Pardo et al.
25 2006; Pardo 2010). *SOS* proteins contribute to different responses to salt stress besides ions
26 homeostasis (Ji et al. 2013). *SOS1* has been also implicated in oxidative stress tolerance by
27 interaction of its cytoplasmic tail with *RCD1* (radical-induced cell death) (Agarwal et al.
28 2006). The role of *SOS1* in K^+ acquisition may be indirect and could possibly arise through
29 H^+ coupling with H^+-K^+ co-transporters (Huang et al. 2012).

30 Sodium efflux by the *SOS1* exchanger is regulated through protein phosphorylation by the
31 alternative *SOS2/SOS3* and *SOS2/CBL10* protein kinase complexes (Qui et al. 2002;
32 Quintero et al. 2002; 2011; Quan et al. 2007). In addition to the *SOS2* kinase, *SOS1* is also
33 phosphorylated in an as yet uncharacterized site by Mitogen-Activated Protein Kinase 6
34 (*MPK6*) (Yu et al. 2010). *SOS2* is a serine/threonine protein kinase belonging to the *SNF1*-

1 related kinase 3 (SnRK3) family (Liu et al. 2000; Gong et al. 2002). SOS3 is a myristoylated
 2 calcium binding protein capable of sensing Ca^{2+} oscillations elicited by salt stress (Liu and
 3 Zhu 1998). Both N-myristoylation and calcium binding are required for SOS3 function in
 4 plant salt tolerance (Ishitani et al. 2000). The C-terminal regulatory domain of SOS2 contains
 5 an auto-inhibitory FISL motif that binds to SOS3 or CBL10, thereby releasing SOS2 from
 6 auto-inhibition (Liu et al. 2000; Guo et al. 2001; Quan et al. 2007). SOS3 acts primarily in
 7 *Arabidopsis* roots under salt stress, whereas the SOS3 homolog SOS3-LIKE CALCIUM
 8 BINDING PROTEIN8 (SCABP8)/CALCINEURIN B-LIKE 10 functions mainly in the shoot
 9 response to salt toxicity (Halfter et al. 2000; Quan et al. 2007). It has been shown that under
 10 salt stress, SOS2 phosphorylates SCaBP8/CBL10 at its C-terminus. This phosphorylation
 11 occurs at the membrane, stabilizes the SCaBP8-SOS2 interaction and enhances plasma
 12 membrane Na^+/H^+ exchange activity of SOS1 (Quan et al. 2007; Lin et al. 2009). SOS2 was
 13 also shown to interact with type 2C protein phosphatase abscisic acid (ABA)-insensitive 2
 14 (ABI2), suggesting a crosstalk between the ABA pathway and SOS pathway (Ohta et al.
 15 2003). In a recent report, the photoperiodical and circadian clock switch *Gigantea* (GI) was
 16 shown to be a negative regulator of the SOS pathway. By physical interaction, GI effectively
 17 sequesters SOS2, thus preventing the activation of SOS1 in the absence of stress (Kim et al.
 18 2013). Recently, it has been demonstrated that SOS3 plays an important role in salt tolerance
 19 by mediating calcium-dependent microfilament reorganization (Ye et al. 2013).
 20 The *Arabidopsis* AtSOS1 is relieved from auto-inhibition upon phosphorylation of the auto-
 21 inhibitory domain by SOS2/SOS3 (Quintero et al. 2011). The auto-inhibitory domain of
 22 AtSOS1 interacts intra-molecularly with an adjacent domain that is essential for activity.
 23 Likewise, the activation mechanism of durum wheat TdSOS1 protein involves the
 24 phosphorylation and inactivation of an auto-inhibitory domain located at the C-terminal end
 25 of the transporter. This region contains the essential residues for its phosphorylation by the
 26 *Arabidopsis* protein kinase SOS2 (Feki et al. 2011). Deletion of the auto-inhibitory domains
 27 of AtSOS1 and TdSOS1 rendered SOS1 proteins that were biochemically hyperactive and
 28 independent of the SOS2/SOS3 regulatory complex. These truncated proteins conveyed much
 29 greater salt tolerance to the budding yeast, but they have not been assayed for salt tolerance in
 30 whole plants. Since the overexpression of *Arabidopsis thaliana* AtSOS1 gene improved plant
 31 salt tolerance in *A. thaliana* and in tobacco (Shi et al. 2003; Yue et al. 2011), the question
 32 addressed here is whether the hyperactive forms of SOS1 could bestow whole plants with
 33 enhanced salt tolerance compared to wild-type proteins. In the present study, durum wheat
 34 TdSOS1 and its hyperactive form TdSOS1^{N972} were expressed under the control of a

1 constitutive promoter and transferred into *Arabidopsis sos1-1* mutant genome by
2 *Agrobacterium*-mediated transformation. The generated transgenic *Arabidopsis* plants were
3 used to investigate which form of durum wheat SOS1 (the wild-type TdSOS1 and/or the
4 hyperactive form TdSOS1^{H972}) improves ionic stress tolerance of *Arabidopsis* plants. Our
5 results demonstrated that the hyperactive form TdSOS1^{H972} conferred a significant ionic
6 stress tolerance to the transgenic *Arabidopsis* that was superior to that imparted by wild-type
7 SOS1 protein. Thus, this hyperactive form can be considered as a salt tolerance determinant
8 that could be screened for in a mutagenized plant population as a way to create salt tolerant
9 crops that are not considered Genetically Modified Organisms (GMO).

10

Materials and methods

Plasmid constructs and *Arabidopsis* transformation

The two durum wheat cDNAs encoding for Na⁺/H⁺ antiporter (TdSOS1) and its hyperactive form (TdSOS1Δ972) were reported in Feki et al. (2011). The open reading frame of *TdSOS1* was amplified by PCR, using the recombinant plasmid pTdSOS1-1 (Feki et al. 2011) as a template, and the primers SX (5'-GTGTCTAGAAATGGAGACGGAGGAGGCCGGCTCCC-3', *Xba*I site underlined) and SE (5'-ATACTAGTTCAGCTGCCTCGCGGTGGGCCGGA-3', *Spe*I site underlined). The resulting fragment was cloned downstream of the constitutive cauliflower mosaic virus (CaMV) 35S promoter into the *Xba*I and *Spe*I sites of the pBI321 binary vector containing the kanamycine resistant selectable marker (Martínez-Atienza et al. 2007). The resultant plasmid was named BSOS. The full length of *TdSOS1*Δ972 open reading frame was amplified using the primers SX and SHE (5'-TAACTAGTTCAGAGGGTTGAAGACAGCAA-3', *Spe*I site underlined) using the recombinant plasmid pTdSOS1-3 (Feki et al. 2011) as a template. The resulting fragment was cloned in the same binary vector using the same restriction enzymes, and the resultant recombinant binary vector was named herein BSOSH. These two recombinant plasmids were introduced separately into *Agrobacterium tumefaciens* strain GV3101 (Konez and Schell 1986). The *Arabidopsis thaliana* transformation was performed using the floral dipping technique (Clough and Bent 1998). Transgenic plants harboring *TdSOS1* or *TdSOS1*Δ972 were selected on Murashige and Skoog (MS) agar medium (Murashige and Skoog 1962) supplemented with 50 mg L⁻¹ kanamycin. The presence and the integrity of the transgene were further confirmed by PCR amplifications using specific primers for *TdSOS1* and for *TdSOS1*Δ972 sequences. Transgenic lines were advanced to T3 generation and homozygous lines were used in this study. The *Arabidopsis sos1-1* mutant line transformed with 35S-*AtSOS1* has been previously described (Shi et al. 2000).

RNA extraction and RT-PCR

Total RNA was extracted from 100 mg of young *Arabidopsis* transgenic lines using the Trizol method (Invitrogen). Total RNA (10 µg) was treated with RNase-free DNase (Promega) at 37°C for 15 min and further incubated at 65°C for 10 min. The reverse transcription was performed at 37°C for 1 h, using the oligo-dT (18 mer) primer and M-MLV reverse transcriptase (Invitrogen). One microlitre of the first strand cDNAs was used as template for PCR amplification with a pair of gene-specific primer KM1 (5'-

GCATCTTATTGGAAGGATTTCTGAA-3') and KM2 (5'-GGAGAGTCCACTCTGACGAT-3') for *TdSOS1* allele and the primers KH1 (5'-AATGCTTGAAGAGGGACGAATAA-3') and KH2 (5'-CCAGTTAATGCCTCATATAGACC-3') for *TdSOS1Δ972* allele. An *Arabidopsis thaliana* β -tubulin gene fragment, used as an internal control, was amplified with the following primers (5'-GTCCAGTGTCTGTGATATTGCACC-3') and (5'-GCTTACGAATCCGAGGGTGCC-3'). Samples were denatured for 3 min at 94°C and then run for 30 cycles of 1 min at 94°C, 45 s at 55°C, 1 min 30 s at 72°C with a final extension of 5 min at 72°C. The PCR products were separated by agarose gel electrophoresis.

Growth conditions and stress treatments

Seeds of each transgenic *Arabidopsis thaliana* line were surface-sterilized and stratified at 4°C for 2-4 days. A germination test was initiated with seeds sown on MS agar medium containing 35 mM or 50 mM NaCl. The seed germination assay was performed by plating 30 seeds of each T3 homozygous seeds of transgenic *Arabidopsis* lines and *sos1-1* mutant expressing *AtSOS1* on MS medium containing NaCl.

For ionic-tolerance experiments, T3 homozygous seeds of transgenic *Arabidopsis* lines were grown on MS agar medium for one week under light/dark cycle condition of 16 h light/8 h dark cycle at 22°C, then transferred to MS medium supplemented with 35, 75 mM NaCl or 15 mM LiCl, and plates were incubated vertically with seedling in the upright position. Plant survival was monitored after 10 days of ionic stress.

For ionic stress treatment under greenhouse conditions, transgenic plants were grown in nutrient solution (Hewitt 1966) for 2 weeks, with a light/dark cycle of 16 h light (23°C) /8 h dark (18°C) cycle at 60-70% relative humidity. Thereafter, the nutrient solution was supplemented with NaCl to a final concentration of 5 mM, or with LiCl to a final concentration of 0.2 mM, and plants were kept under stress for 13 days.

Root elongation test

Seeds of *TdSOS1* or *TdSOS1Δ972* transgenic plants of homozygous T3 generation were surface-sterilized and plated on MS agar medium plates with *Arabidopsis sos1-1* mutant transformed with the empty binary vector (called pBI line) or with the endogenous *AtSOS1* gene (denoted as the AtS line henceforth). Plates were placed vertically in a growth chamber under a 16 h light/8 h dark cycle at 22°C. To evaluate the growth rate under ionic stress conditions, 7 day-old transgenic seedlings were transferred to MS medium supplemented with

different concentrations of NaCl (25, 35 and 75 mM) or LiCl (10 and 15 mM). After 10 days of incubation, root elongation was determined.

Leaf surface determination

Total leaf area of *Arabidopsis* seedlings in nutritive solution containing 5 mM NaCl or 0.2 mM LiCl was calculated using image processing and analysis program UTHSCSA (<http://compdent.uthscsa.edu/dig/itdesc.html>).

Determination of fresh weight and ion contents

Fresh weights were determined immediately at the end of the salt treatment. To determine Na⁺ and K⁺ contents in plant tissues, leaves were carefully separated from roots, rinsed in deionized water, blotted with filter paper and oven dried at 70°C until constant mass was reached. Dry material was mineralized using 0.5 N HNO₃. After centrifugation to remove debris, the supernatants were analyzed by atomic absorption spectrometry.

Determination of relative water content (RWC)

Leaves were harvested and their fresh weight (FW) was determined immediately. They were floated on deionized water overnight at 4°C and the turgescence weight (TW) was recorded. Finally, leaves were dried at 70°C and the dry weight (DW) was recorded. Relative water content was calculated using the following formula: $RWC (\%) = (FW - DW / TW - DW) \times 100$.

Statistical analysis

Data were analyzed using one-way analysis of variance and treatment mean separations were performed using Duncan's multiple range tests at the 5% level of significance.

Results

Molecular characterization of transgenic *Arabidopsis* lines

Mutants of *Arabidopsis* lacking SOS1 are extremely salt sensitive and have combined defects in Na⁺ extrusion and in control of long-distance Na⁺ transport (Qiu et al. 2002; Shi et al. 2002). The *Arabidopsis sos1-1* mutant bears a 14-bp gene deletion that completely abrogates AtSOS1 activity (Shi et al. 2000; Qiu et al. 2002). The durum wheat Na⁺/H⁺ antiporter TdSOS1 (GenBank accession no. EU552490) and its hyperactive form TdSOS1Δ972 were previously characterized (Feki et al. 2011). The difference in the protein structure between these two wheat SOS1 forms is that the hyperactive protein has a shorter C-terminal tail from which the auto-inhibitory domain (AID) and SOS2 phosphorylation site (S2P) have been removed (Fig. 1). To investigate whether the truncated form of durum wheat SOS1 protein complemented the *Arabidopsis sos1-1* mutant, two binary vectors BSOS and BSOSH containing *TdSOS1* or *TdSOS1*Δ972, respectively, were constructed. These vectors contain the *NPTII* gene conferring resistance to kanamycin as a marker for plant transformation. After *Agrobacterium*-mediated transformation of *Arabidopsis* plants and selection with kanamycin, several transformants were produced. For each transformation, four transgenic lines were propagated to the T3 generation from which homozygous plants were isolated for further analyses. The T-DNA region of these two constructs is schematically presented in Figure 2a. Using *TdSOS1* and *TdSOS1*Δ972 specific primers, a fragment of the T-DNA was amplified to verify the presence of the transgene, and the *sos1-1* genetic background was verified by diagnostic PCR of the 14-bp deletion that defines this mutant allele (data not shown).

The expression level of *TdSOS1* and *TdSOS1*Δ972 was analyzed using reverse transcriptase polymerase chain reaction (RT-PCR), performed on young leaves of eight transgenic lines and non-transformed plants. As a control for cDNA amplification, the constitutively expressed *β-tubulin* gene was amplified. As shown in Figures 2b and 2c, specific bands of the expected sizes were obtained for *TdSOS1* transgenic lines (MW1, 7, 8 and 9) and for *TdSOS1*Δ972 transgenic lines (MH1, 3, 4 and 6). However, no amplification was obtained in the case of non-transformed plants (NT). The expression level was almost similar for lines expressing *TdSOS1* or *TdSOS1*Δ972 except MH3 line (Fig. 2b, c). The higher expression level of *TdSOS1*Δ972 observed in line MH3 was probably due to multiple insertions since the *NPTII* marker failed to segregate as a single locus; because of this the MH3 line was not used for further analyses. For other transgenic lines, the corresponding mRNA was stably produced through generations T0-T3. For physiological analyses, we selected lines MW1, MW7 and

MW8 for transgenic plants harboring *TdSOS1* gene, and MH1, MH4 and MH6 for transgenic plants harboring *TdSOS1Δ972*. For these selected lines, genetic segregation data performed using *NPTII* gene gave rise to a 3:1 ratio confirming that this marker segregates as a single copy gene.

Seed germination under salt stress condition

The effect of NaCl on seed germination on MS medium was investigated in the transgenic MW1, MW7, MH1 and MH6 lines and *Arabidopsis sos1-1* mutant transformed with the empty binary vector (pBI line) or with the endogenous *AtSOS1* gene (AtS line). The germination rate of these transgenic lines was not affected by 35 mM NaCl concentration. However, while the transgenic lines expressing durum wheat SOS1 proteins kept growing, seedlings of the pBI line stopped the growth after 7 days of germination in this medium. At 50 mM NaCl, the pBI line failed to germinate at all while *TdSOS1Δ972* transgenic lines presented a higher germination rate (almost 90 %) than *TdSOS1* transgenic lines and AtS lines ($\approx 60\%$) (Fig. 3a). These data show that both wheat alleles, *TdSOS1* and *TdSOS1Δ972*, are functional *in planta* and that the hyperactive form *TdSOS1Δ972* indeed confers a better germination rate than the wild-type *TdSOS1* form and than *AtSOS1*.

The hyperactive form of durum wheat SOS1 confers better ionic stress tolerance to *Arabidopsis* than the wild-type form and *AtSOS1*

One-week-old seedlings were transferred to MS agar medium containing NaCl or LiCl, and plant survival was monitored 10 days after. The toxic effect of NaCl in *Arabidopsis sos1-1* mutant transformed with the empty binary vector (pBI line) was observed by the inhibition of growth. In the presence of NaCl, phenotypic suppression of the *Arabidopsis sos1-1* mutant by durum wheat *TdSOS1* was partial because of the greater growth of congenic lines transformed with *Arabidopsis AtSOS1* (AtS line). By contrast, *TdSOS1Δ972* transgenic lines (MH lines) showed a significantly better salt tolerance phenotype than AtS line in the presence of high concentration of NaCl (75 mM). This concentration was found to be toxic for *Arabidopsis* plants transformed with the wild-type *TdSOS1* form (MW lines) or with the empty binary vector (Fig. 3b).

The *Arabidopsis sos1-1* mutant is hypersensitive to both Na^+ and Li^+ ions (Wu et al. 1996). Li^+ is often used as a Na^+ analog with higher toxicity and hence it can be used at lower concentrations than Na^+ to inhibit plant growth thus avoiding the osmotic effect of high salt concentrations. In medium containing 15 mM LiCl, the toxic effect of Li^+ was significant for

the empty vector pBI line (Fig. 3b). These plants showed chlorotic leaves with clear growth inhibition. The wild-type TdSOS1 protein partially suppressed the Li⁺ sensitivity of the *sos1-1* mutant, albeit less efficiently than the AtSOS1 protein. On the other hand, expression of the TdSOS1Δ972 protein imparted maximal Li⁺ tolerance above the levels achieved by the full-length proteins TdSOS1 and AtSOS1 (Fig. 3b). We quantitated and compared the root elongation of *TdSOS1* and *TdSOS1*Δ972 transgenic lines with that of the *Arabidopsis sos1-1* mutant transformed with endogenous *AtSOS1* gene (AtS line) or with the empty binary vector (pBI line). The root elongation of the pBI line decreased dramatically with the addition of NaCl or LiCl. Roots of *TdSOS1* transgenic lines (MW1 and MW7) showed a modest growth improvement compared to pBI line. By contrast, the *TdSOS1*Δ927 transgenic lines (MH1, MH6) displayed the highest growth rate, which was only slightly affected by the NaCl treatment but reduced about 40-60% by LiCl (Fig. 3c).

A more physiological salt tolerance test was performed using hydroponic cultures. Plants were grown in control nutrient solution for 2-weeks and then the medium was supplemented with 5 mM NaCl or 0.2 mM LiCl. After 13 days, the transgenic line expressing the wild-type TdSOS1 allele (MW1 and MW7) displayed a partial Na⁺- and Li⁺-tolerance phenotype, since it grew better than the pBI line but AtS transgenic plants were bigger under the same conditions. By contrast, the transgenic lines overexpressing the hyperactive form TdSOS1Δ972 (MH1 and MH6) produced the strongest tolerance phenotype in the presence of Na⁺ or Li⁺, demonstrating again that the gain-of-function of the wheat mutant allele is translated into a better salt-tolerance phenotype (Fig. 4a). The salt-induced growth inhibition was analyzed by measuring the total leaf area (TLA) on *Arabidopsis* transgenic lines (MW1, MW7, MH1 and MH6) and the control plants (pBI and AtS) subjected to NaCl and LiCl stress (Fig. 4b). In the absence of stress, similar TLA values were scored for all these lines (≈ 35 mm²). In the NaCl stress condition, MH1 and MH6 lines showed only a slight reduction of TLA relative to control conditions. Moreover, the TLA values registered in MH1 and MH6 lines were higher than those of other lines, including the control line AtS. At 0.2 mM LiCl, the decrease in TLA values was significant for all lines, albeit in the *TdSOS1*Δ972 transgenic lines MH1 and MH6 the TLA was reduced only by 25% alike the control line AtS (Fig. 4b).

We also measured shoots and roots weight of *Arabidopsis* transgenic *TdSOS1* and *TdSOS1*Δ972 lines in comparison with the control plants (pBI and AtS lines). In the absence of stress, similar shoots and roots fresh weight were scored for all plants. At 2.5 mM NaCl, a reduction of ≈ 50% in shoot and root fresh weight was registered in pBI line. However, the reduction in root fresh weight for the two MH lines was slight and no difference was detected

1 in fresh shoot weight compared to control conditions without salt. Concerning the two MW
2 line, only root fresh weight was significantly reduced. At 5 mM NaCl, shoot and root fresh
3 weight was reduced significantly for all the transgenic lines. In fact, the reduction of fresh
4 weight of MW lines was about 50% in shoots and 75% in roots. These reductions were
5 significantly lower for *TdSOS1Δ972* transgenic lines compared to the positive control line
6 AtS (Fig. 4c). Addition of LiCl to the hydroponic solution reduced the shoot fresh weight and
7 root to $\approx 50\%$ for MH1 and MH6 lines. However, shoot and root fresh weight of the MH1 and
8 MH6 lines was still higher than that of *TdSOS1* and AtS transgenic lines (Fig. 4c).
9 Taken together, these results indicate that the wild-type TdSOS1 form of durum wheat SOS1
10 is functional but less efficient than the native AtSOS1 counterpart to complement the
11 *Arabidopsis sos1-1* mutant. This complementation is highly improved by the use of the
12 hyperactive form *TdSOS1Δ972*, which produces plants with better performance under salt
13 stress even when compared with the transgenics that overexpress the wild-type *Arabidopsis*
14 SOS1 protein, suggesting that the truncated wheat SOS1 protein is a superior salt tolerance
15 determinant.

17 **Relative water content of *Arabidopsis* transgenic lines**

18 The relative water content (RWC) of *Arabidopsis* transgenic lines (MW1, MW7, MH1 and
19 MH6) and the control plants (pBI and AtS) subjected to salt stress for 13 days was
20 determined. Values of RWC around 85-95% were found in well-hydrated tissues. A RWC
21 lower than the critical mark of 50% typically results in plant death (Pardo 2010). After
22 application of salt stress, RWC of *TdSOS1* and *TdSOS1Δ972* transgenic plants was higher
23 than 50%. As shown in Fig. 4d, in control conditions, the RWC was almost the same for all
24 tested lines. In the presence of 5 mM NaCl, all transgenic plants showed reduction in their
25 RWC. However, a higher water loss was observed in pBI line compared to the other
26 transgenic lines. The RWC registered in *TdSOS1Δ972* transgenic lines decreased slightly to a
27 value of ≈ 0.8 relative to non-stress conditions. This result indicated a greater water retention
28 capacity in *TdSOS1Δ972* transgenic plants than the other transgenic lines.

30 ***TdSOS1Δ972* transgenic lines accumulate less Na^+ than transgenic lines expressing wild- 31 type SOS1 proteins**

32 The main function of SOS1 is to maintain a favorable Na^+/K^+ ratio in plants under saline
33 stress (Pardo 2010). It has been shown that *sos1-1* mutant plants accumulate Na^+ , inducing K^+
34 loss, and conversely plants overexpressing SOS1 decrease internal Na^+ levels (Wu et al. 1996;

Shi et al. 2003). We analyzed Na⁺ and K⁺ contents in shoots and roots of transgenic plants (MW1, MW7, MH1 and MH6 lines) and in control plants (pBI and AtS lines), subjected to salt stress (5 mM NaCl) in hydroponic solution for 13 days. Contents were determined on a dry matter basis and hence they were independent of the RWC of the samples. As shown in Fig. 5, under control conditions, the Na⁺ and K⁺ contents were almost the same in shoots and roots for all the transgenic plants. When grown at 5 mM NaCl, the Na⁺ content increased in shoots and roots of these plants and especially for *Arabidopsis sos1-1* mutant transformed with the empty binary vector (pBI line). As expected, all other transgenic lines accumulated less Na⁺ in shoot and root than pBI line. However, the Na⁺ content in MW1 and MW7 lines was higher than MH1 and MH6 lines (Fig. 5a), reflecting the different activity of the durum wheat wild-type protein and the hyperactive one. In the presence of 5 mM NaCl, the K⁺ content decreased significantly in shoot and root of pBI line. K⁺ content of *TdSOS1* transgenic lines was slightly higher than in the pBI line in both shoot and root. The retention of K⁺ in shoot and root of *TdSOS1Δ972* transgenic line was significantly higher due to the lower level of Na⁺ accumulation (Fig. 5b). *Arabidopsis sos1-1* transformed with *AtSOS1* gene showed intermediates values of Na⁺ and K⁺ to those observed in *TdSOS1* and *TdSOS1Δ972* transgenic lines (Fig. 5).

Discussion

Arabidopsis sos1-1 mutant plants have been shown to display sensitivity to both Na⁺ and Li⁺ ions (Wu et al. 1996; Shi et al. 2000). Recently, the durum wheat SOS1 has been functionally characterized in a yeast strain lacking endogenous Na⁺ efflux proteins. The auto-inhibitory domain at the C-terminus of TdSOS1 regulates its activation by the protein kinase SOS2. The truncation of the auto-inhibitory domain in the TdSOS1Δ972 form bypasses the need of activation by *Arabidopsis thaliana* SOS2-SOS3 complex and becomes a constitutively active exchanger (Feki et al. 2011). Over-expression of *SOS1* has been shown to enhance the salt tolerance of transgenic *Arabidopsis* (Shi et al. 2003) and tobacco plants (Yue et al. 2011). In this work, we addressed the question of whether the hyperactive form of TdSOS1 was a superior salt tolerance determinant compared to wild-type TdSOS1 protein. To this end, we generated *Arabidopsis sos1-1* mutant transgenic plants expressing *TdSOS1* or *TdSOS1Δ972*. Firstly, our data showed that expression of the wild-type protein TdSOS1 slightly complemented the Na⁺ and Li⁺ sensitivity of *Arabidopsis sos1-1* mutant (Fig. 3, 4). Notwithstanding with these results in planta, TdSOS1 behaved as functionally equivalent to

the *Arabidopsis* AtSOS1 protein in the yeast system (Feki et al. 2011). Alike AtSOS1 (Qiu et al. 2002; Shi et al. 2002), OsSOS1 (Martínez-Atienza et al. 2007) and TaSOS1 (Xu et al. 2008), the TdSOS1 protein catalyzed Na^+/H^+ antiport in yeast (Feki et al. 2011). The functional analysis in the yeast system also showed that the *Arabidopsis* SOS2-SOS3 complex recognized and activated in vivo the wild-type TdSOS1. Similar results were reported with the rice OsSOS1 homologue, which complemented the *sos1-1* mutant of *Arabidopsis* albeit less efficiently than the endogenous AtSOS1 (Martínez-Atienza et al. 2007). By contrast, complementation of the *sos2* and *sos3* mutants with the corresponding OsCIPK24 and OsCBL4 proteins of rice produced near wild-type tolerance to NaCl (Martínez-Atienza et al. 2007). Thus, it appears that activation of heterologous SOS1 proteins expressed in *Arabidopsis* requires of additional event(s) besides phosphorylation by the SOS2-SOS3 complex. One of such regulatory events could be phosphorylation by MPK6 (Yu et al. 2010; Ji et al. 2013). Deletion of the C-terminal auto-inhibitory domain of TdSOS1 either bypasses this requirement or compensates for it owing to the derepressed activity of *TdSOS1Δ972*.

Recently, Feki and colleagues (2011) demonstrated that in yeast the TdSOS1 protein catalyzes efficiently not only Na^+ efflux, but also Li^+ efflux (Feki et al. 2011). This is in contrast to *Arabidopsis*, in which SOS1 appears to have a preference for Na^+ transport over Li^+ , while the shorter but highly related protein AtNHX8 catalyzes specific Li^+/H^+ antiporter. Overexpression of *AtNHX8* complemented the sensitivity to Li^+ , but not to Na^+ , of the *sos1-1* mutant (An et al. 2007). No protein homologous to AtNHX8 has been found in the sequenced genomes of cereals. Here, we confirmed that TdSOS1 is a dual Na^+ , Li^+/H^+ antiporter in planta since *TdSOS1Δ972* transgenic plants showed a tolerance phenotype in medium containing toxic concentration of Na^+ or Li^+ .

Secondly, the tolerance was significant greater for transgenic plants expressing the hyperactive form TdSOS1Δ972 than those who expressed the wild -type form TdSOS1 or AtSOS1. This tolerance was illustrated by both higher seed germination and growth rate in medium containing toxic concentration of Na^+ or Li^+ . It has been demonstrated that the hyperactive form TdSOS1Δ972 confers a significant tolerance to the transformed yeast grown in medium containing high concentration of Na^+ or Li^+ . Yeast cells transformed with the hyperactive form TdSOS1Δ972 showed a vigorous growth independently of the presence or not of the *Arabidopsis thaliana* SOS2-SOS3 protein kinase complex (Feki et al. 2011). This finding can explain the significant tolerance of *TdSOS1Δ972* transgenic *Arabidopsis* lines.

Under the salinity conditions used in this study, the difference in RWC in the control and transgenic plants suggest the possibility of a higher uptake of water and maintenance of turgor for *TdSOS1Δ972* transgenic plants. Increased turgor can allow for maintenance of greater stomatal aperture (hence CO₂ supply to the leaf) as well as increased cell division and expansion (hence leaf growth and development). On the other hand, *TdSOS1Δ972* transgenic plants retained less Na⁺ and more K⁺ than the other transgenic plants (Fig. 5). Maintenance of appropriate intracellular K⁺/Na⁺ balance is critical for metabolic function as Na⁺ cytotoxicity is largely due to competition with K⁺ for binding sites in enzymes essential for cellular functions (Serrano 1996; Maathuis and Amtmann 1999). K⁺ is an essential macronutrient that is required for diverse cellular processes such as osmotic regulation, enzyme activity, protein and starch synthesis, respiration and photosynthesis (Hauser and Horie 2010). The capacity to maintain a low Na⁺ concentration or a high K⁺/Na⁺ ratio is considered as an indicator of potential salinity tolerance (Tester and Davenport 2003; Volkov et al. 2004; Hauser and Horie 2010).

Although considerable progress has been made in our current understanding of plant adaptation to environmental stress tolerance, the goal of improving the resistance of crops to abiotic stresses has seen limited success (Pardo 2010). Numerous genes and proteins have been shown to effect tolerance to environmental stress in an array of plant species, but their implementation into improved crops largely rely on genetic modifications that face administrative hurdles and negative public perception. The use of hardy wild relatives or relatively stress tolerant varieties of crop plants as a source of genetic determinants of stress tolerance is time-consuming and labor intensive. Moreover, classical breeding approaches have revealed that stress tolerance traits are dispersed in various quantitative trait loci (QTLs), which make genetic selection of these traits difficult while undesirable genes are often transferred in combination with desirable ones. Our results demonstrated that the wild-type or the hyperactive form of durum wheat SOS1 suppressed the ionic sensibility phenotype of *Arabidopsis sos1-1* mutant. These two TdSOS1 variants could substitute the endogenous protein AtSOS1 and consequently regulate the Na⁺ and K⁺ homeostasis in cells. Unlike the wild-type form, the hyperactive form TdSOS1Δ972 conferred a significant ionic stress tolerance to the transgenic *Arabidopsis*. Thus, this hyperactive SOS1 form can be considered as a salt tolerance determinant and it should be feasible to screen for this or synonymous mutations in a mutagenized population of a crop by TILLING as a way to create salt tolerant crops that are not considered GMO.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Figure 1. Schematic representation of SOS1 protein structure. TdSOS1 has a pore domain with 12 transmembrane regions, followed by a long hydrophilic tail containing a putative cyclic nucleotide binding domain (CNBD), an autoinhibitory domain (AID) and the SOS2 phosphorylation site (S2P). The TdSOS1 Δ 972 protein has a shorter C-terminal domain than the wild-type TdSOS1, which contains only CNBD domain. Numbers present the position of amino acid residues delimiting each domain.

Figure 2. Molecular analysis of *TdSOS1* and *TdSOS1* Δ 972 transgenic plants. **(a)** Physical map of T-DNA region in the binary vectors BSOS and BSOSH. The transgene was inserted between the CaMV35S promoter (P35S) and NOS terminator (TNOS); the *NPTII* marker is flanked by the NOS promoter (PNOS) and terminator (TNOS). KM1, KM2, KH1 and KH2 indicate the primers used for RT-PCR amplification. Expression analysis by RT-PCR of *TdSOS1* in *TdSOS1* transgenic lines (MW) **(b)** and of *TdSOS1* Δ 972 in *TdSOS1* Δ 972 transgenic lines (MH) **(c)**. Specific PCR products of 0.6 kb for MW transgenic lines and 0.8 kb for MH transgenic lines were detected, which were absent in non-transformed (NT) *Arabidopsis sos1-1* mutant. A 0.5 kb of β -tubulin gene fragment was amplified by RT-PCR as an internal control. (-): negative control without cDNA.

Figure 3. Salt tolerance phenotypes of transgenic lines. **(a)** Seeds of control lines (*Arabidopsis sos1-1* mutant transformed with the empty binary vector pBI321 (pBI), or with the endogenous *AtSOS1* gene (AtS)), the *TdSOS1* Δ 972 transgenic lines (MH1 and MH6) and the *TdSOS1* transgenic lines (MW1 and MW7) were germinated directly on MS medium, and on MS medium supplemented with 35 mM NaCl or 50 mM NaCl, and then grown for 7 days. **(b)** Effect of stress treatments on the growth of *TdSOS1* transgenic lines (MW1, MW7 and MW8), *TdSOS1* Δ 972 transgenic lines (MH1, MH4 and MH6) and *Arabidopsis sos1-1* transformed with the empty binary vector (pBI) or with the endogenous *AtSOS1* gene (AtS). Each line were germinated on MS agar medium for 7 days and then transferred to MS medium, and to MS medium supplemented with 35 mM NaCl, 75 mM NaCl or with 15 mM LiCl. Photographs were taken 10 days after the transfer. **(c)** Root elongation of *TdSOS1* transgenic lines (MW1 and MW7), *TdSOS1* Δ 972 transgenic lines (MH1 and MH6), pBI and AtS lines, on MS medium containing different concentrations of NaCl or LiCl. Values are

means $\pm$ SE (n = 4). Asterisks indicate statistically significant greater mean values compared to *Arabidopsis* transformed with the endogenous *AtSOS1* gene (AtS line) ($P < 0.05$).

Figure 4. Stress tolerance of transgenic lines grown in hydroponic culture. **(a)** Seeds of *TdSOS1* transgenic lines (MW1 and MW7) and *TdSOS1Δ972* transgenic lines (MH1 and MH6) were germinated in distilled water solution for one week and then transferred to nutritive solution, and nutritive solution supplemented with 5 mM NaCl or 0.2 mM LiCl. The response to ionic stress was evaluated in comparison to *Arabidopsis sos1-1* mutant transformed with the empty vector (pBI) or with the endogenous *AtSOS1* gene (AtS). Photographs were taken after 13 days in the hydroponic culture. **(b)** Total leaf area (TLA) of individual leaves from the control (pBI and AtS) and transgenic (MW1, MW7, MH1 and MH6) *Arabidopsis* plants. Values are means of five replicates of one fully expanded leaf per plant. **(c)** Relative water content (RWC) of transgenic and control plants after 13 days in salt stress (5 mM NaCl) on hydroponic solution. **(d)** Shoots and roots fresh weight of *Arabidopsis sos1-1* transgenic lines after 13 days in nutritive solution containing 2.5 or 5 mM NaCl (left) or 0.2 mM LiCl (right). Values are means $\pm$ SE (n = 5). Asterisks indicate significantly greater mean values compared to *Arabidopsis* transformed with the endogenous *AtSOS1* gene (AtS line) ($P < 0.05$).

Figure 5. Ion contents. Sodium **(a)** and potassium **(b)** contents in shoots and roots of transgenic and control plants grown in hydroponic culture under salt treatment (5 mM NaCl) for 13 days. Values are means $\pm$ SE (n = 4). Asterisks indicate statistical differences of either smaller sodium contents or greater potassium contents in the transgenic lines compared to the control *Arabidopsis* line transformed with the endogenous *AtSOS1* gene (AtS line) ($P < 0.05$).

Figures

Fig. 1

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