Regulation of durum wheat Na^+/H^+ exchanger TdSOS1 by phosphorylation

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Abstract We have identified a plasma membrane Na^+/H^+ exchanger from durum wheat, designated TdSOS1. Heterologous expression of TdSOS1 in a yeast strain lacking endogenous Na^+ efflux proteins showed complementation of the Na^+- and Li^+-sensitive phenotype by a mechanism involving cation efflux. Salt tolerance conferred by TdSOS1 was maximal when co-expressed with the *Arabidopsis* protein kinase complex SOS2/SOS3. In vitro phosphorylation of TdSOS1 with a hyperactive form of the *Arabidopsis* SOS2 kinase (T/DSOS2Δ308) showed the importance of two essential serine residues at the C-terminal hydrophilic tail (S1126, S1128). Mutation of these two serine residues to alanine decreased the phosphorylation of TdSOS1 by T/DSOS2Δ308 and prevented the activation of TdSOS1. In addition, deletion of the C-terminal domain of TdSOS1 encompassing serine residues at position 1126 and 1128 generated a hyperactive form that had maximal sodium exclusion activity independent from the regulatory SOS2/SOS3 complex. These results are consistent with the presence of an auto-inhibitory domain at the C-terminus of TdSOS1 that mediates the activation of TdSOS1 by the protein kinase SOS2. Expression of *TdSOS1* mRNA in young seedlings of the durum wheat variety Om Rabia3, using different abiotic stresses (ionic and oxidative stress) at different times of exposure, was monitored by RT–PCR.

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Introduction

Salinization of cropland in the Mediterranean region is a major limitation to crop yields. High concentration of sodium (Na^+) in the soil solution impairs cell metabolism and photosynthesis by imposing an osmotic stress on cell water relations and by the toxicity of Na^+ in the cytosol. To prevent the accumulation of Na^+ in the cytoplasm, plants have developed three mechanisms that function in a cooperative manner, i.e., restriction of Na^+ influx, active Na^+ extrusion at the root-soil interface, and compartmentalization of Na^+ in the vacuole (Tester and Davenport 2003). The HKT family of Na^+ transporters and Na^+/K^+ symporters control Na^+ transport in higher plants and quantitative genetics (QTL) approaches have demonstrated that natural variation in the activity or expression of HKT-like transporters is a genetic resource for enhanced salinity tolerance in cereals (Tester and Davenport 2003; Horie et al. 2009; Pardo 2010). Bread wheat (*Triticum aestivum*) cultivars are able to exclude Na^+ from leaves but most durum wheat (Tri*ticum turgidum* L. subsp. *durum*) cultivars lack this trait (Dubcovsky et al. 1996). The hexaploid bread wheat has three genomes, A, B and D, whereas the tetraploid durum wheat has only the A and B genomes. The *Kna1* locus on chromosome 4DL of bread wheat has been linked to Na^+ exclusion and improved salt tolerance (Dvorák et al. 1994; Shah et al. 1987). Recently, QTL analyses in novel durum wheat varieties derived from a cross with the wheat ancestor *Triticum monococcum* (A genome) identified two loci, *Nax1* and *Nax2*, underpinning

reduced Na^+ accumulation in the leaf blade by two different mechanisms (James et al. 2006). The process controlled by *Nax2* involved reduced Na^+ translocation from root to shoot, while *Nax1* enhanced the retention of Na^+ in the leaf sheath, thereby restricting the passage to the leaf blade (James et al. 2006). High-resolution mapping and sequencing analyses suggested that the *Nax1* and *Nax2* loci are polymorphic *HKT* genes of wheat (Huang et al. 2006; Byrt et al. 2007). The *Nax2* region on 5AL is homoeologous to the region on chromosome 4DL containing the major Na^+ exclusion locus in bread wheat, *Kna1*.

Sodium extrusion at the root-soil interface, as well as some level of Na^+ efflux in every other cell type to achieve ion homeostasis, is presumed to be of critical importance for the salt tolerance of glycophytes (Tester and Davenport 2003; Pardo et al. 2006). In durum wheat roots, high rates of Na^+ efflux were inferred because net uptake was very low relative to unidirectional influx (Davenport et al. 2005). Thus, a very rapid efflux of Na^+ from roots must occur to control net rates of influx. The Na^+/H^+ antiporter SOS1 localized to the plasma membrane is the only Na^+ efflux protein from plants characterized so far. In *Arabidopsis thaliana*, three *salt overly sensitive* genes *SOS1*, *SOS2* and *SOS3* have been found to function in a common pathway (Zhu 2000). Based on sequence analysis, the predicted *SOS1* gene product is a 127-kDa membrane protein with 12 putative membrane-spanning domains and a long hydrophilic C-terminal tail in the cytoplasm (Shi et al. 2000). The *SOS1* locus is necessary for the maintenance of ion homeostasis in saline stress condition. Mutants of *Arabidopsis thaliana* lacking SOS1 are extremely salt sensitive and have combined defects in Na^+ extrusion and in controlling long-distance Na^+ transport from roots to shoots (Qiu et al. 2002; Shi et al. 2002). Under salt or oxidative stress, SOS1 interacts through its predicted cytoplasmic tail with RCD1 (radical-induced cell death), a regulator of oxidative stress responses, and functions in oxidative stress tolerance in *Arabidopsis* (Agarwal et al. 2006). Na^+ efflux by the SOS1 exchanger is regulated through protein phosphorylation by two alternative protein kinase complexes, the SOS2/SOS3 complex (Qiu et al. 2002; Quintero et al. 2002), and SOS2/SCaBP8 (Quan et al. 2007). SOS3 is a myristoylated calcium-binding protein capable of sensing Ca^{2+} oscillations elicited by salt stress (Liu and Zhu 1998). The myristoylation and calcium binding are essential for its function (Ishitani et al. 2000). SOS2 is a serine/threonine protein kinase belonging to the SNF1-related kinase 3 (SnRK3) family (Liu et al. 2000; Gong et al. 2002). Upon Ca^{2+} binding SOS3 undergoes dimerization, recruits SOS2 to the plasma membrane and achieve efficient interaction with SOS1 (Guo et al. 2001; Quintero et al. 2002; Sanchez-Barrena et al. 2005). The interaction with SOS3 occurs within the FISL motif localized in the regulatory domain of SOS2 (Guo et al. 2001). The FISL domain is itself inhibitory and its deletion results in constitutive activation of SOS2 (Guo et al. 2004). Besides its role in the SOS pathway, SOS2 regulates other ion transport processes thought to be instrumental for salt tolerance. SOS2 interacts with subunits forming the cytoplasmic sector of the V-ATPase and this interaction is enhanced under salt stress conditions (Batelli et al. 2007). Using a yeast growth assay, co-expression of SOS2 specifically activated CAX1, whereas SOS3 did not, suggesting that SOS2 regulates the vacuolar Ca^{2+}/H^+ antiporter CAX1 in salt stress (Cheng et al. 2004). Moreover, it has been shown that mutation of *SOS1* or *SOS2* suppressed both cortical microtubule disruption and helical growth of the *spiral 1* mutant of *Arabidopsis thaliana* (Shoji et al. 2006). Transgenic *Arabidopsis* plants overexpressing *AtSOS1* showed enhanced salt tolerance (Shi et al. 2003), similarly to the tolerance of transgenic plants overexpressing the complete set of SOS proteins, SOS1, SOS2, and SOS3 (Yang et al. 2009). In *Oryza sativa*, the functional homologs of the *Arabidopsis* protein kinase SOS2 and its Ca^{2+}-dependent activator SOS3 were identified (Martinez-Atienza et al. 2007). In barley, the *HvNax4* locus lowered shoot Na^+ content to different extents depending on the growth conditions. *HvCBL4*, a close barley homologue of the *SOS3* gene of *Arabidopsis*, co-segregated with the *HvNax4* locus (Rivandi et al. 2011). These results indicate that Na^+ efflux mediated by the ubiquitous SOS system is also a potential source of salinity tolerance in cereals.

In a previous report, the cloning of SOS1 from bread wheat was described and Na^+/H^+ exchange activity was demonstrated in purified plasma membrane vesicles from yeast cells expressing TaSOS1 (Xu et al. 2008). In this study, we describe the molecular cloning of durum wheat *TdSOS1* cDNA and its functional characterization using heterologous expression in a salt-sensitive yeast mutant. Further, we show that the activation mechanism of TdSOS1 involves the phosphorylation and inactivation of an auto-inhibitory domain located at the C-terminal end of the transporter. This region contains the essential residues for its phosphorylation by the *Arabidopsis* protein kinase SOS2. The expression of the *TdSOS1* gene was also analyzed in wheat plants under abiotic stress.

Materials and methods

Plant material and stress treatments

Seeds of durum wheat (*Triticum turgidum* L. subsp. *durum*) cultivar Om Rabia3 were supplied by INRAT, Laboratoire de Physiologie Végétale (Tunis, Tunisia). Seeds were

sterilized in 0.5% NaClO solution for 15 min, then washed three times with sterile water and germinated on Petri dishes with a single sheet of wet Whatman #1 filter paper. Seven-days-old seedlings were transferred to containers with modified half-strength Hoagland's solution (Epstein 1972). Seedlings were grown in a greenhouse at 25 ± 5° C, under photosynthetically active radiation of 280 μmol m^{-2} s^{-1}, 16 h photoperiod and 60 ± 10% relative humidity. When plants reached the third-leaf stage, different stress conditions (200 mM NaCl and 10 mM H_20_2) were applied and samples were taken at different time of exposure (0, 3, 6, 14 and 24 h), the roots and the shoots were collected, blotted on filter paper and frozen immediately into liquid nitrogen.

Isolation and cloning of the *TdSOS1* cDNA

Total RNA was extracted from 100 mg of frozen leaves of 10 days-old wheat seedlings (*Triticum durum* cultivar Om Rabia3) using the TRIZOL reagent (Invitrogen). The full-length cDNA of *TdSOS1* was synthesized from the RNA with the SMARTer^{TM} RACE cDNA amplification kit (Clontech) following the protocol provided by the manufacturer. The open reading frame of *TdSOS1* was amplified by PCR with primers SOSXba (5′-GTGTCTAGAATG GAGACGGAGGAGGCCGGCTCCC-3′, X*ba*I site underlined) and SOSKpn (5′-ATGGTACCTCAGCTGCCTCGC GGTGGGCCGGA-3′, K*pn*I site underlined). The resulting fragment was cloned into the X*ba*I and K*pn*I sites of the yeast expression shuttle vector pYPGE15 (Brunelli and Pall 1993) generating plasmid pTdSOS1-1.

To introduce the point mutations S1126A and S1128A in the TdSOS1 sequence a site-directed mutagenesis by PCR-driven overlap extension (Heckman and Pease 2007) was performed using mutagenic primers AA3 (5′-GAG CATGGCGGGAGCGTCCACTCTGACGATGAC-3′) AA5 (5′-GTGGACGCTCCCGCCATGCTCTCGTTCAATCCA-3′) and flanking primers TBF (5′-CTATGCACGAGGCATTC ATTGGAT) and SOSKpn. First, two independent PCR reactions were carried out with primers TBF together with AA3, or SOSKpn with AA5 to generate intermediate PCR products that overlapped in the sequence mutated. These PCR products were denatured and used as template for a second PCR; strands of each product hybridize at their overlapping complementary regions. Amplification of the final product was driven by primers TBF and SOSKpn. Finally this PCR product was cut with B*amH*I and K*pn*I and ligated to plasmid pTdSOS1-1 cut with the same restriction enzymes, replacing the equivalent fragment in the wild-type cDNA. The resulting plasmid was named pTdSOS1-2.

To produce the truncated *TdSOS1Δ972* allele, a sequence encoding from amino acids 775–972 of TdSOS1 was amplified by PCR with the primers TBF (5′-CTA TGCACGAGGCATTCATTGGAT) and 3′SH (5′-TAGG TACCTCAGAGGGTTGAAGACAGCAA-3′), the latter harboring a K*pn*I restriction site plus stop codon. The amplified fragment was digested with B*amH*I/K*pn*I and cloned in pTdSOS1-1 digested with the same enzymes, resulting in the recombinant plasmid pTdSOS1-3. All the PCR products were fully sequenced.

Yeast expression plasmid pFL32T containing the *Arabidopsis* SOS2 and SOS3 genes and the corresponding empty plasmid pFL have been previously described (Quintero et al. 2002).

Yeast strains and media

The salt sensitive yeast strain AXT3K *(Δena1::HIS3::ena4, Δnha1::LEU2, Δnhx1::KanMX4)* (Quintero et al. 2002) was used to test the function of *TdSOS1* alleles. The GX1 yeast strain *(Δena1::HIS3::ena4, Δnhx1::TRP1)* (Shi et al. 2002) was used as positive control for high potassium tolerance test. Strain GRF167 (*ura3-167, his3Δ200*) (Boeke et al. 1988) was selected for expression of GST fusion proteins. In all cases, yeast cells were transformed using the standard polyethylene glycol (PEG) lithium acetate method (Elble 1992). Transformants were selected for the appropriate prototrophy by plating on SD standard medium. For cation tolerance test, aliquots from saturated yeast cultures (5 μl) and tenfold serial dilutions were spotted onto AP plates (Rodriguez-Navarro and Ramos 1984) containing 1 mM KCl, and supplemented with NaCl or LiCl.

Total intracellular ion content

Yeast cells were suspended in AP medium supplemented with 1 mM KCl and, at intervals after the addition LiCl or NaCl, samples were taken, filtered through 0.8 μm-pore nitrocellulose membrane filters (Millipore) and washed with 20 mM $MgCl_2$. Filters were incubated overnight in 0.1 M HCl, and ionic content was determined by atomic emission spectrophotometry of acid-extracted cells (Haro and Rodríguez-Navarro 2002).

Expression and purification of TdSOS1 peptides

DNA fragments encoding the C-terminal region (residues 1,010–1,142) of the wild-type protein or the double mutant with S1126A/S1128A substitutions were obtained by PCR using the following primers: 5TS (5′-GCGGATCCTTCT CTGCTAGAGCCTTG) and 3TS (5′-CACAAGCTTCA GCTGCCTCGCGGTGG) harboring B*amH*I and H*ind*III restrictions sites, respectively. The PCR products were digested with B*amH*I and H*ind*III and were cloned in-frame with GST (glutathione S-transferase) into the

yeast expression vector pEG(KT) (Mitchell et al. 1993) giving rise to the constructs pEG(KT)-DSPS and pEG(KT)-DAPA. Expression of the recombinant peptides was induced with 2% galactose. Cells were collected by centrifugation and lysed with glass beads in PBS buffer (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.4) supplemented with 1% Triton X-100 and the fusion proteins were purified by binding to glutathione-Sepharose beads (GE Healthcare). Protein quantification was performed using the Bradford method (1976).

Phosphorylation assays

The glutathione S-transferase fusion protein GST::SOS2T/DΔ308 obtained from *E. coli* was purified and used as kinase for in vitro phosphorylation assays as described (Guo et al. 2001; Quintero et al. 2002). One-hundred nanograms of peptides GST::TdSOS1(DSPS) or GST::TdSOS1(DAPA) were incubated with and without 100 ng of kinase GST::SOS2T/DΔ308 in 30 µl of kinase reaction (20 mM Tris–HCl pH7.5, 5 mM $MgCl_2$, 1 mM DTT, 10 µM [γ^{-32}P]ATP). This mixture was incubated at 30°C for 30 min and the reaction was stopped by adding 10 µl of 4 × SDS/PAGE sample buffer (8% SDS, 40% glycerol, 0.4% bromophenol blue, 20% β-mercaptoethanol). Aliquots (10 µl) were resolved by SDS–PAGE and exposed to X-ray film.

Semi-quantitative RT–PCR analyses

Total RNA was isolated from frozen roots and shoots of 10 days-old wheat seedlings using the TRIZOL method (Invitrogen). Total RNA (10 µg) was treated with DNaseI (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). The *TdSOS1* gene was amplified using 1 µl of 1/10th dilution of cDNA as template and two specific primers *TdSOS1*; 5′SOS (5′-GCAAGGGCCATCATATTTGAAAT-3′) and 3′SOS (5′-TTTTGAAGTCGCCACAACCT-3′), the last one was designed in 3′UTR region. The wheat's actin gene (GenBank Accession No. AY663392) was used as an internal control for gene expression. The actin primers were AF (5′-CTGACGGTGAGGACATCCAGCCCCTTG-3′) and AR (5′-GCACGGCCTGAATTGCGACGTACATGG-3′). For PCR, samples were denatured for 2 min at 96°C followed by 30 cycles for 30 s at 96°C, 45 s at 55°C and 1 min at 72°C, followed by a final extension of 5 min at 72°C. The PCR products were then separated by electrophoresis in 1% (w/v) agarose gel. For each stress treatment, RT–PCR experiments were repeated three times to validate the results.

Results

Isolation and molecular characterization of wheat *TdSOS1*

Using a RACE-PCR approach, we isolated a full-length cDNA corresponding to *TdSOS1* from durum wheat (*Triticum turgidum* L. subsp. *durum*) cultivar Om Rabia3. Sequence analysis of the *TdSOS1* cDNA revealed an open reading frame of 3,429 kb encoding a protein that shared high sequence homology with the family of SOS1-like plasma membrane Na^+/H^+ antiporters from different plant species (Quintero et al. 2011; Martinez-Atienza et al. 2007). According to this analysis, all the important domains required for the activity and regulation of SOS1 (Quintero et al. 2011) could be identified in TdSOS1 (Fig. 1a). The putative cyclic nucleotide-binding domain that is essential for transport activity is comprised between residues 744 and 831 (Fig. 1b). The putative auto-inhibitory domain of AtSOS1 and the phosphorylation motif recognized by SOS2 were found in the last 163 amino acids of TdSOS1 (Fig. 1c, d). A BLAST search in the GenBank database subset containing 10,652 ESTs from *T. durum* retrieved a single sequence with homology to *TdSOS1*, suggesting the presence of a single *SOS1* locus in the genome of this wheat variety.

Functional characterization of TdSOS1 and activation by the SOS2-SOS3 complex

In order to investigate the regulation of TdSOS1, the full-length cDNA was expressed in the yeast strain AXT3K. This strain lacks the Na^+ efflux proteins ENA1–4 and NHA1 localized at the plasma membrane, the prevacuolar Na^+/H^+ antiporter NHX1, and is unable to grow in the presence of low concentrations of Na^+ or its analogue for ion transport assays, Li^+ (Quintero et al. 2002). Yeast ENA1-4 and NHA1 proteins also mediate K^+ efflux and therefore, AXT3K is sensitive to high K^+ concentrations. Expression of TdSOS1 restored the capacity of yeast cells to grow in the presence of moderate NaCl concentrations, but did not confer resistance to high levels of K^+ in the medium (Fig. 2).

In *Arabidopsis* and rice, SOS1 activity is upregulated by the protein kinase SOS2 and the calcium binding protein SOS3 (Qiu et al. 2002; Quintero et al. 2002; Martinez-Atienza et al. 2007). To test whether TdSOS1 is regulated in a similar way, *Arabidopsis SOS2* and *SOS3* genes were co-expressed in yeast. As shown in Fig. 2, expression of the three SOS proteins enhanced the cellular resistance to Na^+. Unlike the *Arabidopsis* SOS1, TdSOS1 was able to complement the Li^+ sensitivity of AXT3K, suggesting that TdSOS1 can also transport Li^+. All the SOS1 transporters

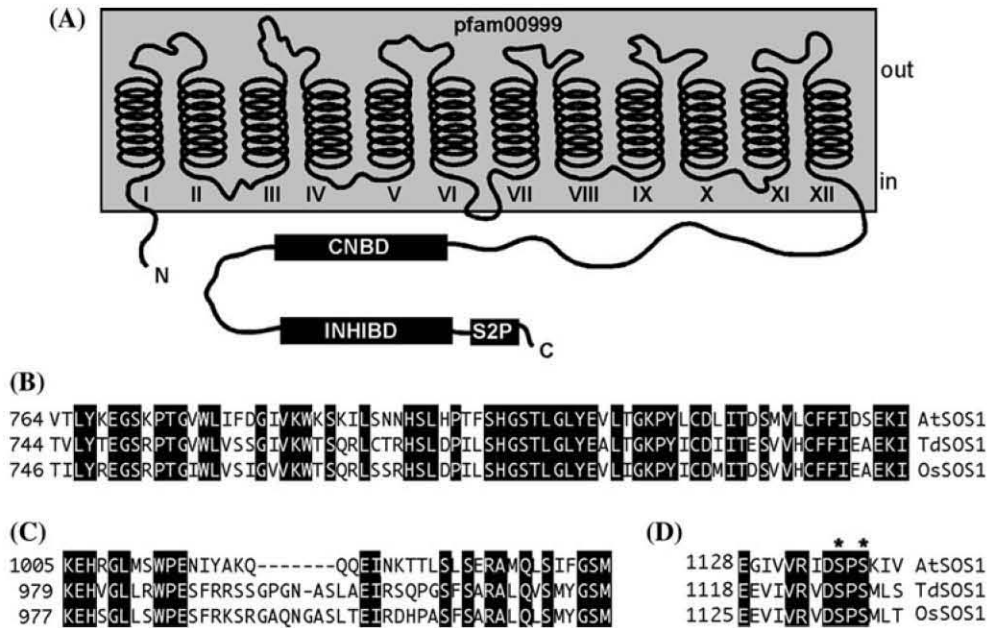

Fig. 1 Predicted topology of TdSOS1 and identification of functional domains. **a** The hydropathy analysis of TdSOS1 protein suggests the presence of the 12 transmembrane regions, which are indicated with *helices*, followed by a long hydrophilic tail. Functional domains are indicated with boxes: Na^{+}/H^{+} exchanger domain (pfam 00999), putative cyclic nucleotide binding domain (*CNBD*), autoinhibitory domain (*INHIBD*) and SOS2 phosphorylation site (*S2P*). Sequence alignment of functional domains in SOS1 proteins. SOS1 sequences of the indicated species were aligned using ClustalW. Shown are the alignments corresponding to the putative cyclic nucleotide binding domain (**b**), the suggested auto-inhibitory domain (**c**), and the SOS2 phosphorylation site, conserved serines are indicated by *asterisks* (**d**). The amino acid residues conserved in the aligned proteins are boxed. Sequence accession numbers: TdSOS1 (ACB47885.1), OsSOS1 (AAW33875.1) and AtSOS1 (AAF76139.1)

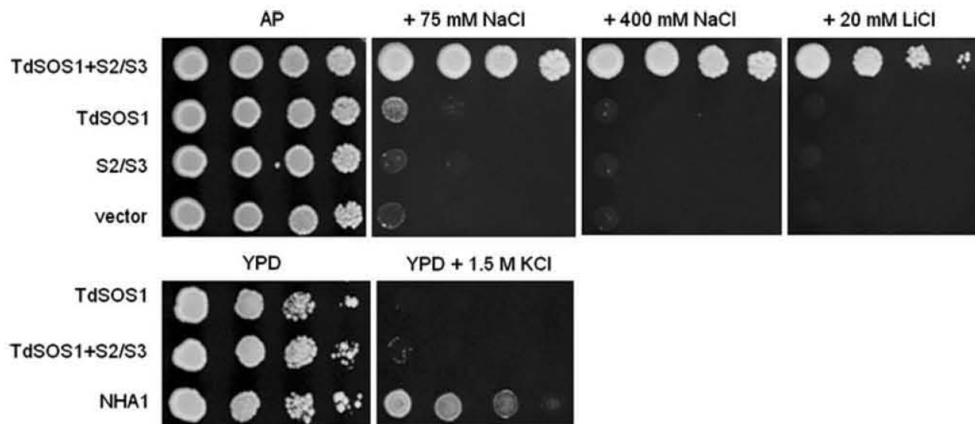

Fig. 2 Functional characterization of wheat TdSOS1. Cells of the AXT3K yeast strain transformed with the empty vectors pYPGE15 and pFL (*vectors*), expressing the kinase complex *SOS2/SOS3* (S2/S3), *TdSOS1* alone (TdSOS1), or co-expressing *TdSOS1* with *AtSOS2* and *AtSOS3* (TdSOS1^{+}S2/S3) were grown overnight. Five microliters of serial decimal dilutions were spotted onto plates of AP medium with 1 mM KCl and supplemented with NaCl (75 and 400 mM) or LiCl (20 mM) and of standard YPD with or without 1.5 M KCl. Plates were incubated at 30°C for 3 days. Experiments shown in this figure were repeated at least three times, with similar results

characterized previously appear to be highly specific for Na^{+} and to have a limited capacity to transport other substrates, including the closely related Li^{+} ion (Quintero et al. 2002 and unpublished results).

To demonstrate that resistance to these cations was due to increased ion efflux driven by TdSOS1, the rate of net ion uptake was measured in AXT3K cells expressing TdSOS1 with and without SOS2-SOS3. The data presented in Fig. 3 show that activated TdSOS1 decreased the net uptake of Na^{+} after 30 min compared to TdSOS1 alone. Similar results were found with Li^{+} uptake rates, in accordance with cellular tolerances (Fig. 2). The observed reduction in net cation uptake is produced by the activation of the Na^{+} efflux, since a reduction of ion influx would

produce instantaneous differences in uptake rates (Haro et al. 1993). Taken together these findings indicate that (i) TdSOS1 mediates the exclusion of Na^+ and Li^+ outside yeast cells, and that (ii) TdSOS1 is a downstream target of the *Arabidopsis* SOS2-SOS3 protein kinase complex.

In vitro phosphorylation of TdSOS1

Activation of *Arabidopsis* and rice SOS1 proteins involves phosphorylation by the SOS2 protein kinase (Quintero et al. 2002; Martinez-Atienza et al. 2007). ClustalW alignment of the C-terminal ends of OsSOS1, AtSOS1 and TdSOS1 proteins showed the conservation of a motif (V,I)(V,I)VR(I,V)DSPS (Fig. 1d) that fits the consensus recognition site for SnRK3/SNF1/AMPK protein kinase family to which SOS2 belongs (Gong et al. 2002). In *Arabidopsis*, two serine residues involved in the phosphorylation of AtSOS1 by the kinase SOS2 were localized at positions S1136 and S1138 embedded in the motif DSPS near the C-terminal end of AtSOS1 (Quintero et al. 2011). To characterize the putative phosphorylation site(s) of wheat TdSOS1, we mutated the two equivalent serine residues in the sequence motif DSPS, i.e. serine S1126 and S1128, to alanine. Recombinant GST-fusion peptides of the last 133 amino acids of TdSOS1 containing either the wild-type sequence (DSPS) or the mutated putative phosphorylation site (DAPA) were purified. To check the purity of the isolated peptides, samples were analyzed by SDS–PAGE electrophoresis. A slight difference in electrophoretic mobility was found between the wild-type and the mutant fusion proteins (Fig. 4a). A possible explanation of this mobility shift is that the wild-type peptide is modified by endogenous yeast kinases. In agreement with this hypothesis, treatment of the DSPS-containing peptide with alkaline phosphatase shifted the band's mobility to that of the mutant peptide DAPA. Wild-type and mutant GST-fusion proteins were subjected to in vitro phosphorylation with the hyperactive form of the *Arabidopsis* SOS2 protein kinase, T/DSOS2Δ308. An intense band corresponding to the phosphorylated form of the wild-type peptide TdSOS1(DSPS) in the presence of the kinase T/DSOS2Δ308 was detected (Fig. 4b). By contrast, phosphorylation of the mutant peptide TdSOS1(DAPA) was dramatically reduced. The bands corresponding to phosphorylated peptides were not detected in the absence of the protein kinase T/DSOS2Δ308. These results indicate that the serine residues in the conserved core sequence DSPS mediate the phosphorylation of TdSOS1 by the kinase SOS2.

To correlate in vitro phosphorylation data with the activity of TdSOS1 in vivo, the mutated phosphorylation site was constructed in the full-length TdSOS1 protein by site-directed mutagenesis as described in Sect. "Materials and methods". In the yeast strain AXT3K, an enhanced tolerance to Na^+ was observed when the wild-type TdSOS1 was co-expressed with the hyperactive kinase T/DSOS2Δ308 (Fig. 5), similarly to the tolerance observed in yeast cells co-expressing wild-type TdSOS1 with the protein kinase complex SOS2-SOS3 (Fig. 2). By contrast, the co-expression of TdSOS1(DAPA) mutant protein with the hyperactive kinase T/DSOS2Δ308 failed to enhance the Na^+ and Li^+ tolerance of the yeast mutant AXT3K (Fig. 5), demonstrating the importance of the two serine residues S1126 and S1128 in the phosphorylation of wheat TdSOS1 and the up-regulation of its transport activity.

Identification of a hyperactive form of TdSOS1

Recently, the presence of an auto-inhibitory C-terminal domain has been demonstrated in the *Arabidopsis* SOS1 protein (Quintero et al. 2011). Phosphorylation at the core motif DSPS relieved AtSOS1 from auto-inhibition.

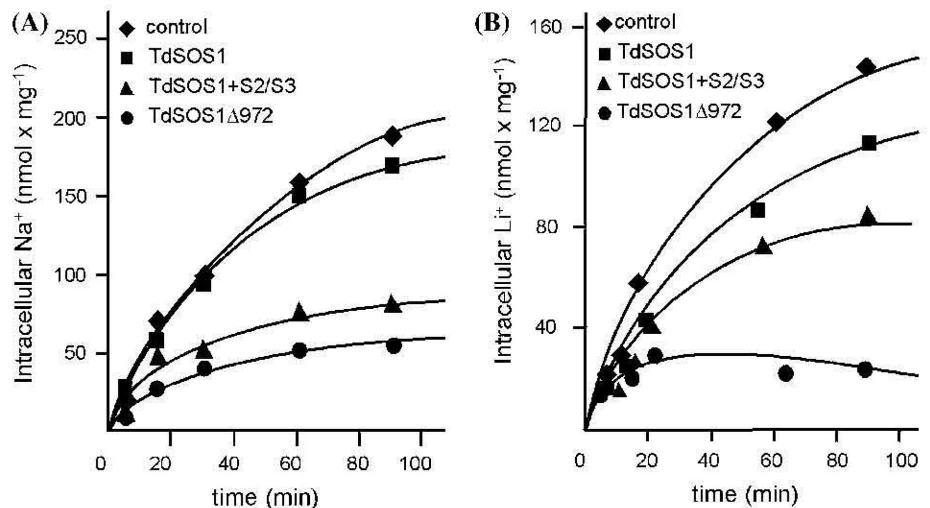

Fig. 3 Ion uptake in yeast cells expressing *TdSOS1* (*squares*), *TdSOS1* and *AtSOS2/AtSOS3* (triangles), *TdSOS1Δ972* (*circles*), or transformed with an empty vector (*diamonds*). Na^+ (**a**), and Li^+ (**b**) contents were measured by atomic emission spectrometry in yeast cells incubated in the presence of 50 mM LiCl and 200 mM NaCl, respectively, for the indicated time intervals

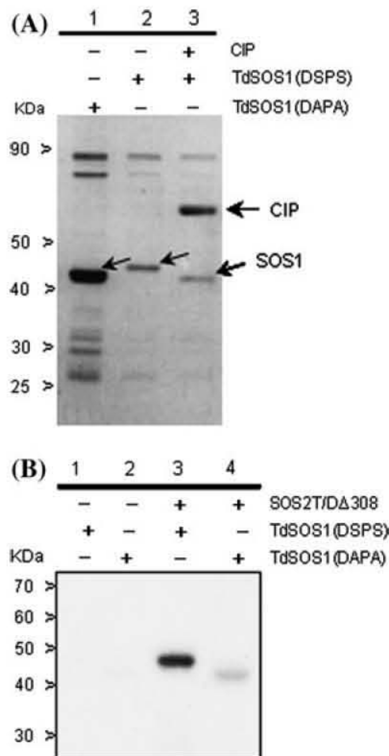

Fig. 4 Phosphorylation of wheat TdSOS1 by the *Arabidopsis* kinase T/DSOS2Δ308. **a** Purification of the TdSOS1 tail (amino acids 1,010–1,142) fused to GST. Aliquots of purified peptides were analyzed by SDS–PAGE and Coomassie stained. *Lane 1*: GST::TdSOS1(DAPA), *lane 2*: GST::TdSOS1(DSPS), and *lane 3*: GST::TdSOS1(DSPS) treated with calf intestinal alkaline phosphatase (*CIP*). Protein bands corresponding to GST::TdSOS1 are indicated by *arrows*. **b** GST::TdSOS1 fusion proteins were incubated with [γ-32P]ATP in the presence or absence of the recombinant protein kinase T/DSOS2Δ308. Aliquots of the reactions were resolved by SDS–PAGE and the gel was exposed to X-ray film. *Lanes 1* and *3* show the GST::TdSOS1(DSPS) protein without or with the kinase T/DSOS2Δ308, respectively. *Lanes 2* and *4* the GST::TdSOS1 (DAPA) protein without or with the kinase, respectively. Molecular masses are indicated on the *left*

Therefore, we deleted 170 amino acids at the end of the C-terminal part of TdSOS1 spanning the phosphorylation motif (residues 1,118–1,131; Fig. 1d) and the adjacent upstream stretch (residues 979–1,025) that show significant sequence conservation with the region that is thought to encompass the auto-inhibitory domain of AtSOS1 (Fig. 1c). This truncated form of the wheat protein TdSOS1Δ972 could complement the sensitivity of the yeast strain AXT3K in medium containing up to 400 mM NaCl or 20 mM LiCl and supported vigorous growth independently of the presence or not of the *Arabidopsis thaliana* SOS2-SOS3 protein kinase complex (Fig. 6). Moreover, TdSOS1Δ972 demonstrated maximal capacity to reduce the net uptake of Na^+ and Li^+ in ion accumulation assays (Fig. 3). Thus, it appears that the presence of *Arabidopsis* SOS2-SOS3 proteins is not essential for the activation of TdSOS1Δ972 protein. This finding also suggests that the last 170 residues of TdSOS1 protein act as an auto-inhibitory domain whose elimination yielded a TdSOS1 protein having maximal transport activity that was insensitive to further activation by *Arabidopsis* SOS2-SOS3 kinase complex.

Expression pattern of *TdSOS1* under different stress conditions

SOS1 gene expression data is available for several plant species, including halophytes. Strikingly, in some cases, salinity produced little or even no alteration of SOS1 transcription (Cosentino et al. 2010; Kant et al. 2006; Mullan et al. 2007; Taji et al. 2004; Wu et al. 2007). By contrast, in other plants the salt challenge induced a clear accumulation of SOS1 mRNA (Martinez-Atienza et al. 2007; Olías et al. 2009; Wang et al. 2010; Tang et al. 2010; Xu et al. 2008). To monitor the expression profile of the *TdSOS1* gene under salt stress, we performed RT–PCR analysis on different organs (roots and shoots) of the salt-tolerant variety Om Rabia3 (Brini et al. 2009) at different times of stress exposure (3, 6, 14, and 24 h). The data presented in Fig. 7 showed that salinity did not elicit significant differences of *TdSOS1* expression, either in roots or shoots. In *Arabidopsis*, *AtSOS1* mRNA is inherently unstable and stress-induced *SOS1* mRNA stability is mediated by reactive oxygen species (ROS) (Chung et al.

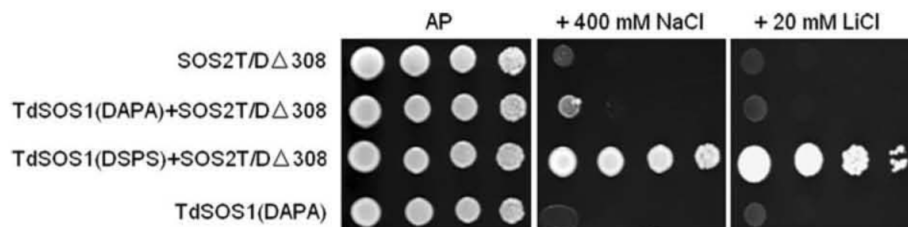

Fig. 5 Functional characterization of the mutated TdSOS1(DAPA) protein. The yeast strain AXT3K was transformed with plasmids directing the expression of the wild-type TdSOS1(DSPS) or mutant TdSOS1(DAPA) proteins with or without the kinase T/DSOS2Δ308. A control strain only with the hyperactive kinase was also used. Yeast cultures were grown overnight and five microliters of serial decimal dilutions of these cultures were spotted onto plates of AP medium with 1 mM KCl and supplemented with 400 mM NaCl or 20 mM LiCl. Plates were incubated at 30°C for 3–4 days

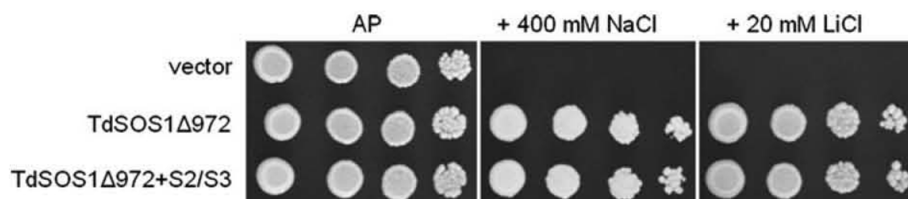

Fig. 6 Enhanced salt tolerance of yeast cells overexpressing *TdSOS1Δ972*. Cells of the yeast strain AXT3K transformed with empty vectors (pYPGE15 and pFL), *TdSOS1Δ972*, or co-expressing *TdSOS1Δ972* with *AtSOS2* and *AtSOS3* (*TdSOS1Δ972* +S2/S3) were grown in AP medium containing 1 mM KCl (AP) and supplemented with 400 mM NaCl or 20 mM LiCl. Plates were incubated at 30°C for 3–4 days

2008). To test if a similar mechanism is operating in wheat, an oxidative (H_2O_2) stress was applied. However, no significant changes in mRNA abundance were found and only a slight reduction of SOS1 transcript accumulation was observed in roots.

Discussion

In cereals, Na^+ toxicity is regarded as the major factor limiting growth in a saline environment, with the leaves being the principal site of accumulation due to the delivery of Na^+ via the transpiration stream (Tester and Davenport 2003). Therefore, the capacity to maintain a low Na^+ concentration or a high K^+/Na^+ ratio in the leaves is considered as an indicator of potential salinity tolerance in cereals. Plant plasma membrane Na^+/H^+ antiporters are thought to be involved in Na^+ homeostasis by extruding the ion from root epidermal cells at the root-soil interface and by regulating the root-shoot distribution of Na^+ in coordination with HKT proteins (Pardo 2010). Indeed, genetic evidence has demonstrated the essential role that SOS1-type proteins play in the salt tolerance of *Arabidopsis thaliana*, its halophytic relative *Thellungiella salsuginea* (a.k.a. *T. halophyla*) and in tomato (Shi et al. 2002; Oh et al. 2009; Olías et al. 2009). Homologous SOS1 proteins from rice and bread wheat have also been characterized, but conclusive evidence for their importance on the salt tolerance of these cereal crops is not yet available (Martinez-Atienza et al. 2007; Xu et al. 2008). However, the ability to limit Na^+ accumulation in barley shoots has been linked to protein polymorphism of HvCBL4, a close homologue of the *Arabidopsis* SOS3 protein (Rivandi et al. 2011). Rice plants may possess a functional orthologue of the *HvNax4* locus (*OsCBL4*) that facilitates vigorous seedling growth in saline filter paper culture (Prasad et al. 2000; Rivandi et al. 2011). In bread wheat—*Lophopyrum elongatum* amphiploid lines, salt tolerance was associated with improved Na^+ exclusion, a trait that was linked to chromosome 3E from *L. elongatum* that contained a *SOS1* gene from this wild relative of wheat (Mullan et al. 2007). Thus, the possibility exists that net Na^+ accumulation in wheat is controlled by the interplay of HKT and SOS proteins, with genetic polymorphisms in these genes underpinning natural variation in salt tolerance (Pardo 2010).

In this study, we report the cloning and characterization of the putative plasma membrane antiporter *TdSOS1* from durum wheat (cv. Om Rabia3). TdSOS1 belongs to the *Arabidopsis* CPA1 family (Cation Proton Antiporter 1) and

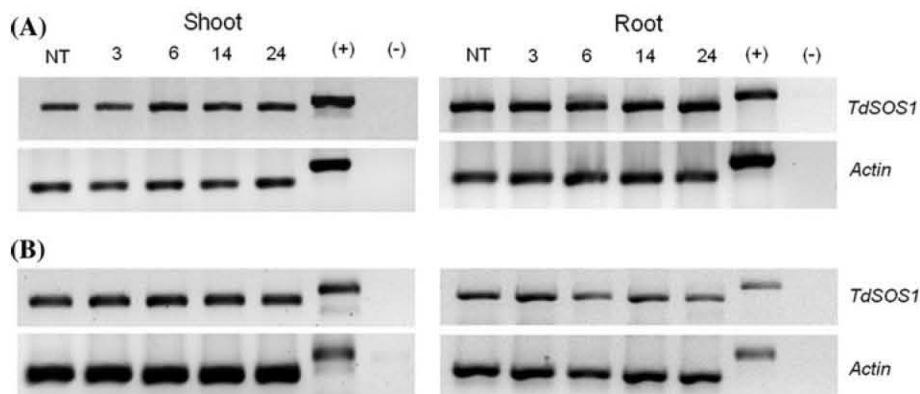

Fig. 7 Abundance of *TdSOS1* transcripts in shoot and root of the durum wheat variety Om Rabia3 in response to salt (**a**, 200 mM NaCl) or oxidative (**b**, 10 mM H_2O_2) stress. A combination of specific gene primers for *TdSOS1* was designated in the 3′ region and used to amplify 753 bp of the *TdSOS1* cDNA. Actin amplification was used as internal control. (+) PCR amplification using the genomic DNA of the wheat as a template; (−) amplification in the absence of templates

presents the same predicted structure than the NhaP-like exchanger's subfamily (Mäser et al. 2001; Pardo et al. 2006). TdSOS1 contains an N-terminal transmembrane region encompassing twelve putative transmembrane domains followed by a long hydrophilic C-terminal part. Heterologous expression in the yeast strain AXT3K, which lacks endogenous Na^+ efflux proteins, indicated that TdSOS1 is a Na^+/H^+ antiporter, like AtSOS1 (Qiu et al. 2002; Shi et al. 2002), OsSOS1 (Martinez-Atienza et al. 2007) and TaSOS1 (Xu et al. 2008). However, in contrast to the best characterized protein AtSOS1 from *Arabidopsis thaliana*, TdSOS1 also catalyses efficient Li^+ efflux when expressed in yeast (Fig. 2). *Arabidopsis sos1* mutant plants have been shown to display sensitivity to both Na^+ and Li^+ ions (Wu et al. 1996), but biochemical tests and functional expression in yeast indicated that AtSOS1 is a poor Li^+ transporter (Quintero et al. 2002; Shi et al. 2002). In this regard, it is important to note that the Na^+ sensitivity of *sos1* mutant increased more than 20-fold relative to wild-type whereas sensitivity to Li^+ merely doubled (Wu et al. 1996), indicating a strong bias toward Na^+ efflux by the SOS1 protein. It is also worth to mention that *Arabidopsis* protein AtNHX8, which is related to AtSOS1 but lacks the C-terminal part downstream amino acid 742 in AtSOS1, is important for Li^+ tolerance but not for tolerance to Na^+ or other toxic ions (An et al. 2007). Cereals like rice and wheat do not appear to have an AtNHX8 homologue and perhaps TdSOS1 has evolved to catalyze the transport of these two alkali cations. This is the first molecular and biochemical characterization of a Na^+ and Li^+ efflux protein from a cereal crop (*Triticum durum*).

The *Arabidopsis* AtSOS1 and the rice OsSOS1 proteins were phosphorylated and activated by the *Arabidopsis thaliana* SOS2-SOS3 proteins complex (Quintero et al. 2002; Martinez-Atienza et al. 2007). In *Arabidopsis thaliana*, the calcium binding protein SOS3 has been shown to interact with and activate the protein kinase SOS2 in the presence of calcium (Halfter et al. 2000; Quintero et al. 2002). In this work, we have demonstrated that the complex SOS2-SOS3 activates the wheat protein TdSOS1. The yeast strain AXT3K co-expressing these three proteins restored a salt-tolerance phenotype in AP medium containing high concentration of Na^+ and Li^+ (Fig. 2). Cellular tolerances were correlative with the relative rates of net Na^+ and Li^+ uptake measured in AXT3K cells expressing TdSOS1 with and without SOS2-SOS3 (Fig. 3). These results strongly suggest that the mechanistic details of the biochemical regulation of SOS1 proteins are conserved among these species and that, as a consequence, functional homologs of the SOS2-SOS3 proteins should exist in wheat.

The deletion of the auto-inhibitory FISL domain in the C-terminus of AtSOS2 together with T/D mutation in amino acid 168 of the activation loop, generated a constitutively active SOS2 kinase (T/DSOS2Δ308) (Gong et al. 2004; Guo et al. 2001, 2004). This kinase T/DSOS2Δ308 positively regulated the activity of TdSOS1 by protein phosphorylation (Fig. 4b). In *Arabidopsis thaliana*, AtSOS1 is activated by AtSOS2 through phosphorylation of serine residue S1138 in the conserved motif (V,I)(V,I)VR(I,V)DSPS (Quintero et al. 2011). This potential phosphorylation site is conserved between AtSOS1, OsSOS1 and TdSOS1 proteins (Fig. 1d). The substitution in TdSOS1 of the two serine residues in the core sequence DSPS to alanine reduced substantially, but it did not completely abrogate, the phosphorylation signal (Fig. 4b). This might be due to the presence of additional, low preference phosphorylation sites unmasked by the use of the hyperactive kinase T/DSOS2Δ308. In spite of this, co-expression of the mutated protein TdSOS1(DAPA) with the hyperactive kinase T/DSOS2Δ308 in yeast resulted in dramatic decrease of salt tolerance compared to wild-type SOS1 protein (Fig. 5). This suggests that the identified sites of phosphorylation of the wheat TdSOS1 protein are essential for its activation and, consequently, for salt tolerance. However, during the GST-fusion protein purification process, it was found that the wild-type TdSOS1(DSPS) was phosphorylated by an unknown yeast kinase(s) whereas the TdSOS1(DAPA) was not. This uncharacterized modification did not modify the in vivo activity of TdSOS1 since yeast transformants containing the wild-type (DSPS) or the mutant (DAPA) TdSOS1 proteins displayed similar low salt tolerance in the absence of SOS2, whereas only the wild-type protein was stimulated by the protein kinase to a significant extent (Fig. 5). Moreover, it is unlikely that this spurious phosphorylation site is shared by SOS2 since the wild-type protein with altered gel mobility was efficiently phosphorylated by SOS2 in vitro (Fig. 4). We surmise that the unidentified yeast kinase might be a MAP kinase since the C-terminus of AtSOS1 has been recently reported to be phosphorylated by the MAP kinase MPK6 in *Arabidopsis* (Yu et al. 2010), and the sequence adjacent to the core motif DSPS fits with a MAP kinase consensus site predicting phosphorylation at he serine residue S1126 preceding the proline in the DSPS motif. This may help explain why the DAPA mutant peptide was recovered unphosphorylated from yeast. In *Arabidopsis*, AtSOS1 is phosphorylated by SOS2 at S1138, equivalent to S1128 in TdSOS1. The residue phosphorylated by MPK6 in Arabidopsis and the effect on SOS1 activity remains to be determined.

Three functional domains have been recently identified in the C-terminal portion (amino acids 742–1,146) of the *Arabidopsis* SOS1 protein (Quintero et al. 2011). The first one expands from amino acids 764–849, has similarity to cyclic nucleotide binding domains (CNBD; Prosite

pf00027), and is essential for protein activity. This is followed by an auto-inhibitory domain located between amino acids 1,005 and 1,044, which is counteracted by the target sequence phosphorylated by SOS2 that is embedded in the last 50 amino acids of the protein. Mutations that removed the CNBD domain (allele *sos1-7*) or changed invariant amino acids (alleles *sos1-8* and *sos1-9*) in this domain generated loss-of-function mutants (Shi et al. 2000; Quintero et al. 2011). Truncations downstream the auto-inhibitory domain that removed the SOS2 phosphorylation site (allele *sos1-11*, stop codon at residues N1047 and L1072) (Shi et al. 2000; Quintero et al. 2011), also produced inactive proteins because these mutations prevented the phosphorylation by SOS2 that relieved SOS1 from auto-inhibition. By contrast, deletion of the auto-inhibitory domain and the SOS2 phosphorylation site together generated very active proteins in the yeast system (Quintero et al. 2011). Equivalent domains were identified in TdSOS1 based on sequence similarity (Fig. 1). Thus, we deleted the last 170 amino acids containing the putative phosphorylation site (S1126, S1128) and the adjacent conserved domain that has been shown to mediate the auto-inhibition of AtSOS1 (Quintero et al. 2011). When the truncated TdSOS1Δ972 was expressed in AXT3K cells, halotolerance was completely recovered and cells could grow in AP medium containing up to 400 mM sodium and 20 mM lithium (Fig. 6). Similar growth and halotolerance were observed in AXT3K cells co-expressing TdSOS1Δ972 with AtSOS2 and AtSOS3, i.e. no further activation of TdSOS1Δ972 was observed. This truncated form of TdSOS1 may by-pass the need of activation by the SOS2-SOS3 complex, and becomes a constitutively active form of the wheat SOS1 protein. These results suggest a conserved mechanism of activation of SOS1 proteins from monocots and dicots. Therefore, we propose a model for the regulation of the activity of wheat TdSOS1 protein involving the phosphorylation by the kinase SOS2 under ionic stress. The SOS2 kinase will phosphorylate TdSOS1 in one or both of the serine residues S1126 and S1128, thereby relieving TdSOS1 from auto-inhibition and stimulating its competence to catalyze ion transport.

In *Oryza sativa*, the expression level of *OsSOS1* increased significantly in roots after 3 h of salt treatment and reached a maximum after 15 h (Martinez-Atienza et al. 2007). In *Triticum aestivum*, the expression level of *TaSOS1* reached the highest level at 3 h after NaCl treatment but returned to pre-stress levels 9 h after treatment (Xu et al. 2008). In *Arabidopsis thaliana*, *AtSOS1* gene expression was up regulated in response to NaCl stress mainly in roots (Shi et al. 2000), due to enhanced mRNA stability. Chung et al. (2008) demonstrated that *SOS1* mRNA is unstable at normal growth conditions but its stability, which is mediated by reactive oxygen species, is considerably increased under salt stress and other ionic and dehydration stresses. Therefore, we investigated the expression profile of *TdSOS1* in the tolerant durum wheat variety Om Rabia3 under ionic (NaCl) and oxidative (H_2O_2) stress. In contrast with previous reports, *Triticum durum TdSOS1* expression remained mostly unchanged under salt and oxidative stress, perhaps reflecting the greater salt tolerance of Om Rabia3. Also worth noting is that *SOS1* orthologous genes on wheat chromosomes 3A and 3D and on *L. elongatum* chromosome 3E did not show differential expression in response to salt stress, while chromosome 3E was genetically linked to salt tolerance and reduced leaf Na^+ content (Mullan et al. 2007).

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