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Comparative functional analysis of two wheat Na^+/H^+ antiporter *SOS1* promoters in *Arabidopsis thaliana* under various stress conditions

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Abstract The bread wheat *TaSOS1* has been previously shown to be induced by salt stress treatment. To further investigate the regulation of the *TaSOS1* gene, the two genomic fragments $Pr_{SOS1\text{-}AB}$ and $Pr_{SOS1\text{-}D}$ have been isolated and sequenced. $Pr_{SOS1\text{-}AB}$ and $Pr_{SOS1\text{-}D}$ are the promoter regions of *SOS1* alleles, which are localised on genomes A and/or B, and on genome D, respectively. Sequence analysis of these two promoters revealed the presence of *cis*-regulatory elements which could be required for abiotic stress and abscisic acid (ABA) responsiveness. Histochemical assays of stably transformed *Arabidopsis* T3 plants showed that $Pr_{SOS1\text{-}AB}$ and $Pr_{SOS1\text{-}D}$ are active in this heterologous system, and their activities were almost the same at early developmental stages (4-, 8- and 12-day-old transgenic *Arabidopsis* seedlings). Nevertheless, β-glucuronidase (GUS) activity was detected only in plants carrying the $Pr_{SOS1\text{-}AB}$–*gusA* construct grown for 20 or 30 days. Furthermore, in these plants, the application of abiotic stress produced an accumulation in *gusA* transcripts. Taken together, these results show that, in this heterologous dicot system and under normal growth conditions, $Pr_{SOS1\text{-}AB}$ and $Pr_{SOS1\text{-}D}$ are age-dependent and organ-specific promoters. However, in the presence of different stress conditions, the activities of these two promoters became different and only $Pr_{SOS1\text{-}AB}$ is an abiotic stress-inducible promoter at different developmental stages. Thus, $Pr_{SOS1\text{-}AB}$ can be used for the development of abiotic stress-tolerant transgenic plants.

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Introduction

Salinity stress negatively impacts agricultural yield throughout the world, affecting production, whether it is for subsistence or economic gain. The plant response to salinity consists of numerous processes that must function in coordination to alleviate both cellular hyperosmolarity and ion disequilibrium (Tester and Davenport 2003). In addition, crop plants must be capable of satisfactory biomass production in a saline environment. Recent progress in the elucidation of salt stress signalling and effector output determinants that mediate ion homeostasis has uncovered some potential biotechnology tactics that may be used to obtain salt-tolerant crop plants, i.e. enhance the yield stability under salinity. However, it has been shown that high constitutive expression of the foreign gene may be detrimental to the host plant, with independent reports of increased sterility, retarded development, abnormal morphology, yield penalty, altered grain composition or transgene silencing (Sinha et al. 1993; Xu et al. 2006). The use of a strong, tissue-specific or inducible promoter to restrict gene expression to the required tissue, at a particular developmental stage and/or in response to a stress, may solve this type of problem (Karim et al. 2007; Pino et al. 2007). Until now, numerous inducible promoters have been isolated from a wide variety of organisms. Among these, the biotic and abiotic stress-inducible *rd29A* promoter has been widely used to minimise the otherwise negative effect on plant growth of transgene expression in plants such as tobacco, sugarcane and potato (Kasuga et al. 2004; Behnam et al. 2006; Wu et al. 2008).

Signal transduction networks for abiotic stress are divided into three major signalling types: osmotic/oxidative stress

signalling involves the generation of reactive oxygen species (ROS)-scavenging enzymes and antioxidant compounds, as well as osmolytes; Ca^{2+}-dependent signalling that leads to the activation of late embryogenesis abundant (LEA)-type genes; and Ca^{2+}-dependent salt overly sensitive (SOS) signalling (Xiong et al. 2002). The SOS signalling pathway, which comprises *SOS3*, *SOS2* and *SOS1*, is a pivotal regulator of, at least some, key transport systems required for ion homeostasis (Sanders 2000; Zhu 2000). Loss-of-function mutations in *SOS3*, *SOS2* and *SOS1* cause hypersensitivity to Na^+ (Wu et al. 1996; Zhu et al. 1998). The direct downstream target of this pathway is *SOS1*, a plasma membrane Na^+/H^+ antiporter (Shi et al. 2000; Qiu et al. 2002; Quintero et al. 2002). *SOS3* is a calcium-binding protein (Liu and Zhu 1998; Ishitani et al. 2000), while *SOS2* is a Ser/Thr kinase. Both the catalytic and regulatory domains are essential for *SOS2* function in salt tolerance. The C-terminal regulatory domain contains an auto-inhibitory FISL motif that binds to *SOS3* or calcineurin B-like 10 (*CBL10*), thereby releasing *SOS2* from auto-inhibition (Liu et al. 2000; Guo et al. 2001; Quan et al. 2007). *SOS3–SOS2* and *CBL10–SOS2* complexes regulate the plasma membrane Na^+/H^+ exchange activity of *SOS1* through phosphorylation, which releases *AtSOS1* from auto-inhibition (Quintero et al. 2002, 2011; Quan et al. 2007). In addition to *Arabidopsis*, the *SOS1* gene has been identified in other plants like rice (Martínez-Atienza et al. 2007), wheat (Xu et al. 2008; Feki et al. 2011), tomato (Olías et al. 2009) and *Thellungiella salsuginea* (Oh et al. 2009). Despite the demonstrated role of some *SOS1* genes in ion homeostasis and in the partitioning of the toxic ion Na^+ between plant organs (Shi et al. 2002; Olías et al. 2009), only experimental functional analysis of *Arabidopsis SOS1* and *Salicornia brachiata SbSOS1* promoter was performed in transgenic *Arabidopsis* and tobacco plants, respectively (Shi et al. 2002; Goyal et al. 2013). *Arabidopsis SOS1*, *SOS2* and *SOS3* promoter regions present the same *cis* elements. However, the predicted *cis* elements present in *SOS2* promoter are higher than *SOS1* and *SOS3*, with the presence of many small RNA target sites. This *in silico* analysis indicates that these three *SOS* promoter regions could be involved in common features of transcriptional regulation, and the *SOS2* gene is regulated by several upstream transcription factors and is involved in other outputs besides Na^+ transport by *SOS1* (Ji et al. 2013). Despite the extensive synteny of the open reading frames (ORFs) and the conservation of gene structures for *SOS1* between *Arabidopsis* and its halophytic relative (*Thellungiella parvula*), the promoter region of this gene is conserved only between the *Thellungiella* species (Oh et al. 2010).

It has been shown previously that *Arabidopsis SOS1*, *SOS2* and *SOS3* genes have different special expression profiles. The first two genes are expressed in both roots and shoots, while the latter is mainly expressed in root tissues (Liu et al. 2000; Shi et al. 2002; Quan et al. 2007). The expression of these genes is induced by salt stress in roots, but only *SOS3* expression displays differential induction in various types of root cells located in different developmental root zones in response to salt stress (Ji et al. 2013). *Arabidopsis AtSOS1* is preferentially expressed in epidermal cells at the root tip and in parenchyma cells at the xylem/symplast boundary of roots, stems and leaves (Shi et al. 2002). Concerning *SOS1* gene expression, salt challenge induces a clear accumulation of *SOS1* mRNA (Martínez-Atienza et al. 2007; Xu et al. 2008; Olías et al. 2009; Tang et al. 2010; Wang et al. 2010). Strikingly, in some cases, salinity produced little or even no alteration of *SOS1* transcription (Taji et al. 2004; Kant et al. 2006; Mullan et al. 2007; Wu et al. 2007; Cosentino et al. 2010; Feki et al. 2011). To our knowledge, the expression profile of wheat *SOS1* has been analysed under different abiotic stresses, but the wheat *SOS1* promoter activity has not yet been experimentally analysed. In this study, we showed that the two isolated wheat *SOS1* promoter regions ($Pr_{SOS1\text{-}D}$ and $Pr_{SOS1\text{-}AB}$) are active, age-dependent and organ-specific promoters in *Arabidopsis* plants. Moreover, we demonstrated that, contrary to $Pr_{SOS1\text{-}D}$, $Pr_{SOS1\text{-}AB}$ is an abiotic stress-inducible promoter at different developmental stages (8, 20 and 30 days old). Thus, the $Pr_{SOS1\text{-}AB}$ promoter will be useful for specific spatiotemporal targeting and accumulation of proteins conferring tolerance to abiotic stresses in transgenic plants.

Materials and methods

Genomic library screening and isolation of wheat *SOS1* promoter regions

The bacterial artificial chromosome (BAC) library from *Triticum aestivum* (cv. Chinese spring) was screened using polymerase chain reaction (PCR) amplifications as described by Isidore et al. (2005). Amplifications were done using wheat *SOS1*-specific primers, which are S1 as a sense primer and S2 as a reverse primer (Table 1). The 5′-flanking region of the wheat *SOS1* gene was isolated using the inverse PCR method as described by Ochman et al. (1988). PCR reactions were carried out with the corresponding recombinant BAC clone as a template, and with the wheat *SOS1*-specific primers IS1 and IS2, designed close to the 5′UTR sequence (Table 1). Two promoter sequences, named $Pr_{SOS1\text{-}D}$ and $Pr_{SOS1\text{-}AB}$ of 2,660 and 2,745 bp, respectively, were obtained by sequencing.

In silico analysis of the wheat *SOS1* promoters

The search for putative *cis* elements in the two promoter sequences was carried out using the signal scan search provided by the PLACE (http://www.dna.affrc.go.jp/PLACE/)

Table 1 Sequences of primers used for genomic library screening, inverse PCR and cloning of the two wheat *SOS1* promoter regions in the pCAMBIA1391Z vector

Primer	Nucleotide sequence (5′–3′)
S1	GCATCTTATTGGAAGGATTTCTGAA
S2	GGAGAGTCCACTCTGACGAT
IS1	TCCTCGTCCTCGGCGTCGCCCTCGGC
IS2	GCCCAGCACGAGGGCCACCCCGAAGA
ES1	ATCTGCAGTCTAGACATGTGTCTAAAA
NS1	TCTCCATGGCCACCGCCGCCGTCGAGG
ES2	ATCTGCAGGAATTCATGAAAATAATTT
NS2	TCTCCATGGCCGCCGCCGTCGCCGTCG

and the PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) databases (Higo et al. 1999; Lescot et al. 2002).

Construction of the binary vectors and *Arabidopsis* transformation

Fragments of the two promoters $Pr_{SOS1\text{-}D}$ and $Pr_{SOS1\text{-}AB}$ were amplified using the two different couples of primers *ES1–NS1* and *ES2–NS2*, respectively. The two forward primers *ES1* and *ES2* harbour the P*st*I restriction site, and the two reverse primers *NS1* and *NS2* harbour the N*co*I restriction site. The two resulting fragments were cloned separately in front of the *gusA* gene into the pCAMBIA1391Z vector (Cambia, Canberra, Australia), using the P*st*I and N*co*I restriction sites. The resulting recombinant binary vectors were named pCAMBIA1391Z–$Pr_{SOS1\text{-}D}$–*gusA* and pCAMBIA1391Z–$Pr_{SOS1\text{-}AB}$–*gusA*. *Agrobacterium tumefaciens* strain GV3101 (Konez and Schell 1986) was transformed separately by the freeze–thaw transformation method (Chen et al. 1994) with these two binary vectors and the binary vector pCAMBIA1301, which were subsequently used for *Arabidopsis* transformation. The *Arabidopsis thaliana* transformation was performed using the floral dipping technique (Clough and Bent 1998). Transgenic plants were selected on Murashige and Skoog (MS) agar medium (Murashige and Skoog 1962) containing 20 μg L^{-1} hygromycin. Seeds of the T2 generation were harvested and the seedlings from the homozygous T3 generation were used for histochemical β-glucuronidase (GUS) staining. The wild-type *Arabidopsis* and 35S-*gusA* transgenic plants were used as negative and positive controls, respectively.

Identification of the transgenic *Arabidopsis* plants

Genomic DNA was extracted as described by Michiels et al. (2003) from the leaves of $Pr_{SOS1\text{-}AB}$–*gusA* and $Pr_{SOS1\text{-}D}$–*gusA* transformed *Arabidopsis*, and was used in PCR amplifications. To detect positive lines, the couples of primers from the *gusA* gene (GR) and flanking the promoter $Pr_{SOS1\text{-}AB}$ region (ABF) or the promoter $Pr_{SOS1\text{-}D}$ region (DF) were used (Table 2). The amplified products were resolved on a 1 % agarose gel and visualised by ethidium bromide staining.

For each *Arabidopsis* transformation, four positive transgenic lines were randomly selected to analyse the expression of the *gusA* gene. From 20-day-old seedlings, total RNA was isolated using the Trizol method (Invitrogen), and to remove contaminating DNA, it was then treated with RNase-free DNaseI (Promega) at 37 °C for 15 min and further incubated at 65 °C for 10 min. DNase-treated RNA samples (0.5 μg) were reverse-transcribed using M-MLV reverse transcriptase (Invitrogen). The reverse transcription (RT) reactions were performed at 37 °C for 1 h and using the oligo-dT (18 mer) primer. One microlitre of each cDNA was used as the template for PCR amplification with 2 units of Taq DNA polymerase (Invitrogen), 200 μM dNTPs and 0.5 μM *gusA*-specific primers (GusF–GusR) (Table 2). An *Arabidopsis thaliana β-tubulin* gene fragment (GenBank accession no. XM_002863542.1), used as an internal control, was amplified with the forward TubF and the reverse TubR primers (Table 2). The PCR products (5 μl) were separated on 1.5 % agarose gel.

Growth conditions, abiotic stress treatment and *gusA* expression analysis

The effect of abiotic stress on *gusA* transcripts accumulation was monitored using seeds of homozygous transgenic lines and the non-transformed plants. Seeds of each T3 homozygous transgenic *Arabidopsis* lines were surface-sterilised and then grown on MS agar medium under light/dark cycle conditions of 16 h light/8 h dark cycles at 22 °C. Seedlings were grown in MS agar medium for 8, 20 or 30 days and then transferred to MS agar medium containing 100 mM NaCl, 100 mM mannitol or 20 μM abscisic acid (ABA), and kept for 2 days in each treatment. Then, the plants were harvested for analysis by histochemical GUS staining and for *gusA* expression analysis. Total RNA was extracted from 20- and 30-day-

Table 2 Sequences of primers used for the analysis of the transgenic *Arabidopsis* plants by PCR amplifications and semi-quantitative RT-PCR

Primer	Nucleotide sequence (5′–3′)
GR	GCTATGTAATATTTACACCATAACC
ABF	CTCCCTCCATTCTACAATGTAGTGC
DF	GTCGATGAACGATCGGGTGCCGTT
GusF	AGACTGTAACCACGCGTCTGTTGA
GusR	CATCAAAGAGATCGCTGATGGTAT
TubF	GTCCAGTGTCTGTGATATTGCACC
TubR	GCTTACGAATCCGAGGGTGCC

old seedlings, and treated with RNase-free DNaseI. The cDNA was synthesised by means of the M-MLV reverse transcriptase (Invitrogen). PCR amplifications were performed using the primers GusF and GusR (Table 2) and the PCR-amplified products were visualised on ethidium bromide-stained 1.5 % agarose gels and quantified using the Gel DocXR Gel Documentation System (Bio-Rad). This software was used to calculate the average band density, which was recorded and used in graphic analyses. The band density was determined by this software and was given in arbitrary units and graphed using Microsoft Excel. The error bars were determined from three separate biologic replicates. Each of the three biological replicates consisted of pooled plants subjected or not to different stress conditions.

Histochemical GUS staining

GUS activity was assayed histochemically by incubating tissue under vacuum infiltration with GUS staining solution [50 mM Na_2HPO_4 buffer (pH 7.0), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.1 % Triton X-100 and 1 mg/l X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide)] for several minutes and then incubated overnight at 37 °C (Jefferson et al. 1987). The pigments and chlorophyll were removed by soaking the *Arabidopsis* tissues for several hours in 70 % (v/v) ethanol. Three to six stained plants from three independent experiments were observed under binocular loupe and photographed using an Olympus W120 digital still camera, and most of the photos showed similar results.

Results

Isolation and *in silico* analysis of the two wheat *SOS1* promoter regions

A genomic library from bread wheat (*Triticum aestivum*) was screened by PCR with specific primers of the wheat *SOS1* gene, which resulted in the isolation of two different BAC clones, BAC-AB and BAC-D, containing the wheat *SOS1* alleles that are localised on genomes A and/or B, and on genome D, respectively. In order to obtain the promoter region of the bread wheat *TaSOS1* gene, we performed an inverse PCR using *SOS1*-specific primers and the two BAC clones obtained as a template. 2,661-bp ($Pr_{SOS1\text{-}D}$) and 2,745-bp ($Pr_{SOS1\text{-}AB}$) genomic DNA fragments were isolated from BAC-D and BAC-AB clones, respectively, sequenced and deposited into GenBank NCBI ($Pr_{SOS1\text{-}D}$: accession no. KF169800, $Pr_{SOS1\text{-}AB}$: accession no. KF169799).

During the inspection of the two promoter sequences, we found that 140 nucleotides at the 3′ end of the $Pr_{SOS1\text{-}D}$ promoter region exhibited similarity with the sequence upstream of the ATG of the *TaSOS1* gene (accession no. AY326952), confirming that this cloned sequence was the upstream region of the wheat *SOS1* allele, which is localised on genome D. Moreover, the $Pr_{SOS1\text{-}AB}$ promoter region was similar to $Pr_{SOS1\text{-}D}$ and contained five supplementary nucleotide sequences (Fig. 1a). Thus, this second *SOS1* promoter region was the upstream region of the wheat *SOS1* allele, which is localised on genomes A and/or B. This finding allowed us to determine the putative transcription start site (+1), which was located 140 and 133 bp upstream of the ATG codon of the $Pr_{SOS1\text{-}D}$ and $Pr_{SOS1\text{-}AB}$ promoters, respectively. Two potential TATA boxes (TAAATAA) were identified at −27 and −40 nucleotides upstream of the transcription start site of the $Pr_{SOS1\text{-}D}$ and $Pr_{SOS1\text{-}AB}$ promoters, respectively (Fig. 1b, c), which was consistent with the regular features of eukaryotic promoters (Ke et al. 1997).

In silico analysis of the two upstream regions from the transcription start site of the *SOS1* allele was performed using the plant promoter databases PLACE and PlantCARE to obtain additional indications about the transcriptional regulation of this gene. *In silico* analysis revealed similarity in the type but differences in the number and the position of the *cis* elements among the two wheat *SOS1* promoter regions. Many regulatory *cis* elements were found and some of them are related to abiotic (dehydration and salt), biotic (fungal elicitor) and hormone (ABA) stress responses. Like *Arabidopsis* and *Salicornia brachiata SOS1* promoters (Goyal et al. 2013; Ji et al. 2013), *in silico* analysis showed also the presence of several potential binding sites for transcription factors such as MYB, DOF and WRKY (Table 3).

Generation of the transgenic *Arabidopsis* plants

In order to study the expression of the *gusA* gene under different abiotic stresses, the two T-DNA of the recombinant binary vectors pCAMBIA1391Z–$Pr_{SOS1\text{-}D}$–*gusA* and pCAMBIA1391Z–$Pr_{SOS1\text{-}AB}$–*gusA* were introduced separately by *A. tumefaciens*-mediated transformation in several independent transgenic *Arabidopsis* lines. These vectors contain the *hptII* gene conferring resistance to hygromycin as a selectable marker for plant transformation. After *Agrobacterium*-mediated transformation of *Arabidopsis* plants and selection with hygromycin, several transformants were produced. From each transformation event, three transgenic lines were propagated to the T3 generation, from which homozygous plants were isolated for further analyses. The two T-DNA regions of these two binary vectors are schematically presented in Fig. 2a.

T-DNA integration and *gusA* gene transcription were confirmed in the putative transgenic events by PCR and semi-quantitative RT-PCR, respectively. As expected, PCR products of 600- or 800-bp fragments were detected in the seven putative transgenic *Arabidopsis* lines carrying the $Pr_{SOS1\text{-}AB}$–

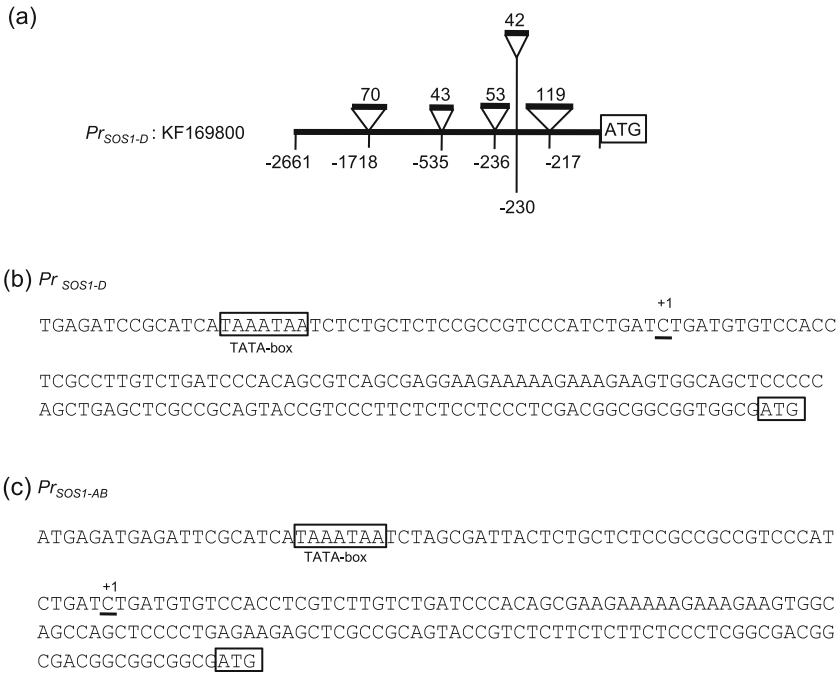

Fig. 1 **a** Schematic presentation of the five gaps in the Pr_{SOS1-D} compared to the $Pr_{SOS1-AB}$ promoter regions. The numbers represent their lengths (*positive numbers*) and their positions (*negative numbers*). The ATG codon is indicated by the *rectangle*. Presentation of the putative transcription start site (designated as *+1*) and the putative TATA box in the Pr_{SOS1-D} (**b**) and $Pr_{SOS1-AB}$ (**c**) promoter regions

gusA construct and the nine putative transgenic plants carrying the Pr_{SOS1-D}–*gusA* construct, respectively (Fig. 2b). The expression level of the *gusA* gene was analysed using RT-PCR, performed on young leaves of four AB lines (*Arabidopsis* carrying the $Pr_{SOS1-AB}$–*gusA* construct), four D lines (*Arabidopsis* carrying the Pr_{SOS1-D}–*gusA* construct) and non-transformed plants. As a control for cDNA amplification, the constitutively expressed *β-tubulin* gene was amplified. Semi-quantitative RT-PCR showed that the *gusA* transcript was expressed in these eight selected transgenic lines, and the expression level was almost similar for the AB and D lines, except the D4 line (Fig. 2c). Two representative transgenic lines AB6 and D9 were selected to further investigate the functional properties of $Pr_{SOS1-AB}$ and Pr_{SOS1-D}, respectively, in T3 homozygous seedlings. Genetic segregation data performed on the selected lines using the *hptII* gene gave rise to a

Table 3 Some putative *cis*-acting elements present at 2,661 bp upstream of the ATG of bread wheat *SOS1* alleles which are localised on genomes A and/or B ($Pr_{SOS1-AB}$) and on genome D (Pr_{SOS1-D}). N^a and N^b: the number of *cis*-regulatory elements present in $Pr_{SOS1-AB}$ and Pr_{SOS1-D}, respectively; (+): positive strand, (−): minus strand

Function/responsive	Motifs	Na	Nb	References
Early responsive elements in dehydration	ABRELATERD1	3 (+) 4 (−)	3 (+) 6 (−)	Simpson et al. (2003)
	ACGTATERD1	6 (+) 7 (−)	9 (+) 10 (−)	Simpson et al. (2003)
	ABRERATCAL	3 (+) 4 (−)	2 (+) 4 (−)	Kaplan et al. (2006)
Transcription factor binding sites	MYBCORE	2 (+) 2 (−)	4 (+) 0 (−)	Urao et al. (1993)
	MYBCOREATCYCB1	2 (+) 1 (−)	0 (+) 2 (−)	Planchais et al. (2002)
	DOFCOREZM	28 (+) 12 (−)	24 (+) 12 (−)	Yanagisawa and Schmidt (1999)
	WRKY7lOS	10 (+) 10 (−)	9 (+) 10 (−)	Zhang et al. (2004)
Pathogen and salt-induced gene	GT1GMSCAM4	7 (+) 3 (−)	8 (+) 3 (−)	Park et al. (2004)
Tissue-specific expression elements	GATABOX	3 (+) 10 (−)	4 (+) 9 (−)	Lam and Chua (1989)
	BS1EGCCR	0 (+) 2 (−)	1 (+) 1 (−)	Lacombe et al. (2000)

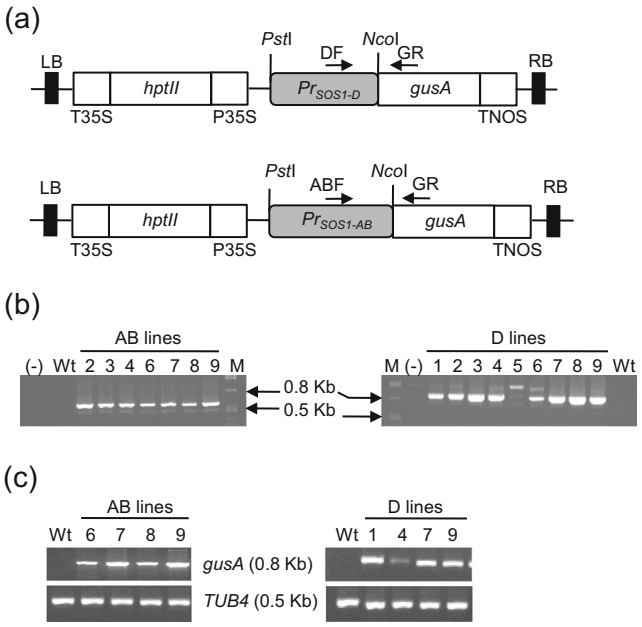

Fig. 2 Molecular analysis of the different transgenic *Arabidopsis* plants. **a** The T-DNA schematic maps of the two binary vectors pCAMBIA1391Z–$Pr_{SOS1\text{-}AB}$–*gusA* and pCAMBIA1391Z–$Pr_{SOS1\text{-}D}$–*gusA* used for *Arabidopsis* transformation. The two wheat *SOS1* promoters were inserted separately in this binary vector at the restriction sites P*st*I and N*co*I, upstream of the *gusA* gene. The *HPTII* marker is flanked by the CaMV35S promoter (P35S) and terminator region (T35S). GR, ABF and DF indicate the primers used for the detection of the positive *Arabidopsis* lines. **b** PCR analysis of the seven and nine T2 transgenic *Arabidopsis* lines carrying pCAMBIA1391Z–$Pr_{SOS1\text{-}AB}$–*gusA* (AB lines) and pCAMBIA1391Z–$Pr_{SOS1\text{-}D}$–*gusA* (D lines), respectively, and the non-transformed *Arabidopsis* plants (Wt). M: molecular weight marker, (−): negative control without DNA. **c** RT-PCR analysis of the four AB lines (AB6, 7, 8 and 9) and the four D lines (D1, 4, 7 and 9) using specific primers for the *gusA* gene. No amplification was detected in the case of the non-transformed plant (Wt). A 0.5-kb *β-tubulin* gene fragment was amplified by RT-PCR as an internal control

3:1 ratio, confirming that this marker segregates as a single copy gene. GUS activity and *gusA* transcript accumulation were monitored by histochemical staining and RT-PCR, respectively.

Activity of $Pr_{SOS1\text{-}AB}$ and $Pr_{SOS1\text{-}D}$ promoters in the transgenic *Arabidopsis* plants

GUS activity was analysed in the transgenic *Arabidopsis* seedlings carrying the $Pr_{SOS1\text{-}AB}$–*gusA* (AB6 line) or $Pr_{SOS1\text{-}AB}$–*gusA* constructs (D9 line) grown in normal MS medium and at different developmental stages. At an early developmental stage (4 days old), clear GUS activity was detected only in the young leaves of these two lines. Histochemical staining of 8- and 12-day-old AB6 and D9 seedlings did not enable the detection of any GUS activity. However, the GUS activity was different between AB6 and D9 lines grown for 20 or 30 days in normal MS medium. Indeed, at these two seedling developmental stages, no GUS activity was observed in the D9 line. Concerning the AB6 line, GUS activity was detected slightly in the roots and leaves of 20-day-old seedlings. In mature plants (30 days old), blue staining was observed in leaves and not in the stems, roots or flowers (Fig. 3). Taken

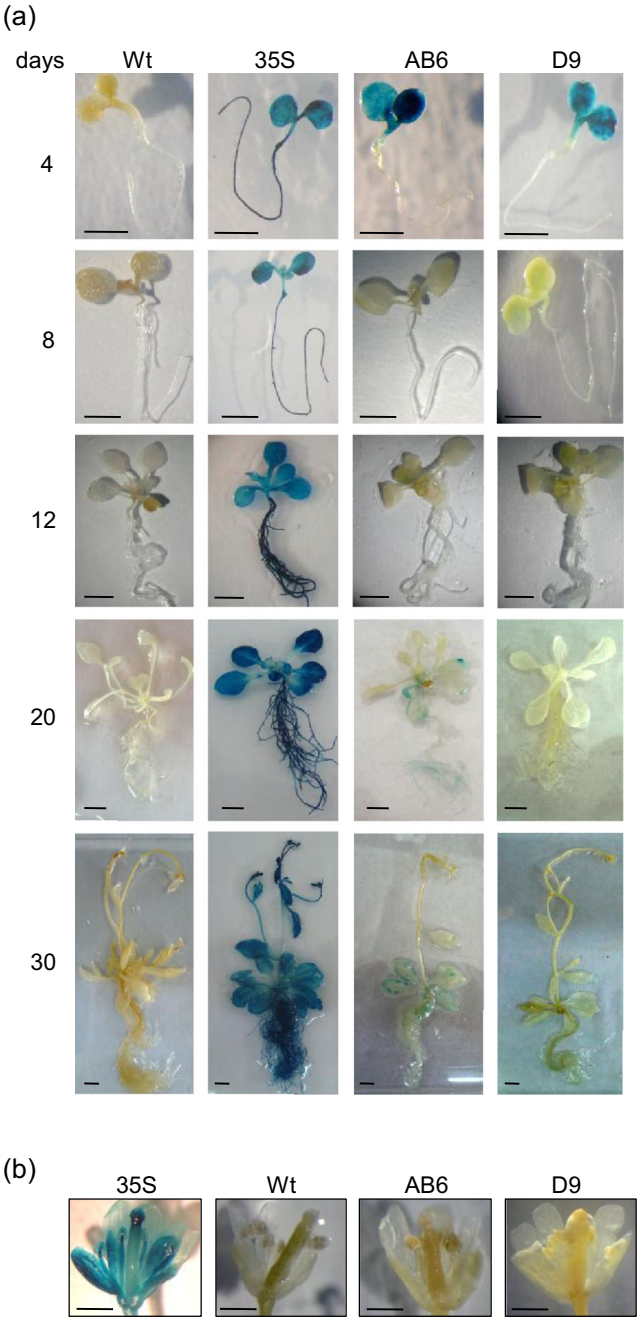

Fig. 3 **a** GUS activity in transgenic *Arabidopsis* plants carrying the pCAMBIA1391Z–$Pr_{SOS1\text{-}AB}$–*gusA* construct (AB6 line) or the pCAMBIA1391Z–$Pr_{SOS1\text{-}D}$–*gusA* construct (D9 line) at different developmental stages (4, 8, 12, 20 and 30 days old). Wt: non-transformed plants, 35S: transgenic *Arabidopsis* plants carrying pCAMBIA1301 (positive control). **b** Binocular observation of GUS staining in flowers of transgenic *Arabidopsis* plants (AB6 and D9) and of the control plants (35S and Wt)

together, these results showed that the $Pr_{SOS1\text{-}AB}$ and $Pr_{SOS1\text{-}D}$ promoters are active in *Arabidopsis* plants; their activities were almost the same only at early developmental stages, showing them to be age-dependent and organ-specific promoters.

$Pr_{SOS1\text{-}AB}$ is an abiotic stress-inducible promoter at different development stages

In a previous work, it has been demonstrated that the expression of the bread wheat *TaSOS1* gene is induced by salt stress (Xu et al. 2008). Here, the activities of the two wheat *SOS1* promoters were analysed in *Arabidopsis* under different abiotic stresses and at different developmental stages. For this, GUS activity was examined in the two transgenic AB6 and D9 lines grown for 8, 20 and 30 days in MS agar medium and then transferred to the same medium containing NaCl, mannitol or ABA for 2 days. At an early developmental stage (8 days old), the application of NaCl, mannitol or ABA produced a blue staining in the leaves of the two transgenic *Arabidopsis* AB6 and D9 lines. Contrary to the D9 line, GUS activity was observed also in the roots of the AB6 line challenged with NaCl, mannitol or ABA (Fig. 4a). Unlike the D9 line, abiotic and hormonal stresses produced a blue staining in a different part of 20-day-old seedlings of the AB6 line. Indeed, NaCl induced deeper blue staining in leaves and roots compared to ABA and mannitol treatment (Fig. 4b). Moreover, NaCl and ABA led to a strong GUS staining in the root tips of the AB6 line compared to mannitol treatment (Fig. 4c). In mature plants (30 days old), blue staining was observed in the stem and root of the AB6 line challenged with NaCl, mannitol or ABA stresses. In the case of the D9 line, no GUS activity was observed after the application of these different abiotic stresses (Fig. 4d). These data show that, contrary to $Pr_{SOS1\text{-}D}$, the activity of $Pr_{SOS1\text{-}AB}$ is induced by different abiotic stresses independently of the vegetative stage of the transgenic *Arabidopsis* plants.

Relation between stress treatment and *gusA* expression in transgenic *Arabidopsis* carrying the $Pr_{SOS1\text{-}AB}$–*gusA* construct

To further ascertain the induced activity of the $Pr_{SOS1\text{-}AB}$ promoter in 20- and 30-day-old seedlings of the AB6 and AB7 lines, RT-PCR was used to detect the presence of *gusA* transcripts under abiotic (salt and osmotic) and hormonal stress (ABA) conditions. Compared to the non-treated plants, an accumulation of *gusA* transcripts was detected and found to be up-regulated by NaCl, ABA and mannitol treatments in whole 20-day-old seedlings and in the shoots and roots of 30-day-old AB6 seedlings (Fig. 5). These results are in complete agreement with those of the histochemical staining assays.

Discussion

The *SOS1* protein has been associated to salt stress response, and loss of function of the *SOS1* gene results in the hypersensitivity of *Arabidopsis* to NaCl (Wu et al. 1996). After the description of the first *SOS1* gene in *Arabidopsis* (Shi et al. 2000), other *SOS1* genes have been identified in different plants, such as rice, wheat and tomato (Martínez-Atienza et al. 2007; Olías et al. 2009; Xu et al. 2008; Feki et al. 2011). So far, only the expression pattern of *Arabidopsis thaliana AtSOS1* and *Salicornia brachiata SbSOS1* have been examined using the *SOS1* promoter GUS (Shi et al. 2002; Goyal et al. 2013). In this work, we report the expression of *Triticum aestivum TaSOS1* in *Arabidopsis* plants under various abiotic stresses (salt, drought and hormonal). It is worth noting that there is a single *SOS1* locus in the genome of the tetraploid durum wheat (*Triticum durum* L. subsp. *durum*) (AA BB) (Feki et al. 2011). Thus, it seems that there are at least two *SOS1* copies in the hexaploid bread wheat (*Triticum aestivum*) (AA BB DD), one localised on genomes A or B, and the other one on genome D. Here, we report the isolation of two novel promoter regions $Pr_{SOS1\text{-}AB}$ and $Pr_{SOS1\text{-}D}$ of the *TaSOS1* gene by screening the genomic library of bread wheat (*Triticum aestivum*). The cloned $Pr_{SOS1\text{-}AB}$ and $Pr_{SOS1\text{-}D}$ sequences exhibited homology with the 5′ end sequence of *SOS1* alleles, which are localised on genomes A or B, and on genome D, respectively. Thus, the isolated sequences were in the upstream region of the two wheat *SOS1* alleles.

Sequence analysis of these two promoter regions revealed the presence of the same potential abiotic stress responsive *cis* elements, transcription factor-binding sites such as DOF and WRKY, ABA responsive element (ABRE) and GT elements (Table 3). DOF factors play an important role in the genes induced by plant hormones and stress signals (Yanagisawa and Sheen 1998). WRKY is required for positive and negative regulatory behaviours of ABA signalling (Zhang et al. 2004). An ABA-responsive *cis*-acting element named ABRE (C/GACGTGGC) is present in the promoter regions of many ABA-inducible genes. ABA plays a central role as a signalling molecule in stress regulatory networks in plants. Several *trans*- and *cis*-acting regulatory elements have been characterised that function in ABA-dependent and/or ABA-independent manners, leading to stress-inducible gene expression. An ABRE-like sequence (ACGTG) is required for the aetiolation-induced expression of the *erd1* (early responsive to dehydration) gene in *Arabidopsis* (Simpson et al. 2003). In addition, ABRE is the most conserved of the dehydration-inducible promoters in *Arabidopsis thaliana*, rice and soybean, suggesting that the transcriptional regulation of dehydration-inducible genes is similar among these species, with an ABRE-dependent transcriptional pathway (Maruyama et al. 2012). The GT-1 motif plays a role in pathogen- and salt-induced expression of the SCaM-4

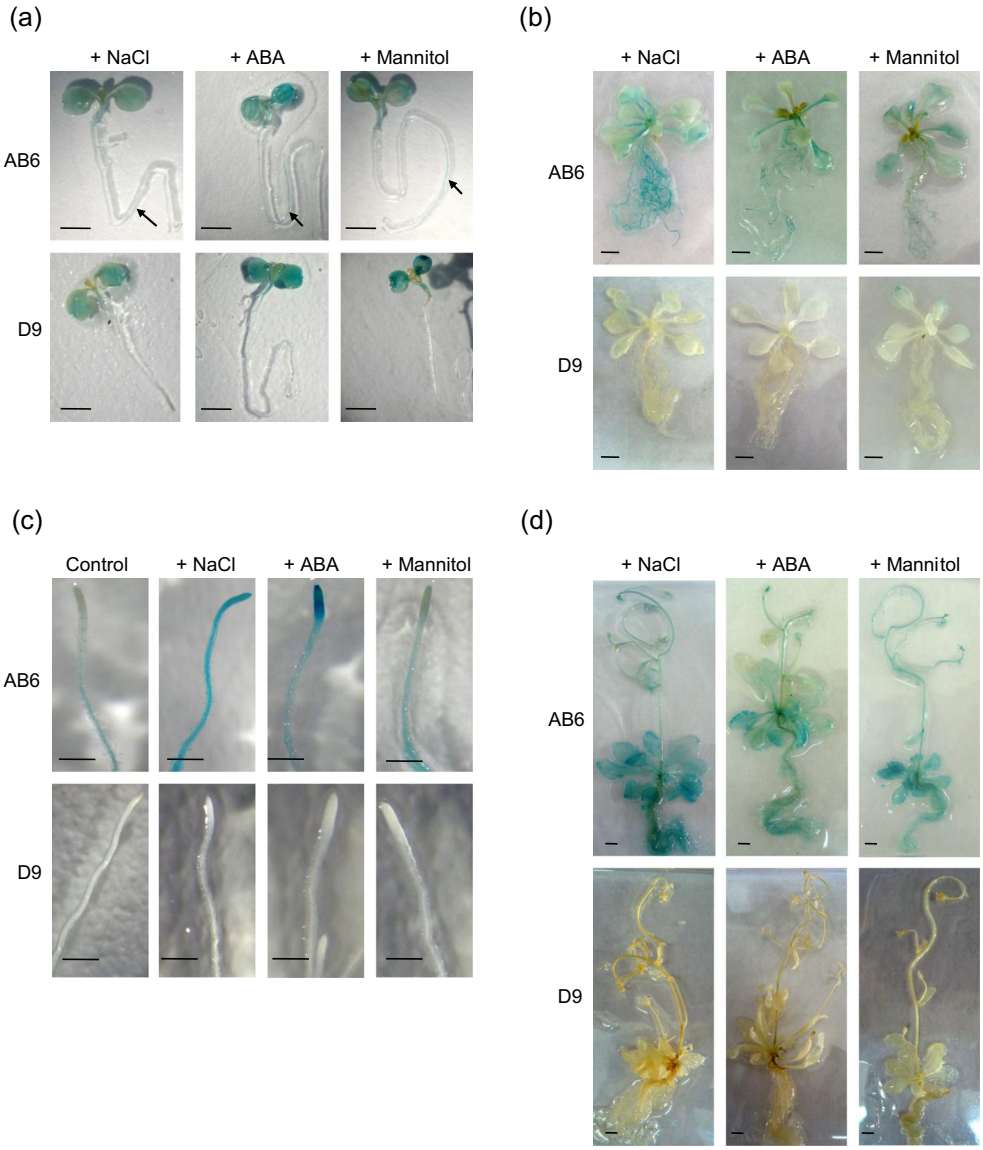

Fig. 4 Histochemical GUS staining of (**a**) 8-, (**b**) 20- and (**d**) 30-day-old transgenic seedlings placed for 2 days in MS agar medium containing 100 mM NaCl, 100 mM mannitol or 20 μM ABA. The *arrows* indicate the blue colouration in 8-day-old seedling roots. **c** Binocular observation of GUS staining in the root tips of 20-day-old transgenic seedlings subjected or not to different stress conditions. AB6 and D9: *Arabidopsis* carrying the pCAMBIA1391Z–$Pr_{SOS1\text{-}AB}$–*gusA* or pCAMBIA1391Z–$Pr_{SOS1\text{-}D}$–*gusA* constructs, respectively

promoter (Park et al. 2004). In the same way, *in silico* analysis of the *SOS1* upstream sequence from *A. thaliana*, *Vitis vinifera* and *Oryza sativa* revealed the presence of almost the same *cis*-regulatory elements (Goyal et al. 2013; Ji et al. 2013).

Many *SOS1* genes were isolated and then characterised in mutant yeast (Shi et al. 2002; Martínez-Atienza et al. 2007; Olías et al. 2009; Oh et al. 2009; Feki et al. 2011). So far, only the regulatory mechanism of the *Arabidopsis SOS1* gene has been analysed. In *Arabidopsis*, *SOS1* mRNA is unstable under normal growth conditions, and its stability is substantially increased upon salt stress treatment (Shi et al. 2003). Chung et al. (2008) identified the *cis* element in *SOS1* mRNA responsible for the stability-regulation and demonstrated that stress-induced *SOS1* mRNA stability is mediated by ROS. As an initial step towards understanding regulatory mechanisms controlling wheat *SOS1* gene expression, the expression pattern of the $Pr_{SOS1\text{-}AB}$ and $Pr_{SOS1\text{-}D}$ promoters was investigated using a *gusA* reporter gene system in transgenic *Arabidopsis* seedlings grown under normal or stressed conditions. In a previous work, it has been demonstrated that monocot gene promoters are functional in a dicots plant (Liu et al. 2003; Iwamoto et al. 2004; Tittarelli et al. 2007). In this study, histochemical staining revealed that the monocotyledonous $Pr_{SOS1\text{-}AB}$ and $Pr_{SOS1\text{-}D}$ promoters are active in this heterologous transgenic system. Moreover, their activities were similar only at early developmental stages (4, 8 and 12 days old). To our knowledge, this is the first isolation and characterisation of two wheat *SOS1* promoters. Histochemical staining revealed the ability of these promoters to direct GUS expression with age-dependent and organ-specific patterns. Indeed, GUS activity was detected only in young leaves of transgenic *Arabidopsis* carrying the $Pr_{SOS1\text{-}D}$-*gusA* construct (D lines). Despite the presence of *gusA* mRNA in D lines, GUS activity was never detected in roots and leaves. In an

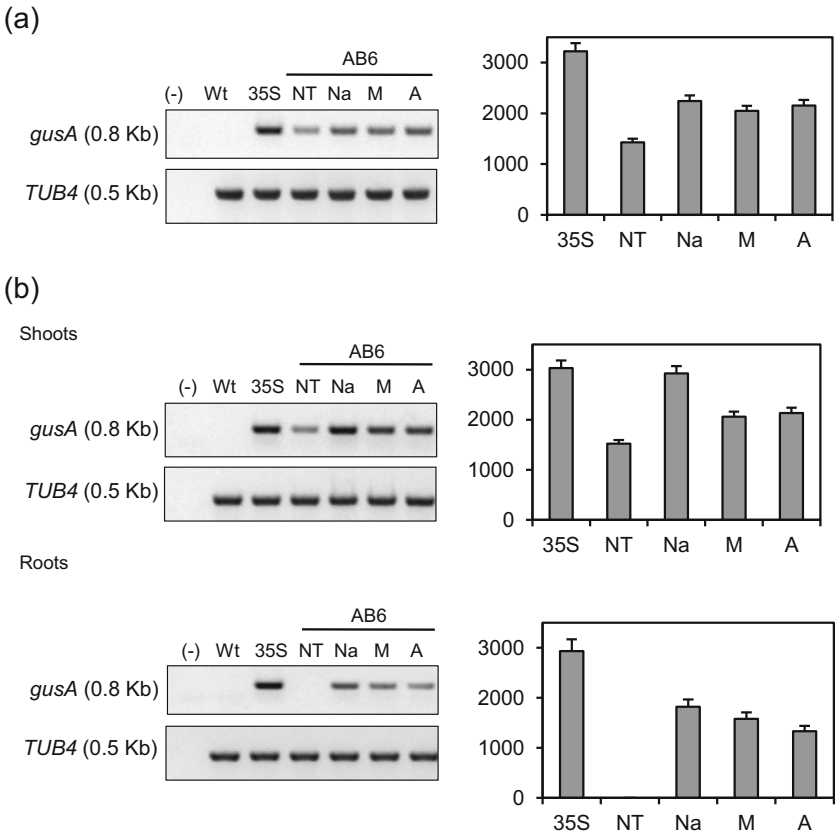

Fig. 5 Analysis of *gusA* expression in the whole plants of 20-day-old seedlings (**a**) and in the shoots and roots of 30-day-old seedlings (**b**), challenged with various abiotic stresses [100 mM NaCl (Na), 100 mM mannitol (M) or 20 μM ABA (**a**)] for 2 days. NT: non-treated plants. 35S: positive control plant. Wt: non-transformed plant. AB6: the transgenic *Arabidopsis* plant carrying the pCAMBIA1391Z–$Pr_{SOS1\text{-}AB}$–*gusA* construct. A 0.5-Kb *β-tubulin* gene fragment was amplified by RT-PCR as an internal control. (−): negative control without cDNA. The histograms correspond to the band densities in the gels, which are expressed in arbitrary units calculated by the Gel DocXR software. The standard errors were determined from three independent biologic replicates

attempt to explain these results, one may speculate that GUS activity is undetectable because of the absence of GUS or an inactive form of the enzyme. Concerning the transgenic *Arabidopsis* carrying the $Pr_{SOS1\text{-}AB}$-*gusA* construct (AB lines), GUS activity was absent only in 8- and 12-day-old seedlings.

The over-accumulation of stress regulators by using strong constitutive promoters has, in many cases, improved stress tolerance (Hsieh et al. 2002; Ito et al. 2006). Nevertheless, this enhanced stress tolerance is sometimes conferred at the expense of plant development and growth (Xu et al. 2006). The use of stress-inducible promoters is expected to be optimised for driving candidate abiotic stress tolerance genes (Rai et al. 2009; Zhu et al. 2010; Ben Saad et al. 2011). In this study, we showed that the two wheat *TaSOS1* promoters are induced by salt, drought and hormonal stresses. This is consistent with the presence in these promoter regions of the different *cis*-regulatory elements related to various stresses (Table 3). Contrary to our result, it has been demonstrated that the *AtSOS1* and *SbSOS1* promoters are induced by salt stress and not by ABA or cold stress (Shi et al. 2000; Goyal et al. 2013). Our data showed that the induction by these different abiotic stresses was different between these two *TaSOS1* promoters. Indeed, contrary to the $Pr_{SOS1\text{-}D}$ promoter, $Pr_{SOS1\text{-}AB}$ is induced by NaCl, ABA and mannitol at different developmental stages (8, 20 and 30 days old). This finding was supported by RT-PCR analysis of steady-state *gusA* mRNA levels in transgenic *Arabidopsis*, which revealed that the reporter gene transcription under the control of $Pr_{SOS1\text{-}AB}$ was highly induced by the different abiotic stresses. Moreover, GUS activity was significant at root tips under NaCl and ABA stresses. These data are in good agreement with the findings of Shi et al. (2002), which showed that *Arabidopsis AtSOS1* is expressed in root epidermal cells, particularly at the root tip and in cells bordering the vascular tissue. Despite the presence of the same *cis*-regulatory elements in the two wheat *SOS1* promoter regions, their activities were different under various abiotic stress conditions. This suggestion could be explained by the difference in the number of *cis*-regulatory elements within the two isolated promoter regions or by the presence of some *cis*-regulatory elements in the nucleotide sequences which are absent in the $Pr_{SOS1\text{-}D}$ promoter region.

This is the first report on the isolation and characterisation of wheat *TaSOS1* promoters ($Pr_{SOS1\text{-}AB}$ and $Pr_{SOS1\text{-}D}$) from *Triticum aestivum*. Histochemical GUS transient analysis validated that $Pr_{SOS1\text{-}AB}$ and $Pr_{SOS1\text{-}D}$ are functional, age-dependent and organ-specific promoters. Interestingly, in this heterologous system, only the $Pr_{SOS1\text{-}AB}$ promoter is induced by NaCl, mannitol and ABA at different developmental stages. These results will lead to more interest in the $Pr_{SOS1\text{-}AB}$ promoter, because it could be an attractive candidate promoter for the development of transgenic crop plants.

Moreover, $Pr_{SOS1\text{-}AB}$ may be an effective and desirable promoter for controlling stress tolerance candidate genes in terms of driving low constitutive transgene expression under normal conditions and high induction in response to salt (NaCl), hormonal (ABA) and osmotic (mannitol) stresses. For these reasons, using the $Pr_{SOS1\text{-}AB}$ promoter could avoid potential harmful effects related to an over-expression of the target gene under the control of constitutive promoters in transgenic plants.

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Conflict of interest The authors declare that they have no conflict of interest.

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