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Constitutive expression of a salinity-induced wheat *WRKY* transcription factor enhances salinity and ionic stress tolerance in transgenic *Arabidopsis thaliana*

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ABSTRACT

The isolation and characterization of *TaWRKY79*, a wheat class II WRKY transcription factor, is described. Its 1297 bp coding region includes a 987 bp long open reading frame. *TaWRKY79* was induced by stressing seedlings with either NaCl or abscisic acid (ABA). When a fusion between an 843 bp segment upstream of the *TaWRKY79* coding sequence and *GUS* was introduced into *Arabidopsis thaliana*, GUS staining indicated that this upstream segment captured the sequence(s) required to respond to ABA or NaCl treatment. When *TaWRKY79* was constitutively expressed as a transgene in *A. thaliana*, the transgenic plants showed an improved capacity to extend their primary root in the presence of either 100 mM NaCl, 10 mM LiCl or 2 μM ABA. The inference was that *TaWRKY79* enhanced the level of tolerance to both salinity and ionic stress, while reducing the level of sensitivity to ABA. The ABA-related genes *ABA1*, *ABA2 ABI1* and *ABI5* were all up-regulated in the *TaWRKY79* transgenic plants, suggesting that the transcription factor operates in an ABA-dependent pathway.

1. Introduction

Soil salinity is detrimental to crop productivity both because it inhibits the extraction of water by the roots and because the uptake of some salt ions are toxic to cellular function [1]. Plants have evolved various means to adapt to salinity stress; these include the accumulation of osmoprotectants and the up-regulation of stress-responsive pathways driven by specific transcription factors [2,3]. Some of these pathways depend on the phytohormone abscisic acid (ABA), while others do not [4]. The majority of crop species are relatively sensitive to salinity, so are unable to thrive on large areas of arable land which have become saline through over-irrigation [5]. Thus an important priority in crop breeding is to increase salinity tolerance. One strategy to achieve this could be to identify appropriate transgenes [6,7], such as the *LEA* gene which, when constitutively expressed, has been shown to improve the level of drought tolerance of rice [8], the transcription factor *OsMYB3R-2* which enhances low temperature tolerance in rice [9], or the nuclear factor Y B subunit which help maize tolerate drought [10]. Transcription factors are particularly useful in this context, as they frequently participate simultaneously in more than one stress signaling pathway [11,12].

The WRKYs form a large family of plant transcription factors [13]. They feature a conserved 60 residue domain which includes the motif WRKYGQK at its N terminus and either a CCHH or a CCHC zinc finger motif at its C terminus [14]. Phylogenetic analysis has identified several classes of WRKY transcription factors, based on these features [15]. Several WRKY transcription factors have been shown to participate in the abiotic stress response [16,17]. For instance, Jiang et al. have shown that the constitutive expression of either *AtWRKY25* or *AtWRKY33* increased the level of salinity tolerance of *Arabidopsis thaliana* [18], the heterologous expression of *OsWRKY08* (from rice) or *VvWRKY11* (from grapevine) improved osmotic stress tolerance in *A. thaliana* [19,20], while the constitutive heterologous expression of *OsRWKY45* in *A. thaliana* enhanced the plants' tolerance of drought [21].

The wheat genome harbors a large number of WRKY transcription factors, and although several have been isolated, only few have been functionally characterized [22,23]. In some cases, the constitutive expression of these transcription factors increased tolerance to abiotic stress [16,17], and in one case improved host resistance against the fungal pathogen *Fusarium graminearum* [24] . The likelihood that some wheat WRKY transcription factors do play a role in the stress response makes them an attractive target for the wheat molecular biologist. Here, we describe the contribution of one such wheat WRKY transcription factor which proved to be inducible by exposure to salinity.

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2. Materials and methods

2.1. Plant materials and stress treatments

Grains of the relatively salinity tolerant wheat cultivar SR3 (Xia et al., 2003) were germinated at 25 °C under a 16 h photoperiod, and two week old seedlings were exposed for set times to half strength Murashige and Skoog [25] liquid medium containing either 200 mM NaCl, 100 μM ABA or 18% w/v PEG6000. Low temperature stress was applied by holding two week old seedlings at 4 °C in a lit chamber.

2.2. Cloning and sequence analysis of TaWRKY79

The full length *TaWRKY79* cDNA was isolated by PCR amplification from a full length cDNA library prepared from SR3 root mRNA. The necessary primer pair was the gene specific sequence Wrky79-1 along with NT3 (listed in Supplemental Table S1), the former being gene-specific, and the latter targeting the pBluescript (+) vector sequence. The genomic copy of *TaWRKY79* was amplified from SR3 genomic DNA using the primer pairs (listed in Supplemental Table S1) , designed from the *TaWRKY79* cDNA sequence. The *TaWRKY79* promoter sequence was isolated using a Universal GenomeWalker kit (Clontech) employing SR3 genomic DNA as templates. The primer pair Wksp1/2 (listed in Supplemental Table S1)was used for nested PCR cloning. The resulting sequences and the predicted WRKY product sequence were analyzed with DNAman v5.2.2 and BLAST software (www.ncbi.nlm.gov/blast). Putative functional *cis* elements in the promoter sequence were identified with PlantCARE software (bioinformatics.psb.ugent.be/webtools/plantcare/html/).

2.3. RNA extraction, cDNA synthesis and transcription analysis

Total RNA was extracted from *A. thaliana* and wheat seedlings using the TRIzol reagent (Invitrogen), and the first cDNA strand synthesized with an RNAiso plus kit (TAKARA). Quantitative RT-PCR was used to assess transcript abundance. The first cDNA strand was synthesized from 2 μg mRNA template in 20 μl in volume using a primerscript RT reagent kit with gDNA eraser (TAKARA), and the subsequent qRT-PCR was performed in a 10 μl volume comprising 1 μl diluted (1:10 v/v) cDNA, 5 μl 2 times SYBR Ex Taq mix (TaKaRa) and 0.2 μM forward and reverse primers (primer sequences given in Table S1). The reactions comprised a denaturation step of 95 °C/60 s, followed by 45 cycles of 94 °C/30 s, 56 °C/30 s, 72 °C/30 s. *TaActin* and *AtTubulin* were used as internal references for wheat and *A. thaliana*, respectively. The relative abundance of transcript was estimated using the $2^{-\Delta\Delta Ct}$ method. Three biological and three technical replicates were performed per sample.

2.4. Plasmid construction and A. thaliana transformation

To constitutively express *TaWRKY79* in *A. thaliana*, the cDNA sequence was first amplified using the primer pair Wrkyo5/Wrkyo3 (listed in Supplemental Table S1). The resulting amplicon was digested with *Xba*I and then inserted into the pCAMBIA super 1300 vector (CAMBIA), under the control of the CaMV 35S promoter and with hygromycin resistance included as a selectable marker. The correct orientation of the insertion was verified by sequencing. To construct a fusion between *TaWRKY79* promoter and *GUS*, an 843 bp 5′-flanking region of the *TaWRKY79* genomic sequence was inserted into the PCAMBIA1391Z vector upstream of *GUS*. The construct was introduced into *Agrobacterium tumefaciens* strain GV3101, and from there into wild type *A. thaliana* Col-0, using the floral dipping method [26].

2.5. Stress tolerance

Two T3 homozygous lines derived from independent transgenic lines each exhibiting a 3:1 segregation ratio (hygromycin resistant:non-resistant) in the T2 generation were selected to test for abiotic stress tolerance. Five day old transgenic and wild type seedlings were plated in the vertical orientation on agar containing MS salts along with either 100 mM NaCl, 10 mM LiCl or 2 μM ABA, and left to grow for a further five days. Then primary root length was calculated.

2.6. Histochemical staining

To test whether the *TaWRKY79* promoter was induced by stress, ten day old T2 transgenic and control seedlings were sprayed with either sterile water, 100 mM NaCl or 100 μM ABA for 3 h. The treated and untreated leaves were bleached by immersing in acetone for 20 min and then incubated overnight at 37 °C in 1 mM X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide), 50 mM sodium phosphate buffer, as described by Jefferson et al. [27]. Then incubation in 75–50 gradient ethanol was performed to remove chlorophyll from the plant material.

3. Results

3.1. The structure and phylogeny of TaWRKY79

The length of the *TaWRKY79* cDNA was 1297 bp, including a 987 bp open reading frame (encoding 328 amino acids) (submitted to GenBank as Accession No JX047374) (Fig. S1A). Comparison with the relevant genomic sequence identified the presence of a 116 bp intron at position 678. The predicted gene product included a characteristic WRKY domain harboring both the oligopeptide WRKYGQK and C-X5-C-X23-H-X1-H (a putative zinc finger motif) (Fig. S1A). On this basis, *TaWRKY79* was classified as a Group II WRKY. The *TaWRKY79* WRKY domain was highly similar to that found in other abiotic stress responsive *WRKY* genes, such as *HvWRKY38*, *OsWRKY71*, *AtWRKY25* and *AtWRKY33* (Fig. S1B and C).

3.2. Transcription of TaWRKY79 in response to abiotic stress

The RT-PCR showed that *TaWRKY79* was strongly and rapidly induced by both 200 mM NaCl and 100 μM ABA, with transcript abundance peaking at 0.5 h after the treatment was initiated (Fig. 1A and B). A peak also occurred after 24 h of exposure to ABA in the root (Fig. 1A). Neither low temperature nor the presence of PEG had a strong effect on *TaWRKY79* transcription (Fig. 1).

3.3. Isolation of the 5′-flanking regulatory region of TaWRKY79 and GUS staining

To investigate the transcriptional regulation of *TaWRKY79*, 843 bp 5′-flanking region upstream of the first codon was isolated. Several abiotic stress-related *cis* elements, including TC rich repeats, and MBS, HSE and ABRE elements were identified in the regulatory sequence of *TaWRKY79* (Table 1). When the *TaWRKY79* promoter::*GUS* fusion was expressed heterologously in *A. thaliana*, histochemical staining demonstrated that the 843 bp segment of the *TaWRKY79* promoter was able to direct the stress-inducible expression of *GUS*. Under non-stressed growing conditions, a moderate level of GUS activity was detectable in the veins of transgenic plant leaves sampled from ten day old seedlings (Fig. 2A). However, the signal was much stronger following a 3 h exposure to either 100 μM ABA or 100 mM NaCl, showing that the *TaWRKY79* promoter can be induced by at least ABA and salinity stress. No

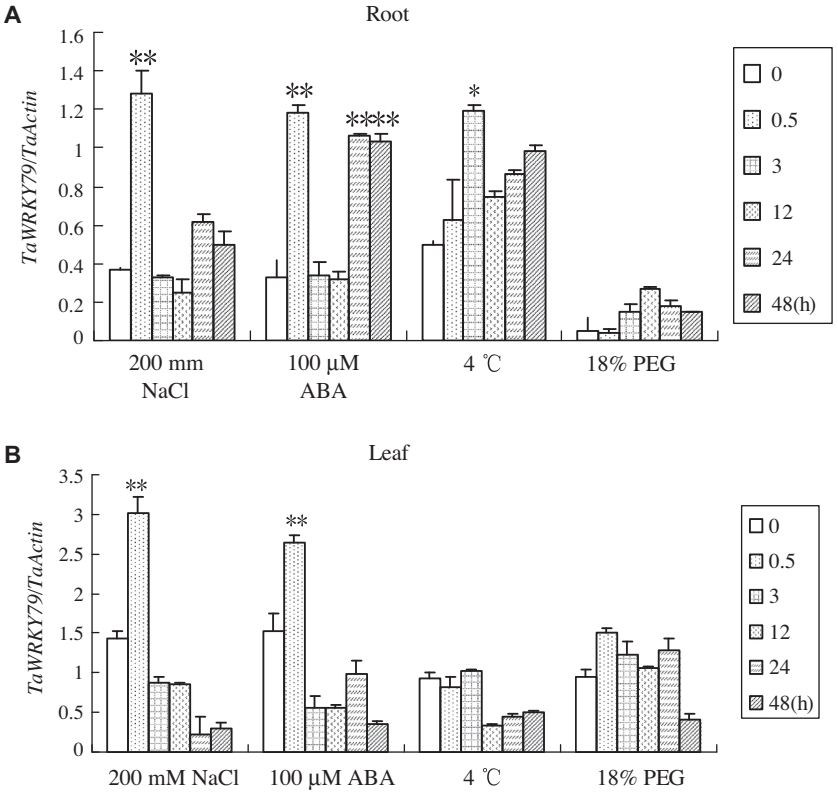

Fig. 1. RT-PCR profiles of *TaWRKY79* in two week old plants subjected to a range of stress treatments. TaActin was used as the internal reference sequence. (A): Expression patterns in roots. (B): Expression patterns in leaves. * indicates $P < 0.05$, ** indicates $P < 0.01$. T-line bar: standard deviation.

Table 1
Putative abiotic stress responsive *cis* elements present in the *TaWRKY79* promoter.

Name of *cis*-elements	Sequence of *cis*-elements	Number of *cis*-elements	Remarks	Reference
ABRE	CGCACGTGTC	1	*cis*-Acting elements involved in the abscisic acid responsiveness	[35]
TC-rich repeats	ATTTTCTTCA	2	*cis*-Acting elements involved in defense and stress responsiveness	[36]
MBS	CAACTG	2	MYB binding site involved in drought inducibility	[37]
CGTCA motif	CGTCA	1	*cis*-Acting regulatory element involved in the MeJA-responsiveness	[38]
TGACG motif	TGACG	1	*cis*-Acting regulatory element involved in the MeJA-responsiveness	[38]
HSE	AAAAAATTTC	1	*cis*-Acting element involved in heat stress responsiveness	[39]

obvious induction was detected after PEG and low temperature were imposed (date not show).

3.4. Constitutive expression of TaWRKY79 enhanced salinity and ionic stress tolerance, while reducing sensitivity to ABA

The phenotype of the two selected *CaMV35S*::*TaWRKY79* transgenic *A. thaliana* lines did not differ from that of the wild type in the absence of stress (Fig. 3B CK). However, when challenged with either NaCl, LiCl or ABA, the primary roots of the transgenic plants grew more freely than those of the wild type control plants (Fig. 3B and C). The phenotype of the transgenic seedlings was similar to that of wild type *A. thaliana* when the seedlings were provided with 150 mM mannitol (Fig. 3B, C). Thus the constitutive, heterologous expression of *TaWRKY79* reduced the sensitivity of the plant to ABA and increased its salinity tolerance via an enhancement of its ionic stress tolerance.

3.5. Altered transcription of abiotic stress responsive genes in TaWRKY79 transgenic plants

The transcript abundance of *ABA1*, *ABA2*, *ABI1* and *ABI5* was higher in the *CaMV35S*::*TaWRKY79* transgenic plants than in wild type ones. However, there was no convincing effect on transcript abundance with respect to the genes *ERD15, SOS1, SOS3, RD19A* or *DREB2B* (all of which are active in an ABA-independent pathway (Fig. 4).

4. Discussion

Here, the salinity-induced wheat class II WRKY transcription factor *TaWRKY79* was isolated and characterized. Consistent with conclusions drawn from microarray experiments, *TaWRKY79* was rapidly induced by both salinity and exogenously applied ABA (Fig. 1A and B). The induction was driven by the activity of the promoter region, since the promoter::*GUS* fusion transgene was substantially more active when the *A. thaliana* plants were challenged by either 100 mM NaCl or 100 μM ABA (Fig. 2). However, there were differences between the localization of expression of the native *TaWRKY79* and the promoter::*GUS* transgene, since *TaWRKY79* transcript was detectable in both the wheat leaf and root, while the GUS signal was only generated in the leaf of the transgenic *A. thaliana* plants. This origin of this difference may be connected with the fact that wheat is a monocotyledonous species, while *A. thaliana* is a dicotyledonous one [28]. Thus, for instance, it has been noted that the level of activity of certain maize promoters

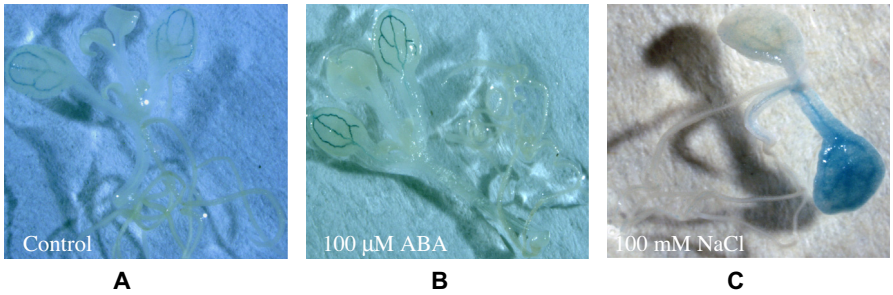

Fig. 2. *TaWRKY79* promoter activity in transgenic *A. thaliana* carrying a *TaWRKY79* promoter fused to *GUS*. GUS staining of ten day old transgenic *A. thaliana* seedlings which had been (A) non-stressed, (B) exposed to 100 μM ABA for 3 h, (C) exposed to 100 mM NaCl for 3 h.

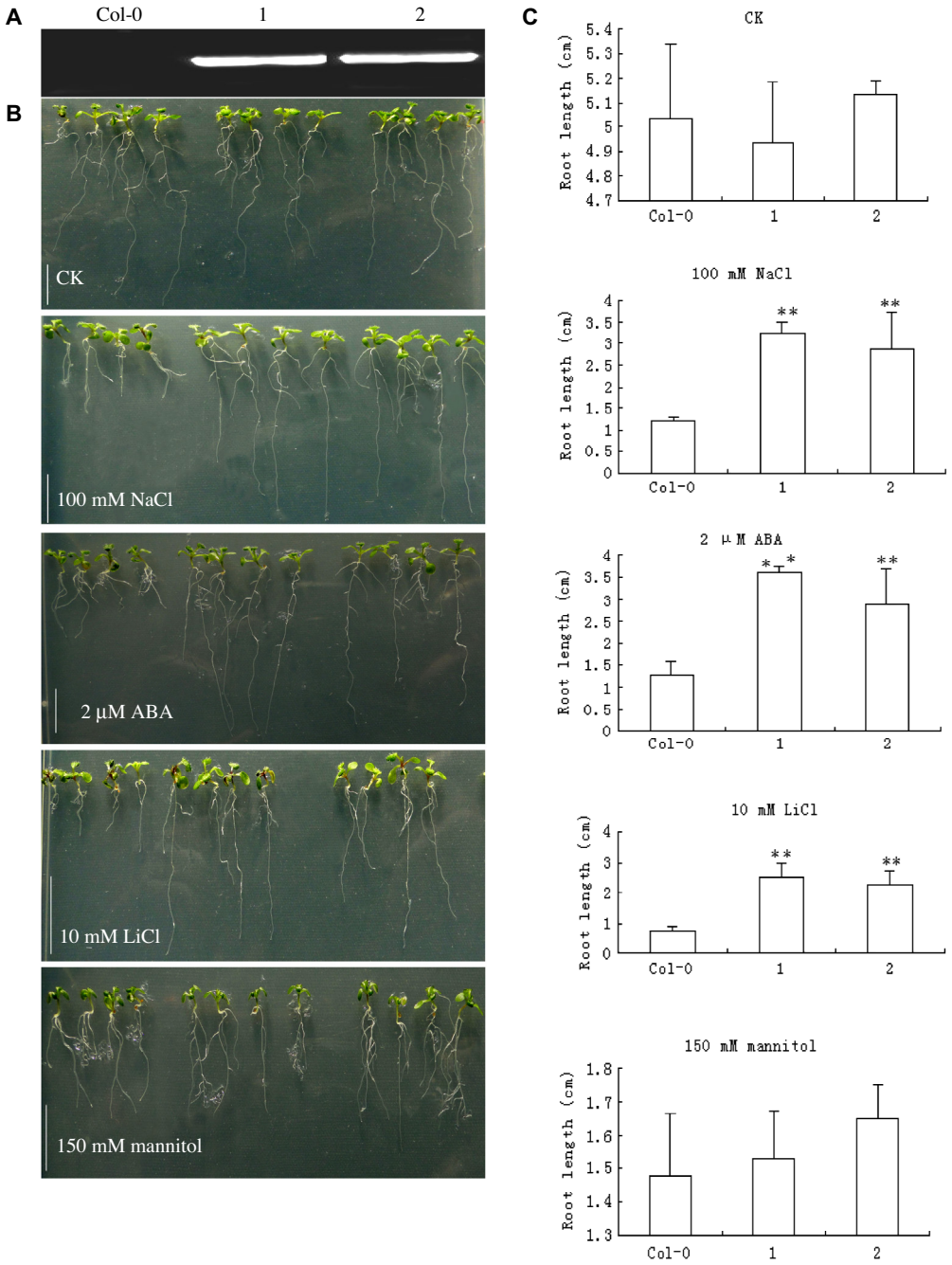

Fig. 3. Phenotype of *A. thaliana* plants constitutively expressing *TaWRKY79*. (A) Genotypic validation of transgenic status, based on the amplification of cDNA. (B) Wild type and transgenic seedlings exposed to non-stressed and stressed conditions. (C) Analysis of the root length of the plants illustrated in (B). Col-0: wild type *A. thaliana*. 1, 2: independent transgenic lines. **: $P < 0.01$. Standard deviations indicated by whiskers.

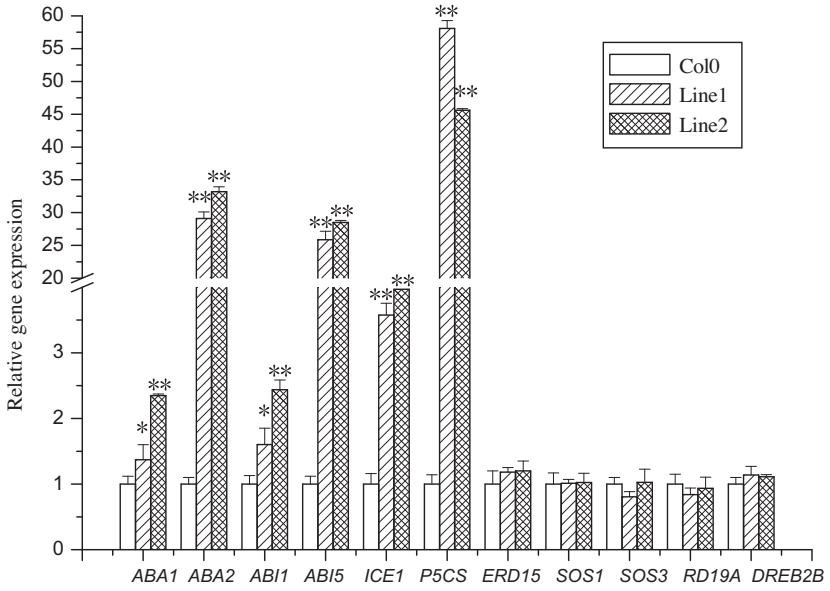

Fig. 4. Transcript abundance of various stress-associated genes in ten day old seedlings grown in the absence of stress. * indicates $P < 0.05$, ** indicates $P < 0.01$. T-line bar : standard deviation.

differs between the endogenous situation and the heterologous one when the transgene host is a dicotyledonous species [29].

The *TaWRKY79* transgenic *A. thaliana* plants produced longer primary roots than the wild type in the presence of either NaCl or ABA (Fig. 3), which demonstrated that the expression of *TaWRKY79* enhanced salinity stress tolerance, while simultaneously reducing the plant's sensitivity to ABA. The phytohormone ABA is typically accumulated by plants exposed to abiotic stress [30,31], and the build-up of ABA helps to activate a number of ABA-dependent transcription factors and downstream stress-response genes [32] . In the *A. thaliana* plants constitutively expressing *TaWRKY79*, the ABA synthesis genes *ABA1* and *ABA2* were both induced, implying an acceleration of ABA production. Consistent with this, the transcript abundance of both *AtABI1* and *AtABI5* was also raised (Fig. 4), demonstrating that *TaWRKY79* likely increases the level of salinity tolerance by increasing traffic through the ABA synthesis and transduction pathways. It has been reported that the presence of a single copy of ABRE is insufficient for the full ABA response to be mounted [33]. Only a single ABRE *cis* element was placeable within the *TaWRKY79* promoter, which may explain the inconsistency noted between the strong up-regulation of *TaWRKY79* in wheat itself and the much more moderate enhancement of GUS expression induced by the presence of 100 μM ABA in the transgenic *A. thaliana* plants (Figs. 1 and 2). The level of inducement of *TaWRKY79* in wheat suggests the presence of additional ABRE regulatory elements outside the promoter regions characterized here.

Low temperature stress is known to adversely affect plant performance in the presence of DRE/CRT *cis*-element in the promoter of genes within an ABA independent signaling pathway [34]. No DRE/CRT *cis* element was present in the *TaWRKY79* promoter region, in line with the minor effect on *TaWRKY79* transcription of low temperature (Fig. 1). The transcript abundance of *AtDREB2B* and *AtRD19A* (both involved in an ABA independent pathway) was unaffected by the presence of the *TaWRKY79* transgene (Fig. 4), indicating that the gene's enhancement of salinity tolerance is probably unrelated to an ABA-independent pathway. Nevertheless, the gene *AtICE1* was up-regulated in the *TaWRKY79* transgenic plants (Fig. 4). This gene is known to regulate the expression of *AtCBF3* via its interaction with *AtMYB15* (Agarwal et al., 2006); the low temperature-responsive *AtCBF3* controls the transcription of certain genes which are also acted upon by DREB transcription factors [3]. The inference is that *TaWRKY79* has a role in the response to a broad range of abiotic stresses.

The level of *TaWRKY79* transcription was not highly responsive to the stress induced by the presence of PEG, just as the phenotype of the transgenic *A. thaliana* lines was similar to that of the wild type control seedlings subjected to osmotic stress by the presence of mannitol (Fig. 3). Thus, the enhanced salinity tolerance shown by the *A. thaliana* plants constitutively expressing *TaWRKY79* could not have been due to the improvement in osmotic stress tolerance. When challenged by LiCl, root growth of the same lines was superior to that of wild type plants (Fig. 3), indicating that *TaWRKY79* has a positive role in the response to ionic stress. Overall therefore, *TaWRKY79* appears to enhance salinity stress tolerance via its positive effect on ionic stress tolerance, acting in an ABA dependent pathway.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.088.

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