Biochemical and Biophysical Research Communications 441 (2013) 476–481

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

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ARTICLE INFO

Article history:
Received 16 October 2013
Available online 26 October 2013

Keywords:
WRKY transcription factor
Wheat
Abiotic stress
Salinity stress
Transgene

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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0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.10.088

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