Biochemical and Biophysical Research Communications xxx (2016) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Identification and function analyses of senescence-associated *WRKYs* in wheat

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ARTICLE INFO

Article history:
Received 4 May 2016
Accepted 6 May 2016
Available online xxx

Keywords:
Wheat
Leaf senescence
WRKY
Senescence-associated gene

ABSTRACT

Leaf senescence is a positive, highly regulated, complex process, and transcription factors play important roles in the regulation of this process. We identified and characterized 116 *WRKYs* from the wheat genome database. Thirteen *TaWRKYs* were confirmed as senescence-associated genes. We focused on *TaWRKY7*, which is up-regulated in the natural leaf senescence process. *TaWRKY7* is expressed in different tissues of wheat and is localized in the nucleus. It shows transcriptional activation activity in yeast cells. The ectopic over-expression of *TaWRKY7* in Arabidopsis (*Arabidopsis thaliana*) significantly promoted early leaf senescence under darkness treatment and prevented leaf moisture losses. *TaWRKY7* played important roles in the senescence process and was involved in abiotic stress responses. Our transcriptomic and genetic studies on *WRKYs* suggest that *WRKY* transcription factors are a type of vital regulator in leaf senescence in wheat (*Triticum aestivum* L.).

1. Introduction

Leaf senescence is the final stage of leaf development [1]. During this process, different macromolecules undergo degradation, and the resulting components are transported to developing and storage tissues of the plant accompanied by programmed cell death in the plant life cycle [2]. Subsequently, the mesophyll cells are dismantled in a coordinated manner to remobilize nutrients and secure reproductive success during leaf senescence [3].

The complex senescence process mainly develops in an age-dependent manner and is triggered by various environmental stresses and hormones. Abscisic acid (ABA) is a key hormone that responds to stress and is well known for promoting leaf senescence [4]. *OsNAP* is induced by ABA, and over-expression of *OsNAP* significantly promotes leaf senescence [5]. Recent studies have indicated that when *pRD29A::PYL9* is used as an ABA receptor, transgenic lines show a dramatic increase in drought resistance and drought-induced leaf senescence in both Arabidopsis and rice (*Oryza sativa*) [6].

Leaves undergo changes in cell structure, metabolism, and gene expression during senescence. Altering the expression level of genes is one of the methods to control ageing [7]. A group of senescence-associated genes (*SAGs*) that is up-regulated during leaf senescence elicited our attention [8]. Through microarray detection, we confirmed the existence of subsets of *SAGs* in Arabidopsis. Alteration of 21% of the transcription factors as key regulators was induced by the process of leaf senescence in Arabidopsis [9].

Wheat is one of the most important crops in the world. Leaf senescence is an important agricultural characteristic that limits plant productivity [10]. Therefore, increasing the duration of leaf photosynthesis during grain filling through delay senescence is a possible means to increase grain yields in wheat. Senescing involves the grain filling stage accompanied by wheat flag leaves [11]. A well-known example of the association between senescence and quality is the NAC-domain (NAM;ATAF1,2;CUC2) transcription factor, which regulates senescence and improves grain protein, zinc, and iron contents in wheat [12]. *TaNAC-S* is a negative regulator of leaf senescence, and delayed leaf senescence may lead to increased yields and grain protein [13].

WRKY transcription factors (TFs) are key regulators in plants. They are involved in various processes. These transcription factors that include at least one WRKY domain at the N-terminus with sequences of about 60 amino acids are highly conserved in all members. Moreover, they have a typical zinc-finger structure at the C-terminus [14]. According to the number of WRKY domains or zinc-finger structures, WRKY are divided into three groups. Group I

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http://dx.doi.org/10.1016/j.bbrc.2016.05.034
0006-291X/

contains two WRKY domains, including a $CX_{4-5}CX_{22-23}HXH(C_2H_2)$ zinc-finger. Group IIa-e has only one WRKY domain and a C_2H_2 motif. One WRKY domain and zinc finger motif-$CX_7CX_{23}HXC(C_2HC)$ are present in Group III. The WRKY domain can bind specifically to the W-box ([T][T]TGAC[C/T]) in the promoter of the target gene to control transcription [14]. Recent reports have indicated that several *WRKY* transcription factors play important roles in the regulatory networks of leaf senescence. They show high expression levels in senescing leaves. In rice, up-regulated TFs comprise 47 *WRKYs* [15]. *AtWRKY6* binds directly to the W-box to control the transcriptional activation of the senescence-induced receptor-like kinase gene. *atwrky6* and over-expression *AtWRKY6* respectively exhibit premature and delayed-senescence phenotypes [16]. *AtWRKY53* can be induced by H_2O_2, which regulates SAG expression [17]. *AtWRKY53* interacts with At*WRKY30*. *AtWRKY30*, which is silenced by microRNA, does not show a senescence phenotype [18]. *AtWRKY54*, *AtWRKY57*, and *AtWRKY70* also play roles in leaf senescence [19].

Several studies on *WRKY* genes that regulate senescence have focused on model plant species, such as Arabidopsis and rice. However, little attention has been devoted to *WRKYs* related to senescence in wheat. In this study, we found a total of 116 *TaWRKY* members in the wheat database, and 13 *TaWRKYs* were induced by senescence in wheat flag leaves. The functions of these genes were predicted. Over-expression of *TaWRKY7* in transgenic Arabidopsis showed early leaf senescence. *TaWRKY7* was induced by ABA in wheat, and *TaWRKY7* overexpression lines led to enhanced tolerance to drought. These results suggest that *TaWRKY7* plays important roles in senescence and is involved in abiotic stress responses.

2. Materials and methods

2.1. Plant materials and growth conditions

Common wheat (*Triticum aestivum* L.) variety Shiluan 02-1 was utilized in the experiment. Plants with wheat flag leaves were excised at different growth stages, and different wheat tissues were grown in a field. For the ABA treatment, the plants were grown in an environmentally controlled growth room at 22°C with a 16/8 h cycle. The second leaves of two-week-old wheat were excised and placed in 100 μM ABA. The detached leaves were harvested 0, 1, 2, 3, 5, and 7 h after treatment.

Arabidopsis ecotype Columbia was utilized as the wild type and for transgenic analysis of *TaWRKY7*. The growth conditions and planting methods of Arabidopsis were similar to those previously described in a study [20].

2.2. Construction of phylogenetic tree for TaWRKYs

A hidden Markov model (HMM) profile for the WRKY domain (PF03106) was downloaded from the PFAM (http://pfam.sanger.ac.uk/). Then, we used BLASTP and TBLASTN to scan for WRKY domains in the wheat protein, cDNA, and RNA of the ENSEMBL database (http://plants.ensembl.org/index.html).

The WRKY sequences of *Arabidopsis thaliana, Oryza sativa,* and *Brachypodium distachyon* were downloaded from PlantTFDB (http://planttfdb.cbi.pku.edu.cn/). TaWRKY sequences were aligned with ClustalW or Muscle. The phylogenetic tree was constructed with the neighbor-joining (NJ) method with a bootstrap test (1000 replicates) using the MEGA5 program.

MEME Suite (http://meme.nbcr.net/meme) was utilized to analyze the conserved motifs of the TaWRKYs [21].

2.3. Subcellular localization of TaWRKY7 in Nicotiana benthamiana

The *TaWRKY7* sequence was cloned into the expression vector pCAMBIA2300-35S-GFP-OCS. An empty pCAMBIA2300 vector was used as the control. The leaves of six-week-old tobacco plants growing at normal conditions were infiltrated by *Agrobacterium.* After 42–48 h, the infected leaves were placed on slides and visualized under a laser confocal microscope at an excitation wavelength of 488 nm.

2.4. Transcriptional activation assay in yeast

The coding sequence of *TaWRKY7* was fused to the *pGTKT7* vector, which encodes a DNA-binding domain. Positive control *TaNAC6* and negative control *pGTKT7* were transformed into the yeast strain AH109. Yeast cells were grown on SD/His^-/Ade^-/Trp^- medium. The transformed yeast cells were grown on SD/Trp^- media or SD/His^-/Ade^-/Trp^- media for 3 d at 30 °C. β-galactosidase activity was detected.

2.5. Generation of TaWRKY7 transgenic Arabidopsis plants

The *TaWRKY7* coding sequence was inserted into PCAMBIA1300:35S. The constructs were then transformed into Col-0, which was mediated by *Agrobacterium*. Transgenic plants were selected with hygromycin.

2.6. Semi-quantitative and quantitative reverse transcription polymerase chain reaction analysis

These analyses were performed as reported previously [22]. All primers used in this study were listed in Supplementary Table S1.

3. Results

3.1. Extensive analysis of leaf senescence-associated WRKYs in wheat

To obtain *WRKYs* in wheat, we used the WRKY domain from PFAM (http://pfam.sanger.ac.uk/) to perform BLASTP/TBLASTN. A total of 116 members of *TaWRKYs* were identified. MEGE was utilized to analyze the conserved WRKY domains whose major was WRKYGQK. Only *TaWRKY114* and *116* possessed WRKYGEK. *TaWRKY4, 6, 19, 25, 74, 77, 79, 86,* and *94* had WRKYGKK (Supplementary Fig. S1).

The phylogenetic relationship of 116 *TaWRKYs*, 72 *AtWRKYs*, 101 *OsWRKYs*, and 81 *BdWRKYs* was analyzed through NJ using MEGA5. The phylogenetic analysis revealed that among the homologous *WRKY* genes from related species, *TaWRKYs* always clustered with those from *Oryza sativa* and *Brachypodium distachyon* possibly because of the close evolutionary relationship among the three species (Supplementary Fig. S2).

To date, senescence-associated *WRKY* in wheat has not been reported. Based on microarray detection conducted in our laboratory (not published), 21 *TaWRKYs* that contain complete ORF were up-regulated in flag leaves during the senescence process. The 21 *TaWRKYs* were *TaWRKY7*(I), *TaWRKY11*(IId), *TaWRKY14*(I), *TaWRKY16*(III), *TaWRKY18*(III), *TaWRKY24*(IId), *TaWRKY36*(IIa), *TaWRKY39*(III), *TaWRKY45*(IId), *TaWRKY48*(I), *TaWRKY52*(I), *TaWRKY54*(IIc), *TaWRKY60*(IIc), *TaWRKY68*(IIb), *TaWRKY71*(IIe), *TaWRKY74*(IIc), *TaWRKY89*(IIc), *TaWRKY96*(I), *TaWRKY114*(III), *TaWRKY115*(III), and *TaWRKY116*(III). To identify the senescence-associated *WRKYs*, we used a separate phylogenetic tree to analyze the relationship between wheat WRKYs and SAGs (Supplementary Fig. S3). High homology exists between *TaWRKYs*

Please cite this article in press as: H. Zhang, et al., Identification and function analyses of senescence-associated *WRKYs* in wheat, Biochemical and Biophysical Research Communications (2016), http://dx.doi.org/10.1016/j.bbrc.2016.05.034