Plant Science 261 (2017) 38–49

Contents lists available at ScienceDirect

Plant Science

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

Transcription factor ThWRKY4 binds to a novel WLS motif and a RAV1A element in addition to the W-box to regulate gene expression

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ARTICLE INFO

Keywords:
WRKY transcription factors
ThWRKY4
cis-Acting elements
Gene expression regulation
Yeast one-Hybrid

ABSTRACT

WRKY transcription factors play important roles in many biological processes, and mainly bind to the W-box element to regulate gene expression. Previously, we characterized a *WRKY* gene from *Tamarix hispida*, *ThWRKY4*, in response to abiotic stress, and showed that it bound to the W-box motif. However, whether ThWRKY4 could bind to other motifs remains unknown. In this study, we employed a Transcription Factor-Centered Yeast one Hybrid (TF-Centered Y1H) screen to study the motifs recognized by ThWRKY4. In addition to the W-box core *cis*-element (termed W-box), we identified that ThWRKY4 could bind to two other motifs: the RAV1A element (CAACA) and a novel motif with sequence of GTCTA (W-box like sequence, WLS). The distributions of these motifs were screened in the promoter regions of genes regulated by some WRKYs. The results showed that the W-box, RAV1A, and WLS motifs were all present in high numbers, suggesting that they play key roles in gene expression mediated by WRKYs. Furthermore, five WRKY proteins from different WRKY subfamilies in *Arabidopsis thaliana* were selected and confirmed to bind to the RAV1A and WLS motifs, indicating that they are recognized commonly by WRKYs. These findings will help to further reveal the functions of WRKY proteins.

1. Introduction

The WRKY transcription factor (TF) family constitutes a large family in plants. For example, there are 103 WRKY proteins in *Oryza sativa* [1], 74 in *Arabidopsis thaliana* [2], and 100 in *Populus trichocarpa* [3]. WRKY family proteins are characterized by the presence of one or two conserved WRKY DNA-binding domains. The WRKY DNA-binding domain comprises approximately 60 amino acids residues, with a highly conserved WRKYGQK heptapeptide sequence at the N-terminus; however, some WRKY proteins have variants of the WRKY amino acid sequence, including WRRY, WSKY, WKRY, WVKY, or WKKY. The WRKY domain also has an atypical zinc-finger structure, in the form of either Cx4-5Cx22-23HxH or Cx7Cx23HxC at the C-terminus [4,5]. According to the number of conserved WRKY domains and the composition of the zinc finger structure, WRKY transcription factors are categorized into three distinct groups (I, II, and III) [6,7]. Group I members include two C2H2 zinc fingers and two conserved WRKY (WRKYGQK) domains; group II members have one C2H2 zinc finger and one WRKY domain; and group III members contain one C2HC zinc finger and one WRKY domain [8,9].

WRKY proteins have functions in various biological processes, such as pathogen defense, leaf senescence, seed germination and dormancy, the development of the root and trichome, cell wall formation, and metabolites synthesis [10–15]. For example, mutants of *A. thaliana WRKY18, WRKY40,* and *WRKY60* improve resistance against *Pseudomonas syringae*, especially the double and triple mutants, *wrky18 wrky40, wrky18 wrky60,* and *wrky18 wrky40 wrky60*. This suggested that these WRKY proteins play roles in pathogen defense [12]. Choi et al. found that OsWRKY6 could activate the expression of *OsICS1* (*Oryza sativa isochorismate synthase 1*), and stabilization of OsWRKY6 regulates defense responses positively [13]. WRKY41 from *A. thaliana* is induced significantly during seed development and its mutant reduces primary seed dormancy, suggesting its involvement in seed development [14]. *A. thaliana* WRKY57 plays a key role as a negative regulator in jasmonic acid (JA)-induced leaf senescence [15]. Additionally, WRKY TFs are associated closely with various plant abiotic stress responses, such as drought, salinity, heat, cold, osmotic stress, and abscisic acid (ABA) signaling. For example, *AtWRKY25* and *AtWRKY33* are involved in heat and salt stress [16–18]. Rice *OsWRKY47* mutant plants are more sensitive to water deficit stress [19], and overexpression of *MtWRKY76* increases tolerance to NaCl and drought stresses [20]. The knockout of *AtWRKY46* in *A. thaliana* plants causes increased sensitivity to salt and drought stresses by regulating stomatal movement [21]. *CmWRKY10* in chrysanthemum mediates drought stress

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http://dx.doi.org/10.1016/j.plantsci.2017.04.016
Received 13 January 2017; Received in revised form 27 April 2017; Accepted 28 April 2017
Available online 04 May 2017

tolerance positively by regulating the expression of stress tolerance genes, such as *DREB1A*, *DREB2A*, *NCED3B*, and *CuZnSOD* [22]. In addition, WRKY proteins are also involved in biotic stress. For instance, OsWRKY45 is controls resistance to the Brown Planthopper *Nilaparvata lugens* negatively in rice [23].

WRKY proteins can bind directly to W-box motifs with the core sequence (C/T)TGAC(C/T) in promoters to regulate the expression of target genes [24–27]. For example, WRKY7, 8, 9, and 11 activate *RBOHB*, encoding an NADPH oxidase, by binding to W-box *cis*-acting elements present in its promoter [24]. The *A. thaliana* WRKY42 protein regulates directly the transcription of the *PHT1;1* (*PHOSPHATE TRANSPORTER1;1*) gene by binding to its promoter containing a W-box motif [25]. Overexpression of *FcWRKY70* improved the expression level of the *arginine decarboxylase* gene via binding to the sequence TTGACC [26]. *GhWRKY34* from cotton (*Gossypium hirsutum*) regulates the expression of *AtSOS2* (*A. thaliana Salt Overly Sensitive 2*) and *AtABF4* (*Abscisic Acid Responsive Elements-Binding Factor 2*) by binding to the TTGACC/T (W-box *cis*-element) in their promoters in response to salt stress [27]. In addition to the W-box, WRKYs also bind to WLE (TGACA) motif [13], PRE4 element (TGCGCTT) [28], sugar-responsive (SURE) element (TAAAGATTACTAATAGGAA) [11,29,30], and WK box (TTTTCCAC) [31]. For instance, the transcription factor, SUSIBA2 (sugar signaling in barley), is a WRKY protein that can not only bind to W-box, but also binds to SURE element as an activator [11]. *Oryza sativa* WRKY6 (OsWRKY6) regulates the expression of *OsPR10a* (*O. sativa pathogenesis-related 10a*) positively by binding to WLE1 (W-box-like element 1) (TGACA) [13]. Machens et al. found that WRKY70 from *A. thaliana* could bind to a novel *cis*-sequence "GACTTTT" to activate gene expression [32].

TFs regulate gene expression by binding to specific *cis*-acting element; therefore, determination of the specific *cis*-acting element recognized by a TF is quite important to reveal its function. Although the functions of WRKYs have been studied extensively, the *cis*-acting elements recognized by WRKYs are incompletely understood, and whether they can bind to other *cis*-acting elements besides the W-box, WLE, PRE4, SURE, and WK-box remains unknown.

Previous studies showed that ThWRKY4 was a Group II WRKY that could increase abiotic stress tolerance, and overexpression of *ThWRKY4* conferred tolerance to salt, oxidative stress, and ABA treatment in the transgenic plants [33]. Further studies showed that ThWRKY4 could improve tolerance to salt and ABA treatment by increasing the activities of superoxide dismutase and peroxidase, decreasing levels of $O_2 \cdot^-$ and H_2O_2, reducing electrolyte leakage, inhibiting the loss of chlorophyll, and protecting cells from death [33]. Microarray analyses showed that overexpression of *ThWRKY4* in *A. thaliana* leads to 165 genes being upregulated significantly. Promoter scanning analysis revealed that ThWRKY4 regulated the gene expression *via* binding to W-box motifs present in their promoters [33]. In the present study, we studied the *cis*-acting elements bound by ThWRKY4 using a Transcription Factor-Centered Yeast one-Hybrid (TF-Centered Y1H) assay [34]. We found that in addition to binding to the W-box core *cis*-element (termed W-box). ThWRKY4 also could bind to a known motif (RAV1A) and an unknown motif (W-box like sequence, WLS). Additionally, screening the distribution of these motifs in the promoters of genes targeted by WRKYs also indicated that RAV1A, W-box, and WLS were all abundant, suggesting that they all play roles in expression regulation mediated by WRKYs. This study will help to reveal the functions of WRKYs in depth.

2. Materials and methods

2.1. TF-Centered Y1H analysis

A random short DNA sequence insertion library was constructed according to Ji et al. as the prey library [34]. The coding sequence (CDS) of *ThWRKY4* was cloned into vector PGADT7-Rec2 as the bait (Clontech, CA, USA) (see Supporting information Table S1 for primers). Y1H screening was performed to determine the *cis*-acing elements bound by ThWRKY4 (GenBank number: JX416193). The positive clones were further subjected to high stringency selection, after which the inserts in the pHIS2 plasmids were sequenced. The insertion sequences, together with the left and the right insertion flanking sequences ("GGG" and "CCC"), were analyzed to determine whether they are known *cis*-acting elements, using the program PlantCARE http://bioinformatics.psb.ugent.be/webtools/plantcare/html/.

2.2. Analyzing the core sequence of the novel motif

The insertion sequence "CCCTTGTCTACGGG" (the insertion flanking sequences are underlined) was not a known motif; therefore, the serial sequences of "TTGTCTAC", "TGTCTAC", "GTCTAC", "TCTAC", "CTAC", "TTGTCTA", "TTGTCT", "TTGTC" and "TTGT", respectively, were interacted with ThWRKY4 to determine the core motif. Three tandem copies of these sequences were cloned into the pHIS2 vector separately as baits. Briefly, pHIS2 was digested with *EcoRI* and *SacI* to generate cohesive ends, and the forward and reverse oligonucleotides containing three tandems of motifs or their mutants were so designed that after annealing they could generate the *EcoRI* and *SacI* cohesive ends, and were then ligated with pHIS2 (see Table S2 for the forward and reverse oligonucleotides), The CDS of *ThWRKY4* was inserted into vector pGADT7-rec2 (Clontech) as the prey according to the user manual (Matchmaker™ One-Hybrid Library Construction & Screening Kit) (see Supporting information Table S1 for the primers). The baits were separately co-transformed with the prey vector into yeast Y187. The transformed yeast cells were selected screened on SD/-Leu/-Trp (DDO) or SD/-His/-Leu/-Trp (TDO) medium supplied with 3-AT (3-Amino-1, 2, 4-triazole).

2.3. Determination the specificity of the bindings of ThWRKY4 to motifs and promoters

To study whether ThWRKY4 could bind to the predicted DNA motifs, three tandem copies of the studied motifs, or their mutants, were inserted separately into vector pHIS2, using the method described in Section 2.2 (see Supporting information Tables S3 and S4 for the forward and reverse oligonucleotides used in the Y1H assay). The motifs were mutated using the following principle: "A/T" was mutated to "C", and "C/G" was mutated to "A". The binding of ThWRKY4 to these sequences was studied using Y1H.

According to the microarray data [33], the genes regulated by ThWRKY4 whose promoters contained RAV1A or WLS motifs were selected for subsequent experiments. Briefly, the promoter sequences of the studied genes (*AT2G41240* and *AT5G50915*) were retrieved from Tair (http://www.arabidopsis.org/), and searched for motifs using the BioEdit program or the Patmatch program on Tair (https://www.arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl). Truncated promoters containing only one of the studied motifs, and truncated promoters lacking the corresponding motif were cloned separately into pHIS2 (see Supporting information Table S1 for primers). Y1H was performed to study the bindings of ThWRKY4 to these truncated promoters.

2.4. Binding assay in tobacco

To further confirm the Y1H results, the bindings of ThWRKY4 to the studied motifs were assayed in tobacco plants [35]. To eliminate the effects of the 35S promoter that controls hygromycin resistance on the expression of the *GUS* gene, the vector pCAMBIA1301 was reformed. The 35S:hygromycin region was removed; at the same time, a 46-bp minimal promoter was used to replace the 35S promoter to drive the *GUS* reporter gene as the reporter constructs. Three tandem copies of the studied motifs, and their mutants were fused with the 46-bp minimal promoter, and cloned separately into reformed vector pC-