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Original Paper

Isolation and molecular characterization of a SoWRKY1 transcription factor from spinach (*Spinacia oleracea*)

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Abstract *WRKY* genes encode proteins which belong to a large family of transcription factors that are involved in various developmental and physiological processes, response to pathogen infections and wound stress in plants. The molecular characteristics of *WRKY* genes involved in these are becoming clear in model plants, like *Arabidopsis*. However, knowledge of *WRKY* genes in other plants, for instance vegetable, is still not enough. In the present studies, using a yeast one-hybrid system we isolated a cDNA from a spinach cDNA library and characterized its function. The cDNA, designated *SoWRKY1*, encodes a putative polypeptide of 362 amino acids which is highly homologous to *Arabidopsis* WRKY40 (AtWRKY40). The putative primary structure of SoWRKY1 contains a single WRKY domain of Cys2His2 zinc finger motif and a potential nuclear localization signal (NLS), whose structure is characteristic of group IIa type WRKY protein. SoWRKY1-sGFP fusion protein was localized to the nucleus when the protein was expressed in onion epidermal cells. SoWRKY1 showed high binding affinity to DNA molecules containing TTGAC(C/T) W-box sequences and transcriptional activation activity in yeast. *SoWRKY1* transcripts in spinach leaves were transiently induced by wounding treatment and salicylic acid (SA). The transcripts accumulated following treatment with cycloheximide (CHX), a protein biosynthesis inhibitor. Functional analysis of SoWRKY1 *in vivo* was performed by overexpression in *Arabidopsis*, and pathogenesis-related *PR1* and *PR2* gene expression level increased in the transgenic plants. These results suggest that SoWRKY1 might be involved as a transcription factor in defense-related signaling transduction pathways of spinach.

Key words: Nuclear localization, plant defense, transactivation, W-box element, WRKY.

In the open air, plants are subjected to constant attack by a variety of pathogens and herbivores, and they have evolved complex defense mechanisms to respond to multiple defense signaling pathways. In plants, many developmental processes and responses to different stress stimuli underlie a complex regulatory mechanism operating at the level of gene expression (Shinozaki and Dennis 2003). Induction of plant defense responses occurs through a highly complex signaling network (Glazebrook et al. 2003; Kunkel and Brooks 2002). Many transcription factors are involved in defense response, in controlling the expression of genes encoding proteins with direct anti-microbial activities or enzymes involved in biosynthesis pathways of defense-related compounds (Eulgem 2005; Singh et al. 2002). WRKY proteins are a family of transcription factors involved in plant response to pathogen infection and a variety of environmental stresses. WRKY transcription factors have been identified in many plants and are encoded by a super family, with more than 74 members in the *Arabidopsis* genome (Ulker and Somssich 2004) and 100 members in the rice genome (Wu et al. 2005).

The name of the WRKY transcription factors is derived from a 60 amino acid-long region, termed the WRKY domain. The WRKY domains contain a conserved WRKYGQK sequence followed by a Cys2His2 or Cys2HisCys zinc-binding motif. Based on the number of WRKY domains and their type of zinc-finger motif, the WRKY proteins were classified into three distinct groups (Eulgem et al. 2000). Group I members contain two WRKY domain and group II and group III members contain one WRKY domain. Group I

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; CaMV35S, cauliflower mosaic virus; CHX, cycloheximide; GFP, green fluorescent protein; GST, glutathione *S*-transferase; JA, jasmonic acid; MCS, multiple cloning site; MeJA, methyl jasmonate; MS, Murashige and Skoog; PCR, polymerase chain reaction; PR, pathogenesis related; SA, salicylic acid; SDS, sodium dodecyl sulfate; 3-AT, 3-aminotriazole; UTR, untranslated region

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and group II members contain a Cys2His2 zinc-finger motif, while group III members contain a Cys2HisCys zinc-finger motif. According to the functional domain, group II splits up into five distinct subgroups (Eulgem et al. 2000). In both *in vivo* and *in vitro* experiments, all WRKY proteins bind specifically to a *cis*-element TTGAC(C/T), termed the W-box (Eulgem et al. 2000). The W-box is frequently present in the promoters of a large number of plant defense-related genes, including *WRKY* itself (Dong et al. 2003) and *PR* genes (Maleck et al. 2000).

The expression of some WRKY factors is regulated by pathogen infection and wounding or treatment with pathogen-derived elicitor, SA (salicylic acid) and JA (jasmonic acid) (Chen and Chen 2002; Mzid et al. 2007; Liu et al. 2007; Oh et al. 2006). Some members have been reported to confer resistance upon pathogen (Knoth et al. 2007; Zheng et al. 2006). On the other hand, several WRKY factors act as negative regulator in pathogen resistance (Journot-Catalino et al. 2006; Wei et al. 2008). WRKY factors act as transcriptional activators or repressors of genes encoding proteins with regulatory functions. *Arabidopsis* WRKY18 (AtWRKY18) increases the expression of *PR* genes (Chen and Chen 2002). AtWRKY6 is reported to negatively repress its own expression, but positively activate *PR1* gene expression, suggesting that AtWRKY6 can modulate both positive and negative transcriptional regulator (Robatzek and Somssich 2002).

The molecular characteristics of *WRKY* genes are becoming clear in model plants, like *Arabidopsis*. Although clarification of role of *WRKY* genes in economic plants is important, knowledges of the genes in plants other than model plants, for instance vegetable, were still not enough. In this study, we isolated a cDNA, designated as *SoWRKY1*, from spinach (*Spinacia oleracea*), which is a homologue of AtWRKY40 using a yeast one-hybrid system. The ability as a transactivator of SoWRKY1 proteins was demonstrated *in vivo* and *in vitro*. These results indicate that SoWRKY1 might also be involved in spinach defense response as a transactivator.

Materials and methods

Plant materials and growth conditions

Suspension-cultured spinach (*Spinacia oleracea* L. cv. Hoyo) cells (Nakagawa et al. 1985) were cultured in conical flasks on a rotary shaker at 140 rpm at 25°C under continuous light conditions. Every 8 days, 10 ml of the suspension culture was subcultured in a 40 ml Murashige and Skoog (MS) medium containing 0.5 mg l^{-1} α-naphathaleneacetic acid and 1 mg l^{-1} benzyladenine. Spinach plants were used for expression analysis of *SoWRKY1 in vivo*. Spinach plants were grown in plastic pots containing humus in growth cabinet controlled for 12 h light (150 μE m^{-2} s^{-1}) at 23°C and 12 h dark at 18°C.

Yeast one-hybrid screening

Total RNA was extracted from one-day-old suspension culture cells using the standard acid guanidium thiocyanate-phenol-chloroform method (Chirgwin et al. 1979). Poly(A)$^+$RNA was isolated from total RNA using Oligotex^{TM}-dT30 <Super> mRNA Purification Kit (TaKaRa Bio Inc., Otsu, Japan). A cDNA library was constructed in pAD-GAL4-2.1 phagemid vector (Stratagene, CA, USA) using a poly(A)$^+$RNA mixture from cultured spinach cells. A synthesized DNA fragment containing 10 tandem copies of the 5 bp W-box sequence 5′-AATTCAGTCAAGTCAAGTCAAGTCAAGTCATCAGTCA AGTCAAGTCAAGTCAAGTCAT-3′ was ligated at *Eco*RI and *Xba*I site into the multiple cloning site (MCS) of the pHISi vector (Clontech, CA, USA). This plasmid was introduced into the yeast YM4271 strain. Library plasmids were introduced into yeast containing the *HIS3* reporter gene and screened for clones on selection medium plates lacking histidine (–His) and leucine (−Leu) containing 15 mM 3-aminotrizole (3-AT). The positive clones were isolated, and the nucleotide sequences of the inserted cDNAs were determined.

Subcellular Localization of SoWRKY1-sGFP

The *SoWRKY1* cDNA was subcloned between the cauliflower mosaic virus (CaMV) 35S promoter and the green fluorescent protein (sGFP, which is a substitution of serine for threonine at position 65 residues in the primary structure of GFP) reporter gene in pUC119 (TaKaRa Bio Inc.) to express sGFP fusion protein. The CaMV35S : sGFP vector without *SoWRKY1* was used as a control. The resulting constructs were introduced into onion (*Allium cepa*) epidermal cells by the particle delivery method with a PDS1000/He (Biolistic Particle Delivery System, Bio-Rad, CA, USA). After bombardment, onion pieces were incubated for 12 h at 22°C in the dark. Then the peels were observed through a green fluorescence microscope, ECLIPSE TE300 (Nikon, Tokyo, Japan).

Recombinant protein and Random oligonucleotide selection

The *SoWRKY1* cDNA was cloned into the expression vector pGEX-5X-1 (Amersham Biosciences, NJ, USA). *Escherichia coli* strain DH5α was then transformed with the vector. The GST-SoWRKY1 fusion protein was induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) when the cells were grown at log phase at 37°C. After 3 h, the cells were collected by centrifugation and suspended in ice-cold PBS (phosphate-buffered saline) containing 1 mM DTT (dithiothreitol) and 1 mM PMSF (phenylmethylsulfonyl fluoride). The cells were lysed by sonication on ice. The GST-SoWRKY1 fusion proteins were purified using MicroSpin GST Purification Module (Amersham Biosciences). The protein concentration was determined by Bradford protein assay using bovine serum albumin as a standard.

The single-stranded 57-mer oligonucleotide, 5′-CGCGAA-TTCGGATCCTAGCN$_{19}$CGTCGTCGACTCGAGTCGA-3′ containing 19-mer random nucleotides flanked by 19 bases of defined sequences on either side, was used as a template for PCR. The PCR primer nucleotides used for amplification of the selected sequences were 5′-CGCGAATTCGGATCCTAGC-3′ and 5′-TCGACTCGAGTCGACGACG-3′. The binding-site selection was performed as described bellow. The purified PCR

products were incubated with GST-SoWRKY1 and glutathione-Sepharose beads, and the mixture was gently mixed at 4°C for 30 min. The mixture was then washed seven times using 1×binding buffer (25 mM HEPES-KOH, pH 7.5, 100 mM KCl, 10 μM $ZnSO_4$, 0.1% (v/v) NP40, 1 mM DTT and 5% (v/v) glycerol). The mixture was suspended with the 1×binding buffer and treated with proteinase K. The proteinase K-treated liquid was subjected to phenol-chloroform extraction and then ethanol precipitation. The purified DNA fragment was separated in a 10% (w/v) polyacrylamide gel and DNA was extracted from approximately 57 bp bands and purified according to the QIAEX II Handbook (QIAGEN). These processes (binding DNA, amplification of DNA fragments by PCR and purification of DNA fragments from gels) were repeated eight times. The purified DNA fragments were cloned into pGEM-T vector. The nucleotide sequences were analyzed by using DNA sequencing kit and DNA sequencer (Applied Biosystem).

DNA-binding affinity: β-galactosidase assay in yeast one-hybrid system

The five clones containing W-box in the pGEM-T vector (Promega) selected by random oligonucleotide selection were digested with *Eco*RI and *Sal*I, then subcloned into the corresponding restriction sites of pHSG398 (TaKaRa Bio Inc.) vector, then subcloned into the corresponding restriction sites of pLacZi (Clontech) reporter vectors. On the other hand, the same five clones containing W-box in T-vectors were amplified by PCR with modified primers with *Hin*dIII and *Kpn*I restriction sites (*Hin*dIII site primer 5′-TAAAGCTTT-CGACTCGAGTCGACGACG-3′, and *Kpn*I site primer 5′-TCGGTACCCGCGAATTCGGATCCTAGC-3′), respectively. The resulting PCR products were cloned into the *Hin*dIII and *Kpn*I sites of the pLacZi vectors in the same way. As a result, five DNA fragments were individually ligated into the MCS of the pLacZi vectors in one direction or opposite direction, and their constructs were integrated into the yeast YM4271 strain. The vector pGAD424 (Clontech) containing *SoWRKY1* cDNA (pGAD424-*SoWRKY1*) was also introduced into the yeast. The transformed yeast clones were selected in Synthetic Defined (SD) (−Ura, −Leu) medium. For the control plasmid, pGAD424 without *SoWRKY1* cDNA was introduced into the yeast. β-galactosidase activity in yeast transfomant was assayed in liquid culture using *o*-nitrophenyl-β-D-galactopyranoside as a substrate according to the Yeast Protocols Handbook (Clontech).

Transactivation analysis of SoWRKY1 protein

Transactivaton activity of SoWRKY1 proteins was analyzed by the two-hybrid method with modification. The pBD-GAL4 Cam vector (Stratagene) containing a *SoWRKY1* cDNA (pBD-*SoWRKY1*) was introduced into the yeast YRG-2 strain, which contains the *HIS3* and *lacZ* reporter genes in its genome. The clone was used in the 3-AT dropout analysis and β-galactosidase assay. The 3-AT dropout analysis was carried out to determine the 3-AT concentration at which the yeast transformant remained viable on alimentative selection medium plates lacking histidine (−His) and tryptophan (−Trp) containing various concentrations of 3-AT (0–40 mM).

The expression of SoWRKY1 gene response to various chemicals and wounding

For wounding treatment, detached young leaves from spinach plants grown for 4 weeks were pierced randomly with a needle and kept on the moist filter paper in petri dishes. Control samples without wounding were also kept in petri dish at the same condition. For chemical treatment, leaves of intact plants were sprayed with solutions of 5 mM SA in water, 200 μM MeJA in 2% (v/v) ethanol, 200 μM ACC (1-aminocyclopropane-1-carboxylic acid, the immediate precursor of ethylene) in water, 100 μM CHX in water. Mock treatments were performed by spraying plants with water. Control leaves (0 h) were harvested before treatment. Leaves were then harvested at various time intervals after treatment, respectively. The harvested leaves were used immediately for RNA extraction. RNA was extracted using the conventional SDS-phenol method and stored at −80°C (Mizuno et al. 2006).

Northern blot analysis was performed using a DIG Northern Starter kit (Roche, Basel, Switzerland). DIG-labeled RNA probe was prepared from full-length *SoWRKY1* cDNA by a run-off transcription method using DIG Starter Kit (Roche). Hybridization was carried out overnight at 68°C in a hybridization solution (5×SSC, 1% (v/v) blocking solution, 0.1% (v/v) *N*-lauroyl sarcosinate, 0.02% (w/v) SDS and 50% (v/v) formamide) with the probe. The transcripts hybridized with the probe were detected using LAS-1000 (FUJIFILM, Tokyo, Japan).

Transformation of Arabidopsis plants and expression analysis of PR genes

The pGEX-5X-1 vector (Amersham) harboring the full coding sequence cDNA sequence of *SoWRKY1* was digested by *Bam*HI and *Not*I and subcloned into the same restriction sites of the vector s221n, under the transcriptional control of the CaMV35S promoter and further subcloned into the binary Ti plasmid vector pEKH (Takesawa et al. 2002) containing a neomycin phosphotransferase II gene (NPT II, kanamaycin resistance). The resulting vector pEKH-*SoWRKY1* containing the full coding sequence of *SoWRKY1* in the sense orientation downstream of the CaMV35S promoter was mobilized into *Agrobacterium tumefaciens* EHA105 strain. The *Arabidopsis* (*Arabidopsis thaliana*) ecotype used was *Columbia*-0 (*Col*-0). The infection was performed according to a published protocol (Glazebrook and Weigel 2002). The seeds were collected from the infiltrated plants and transformats were selected on 1% (w/v) agar plates with half the concentration of MS medium, 1.5% (w/v) sucrose and 50 μg ml^{-1} kanamycin. 20 kanamycin-resistant plants were transferred to pots 10 days after germination and grown in a growth cabinet at 23°C under light (100 μE m^{-2} s^{-1}) with short day photoperiod (8h-light/16h-dark). Expression levels of exogenous *SoWRKY1* were analyzed in independent transgenic T3 plants carrying single transgene insertion loci per construct. Two lines (L7 and L18) expressing high levels of *SoWRKY1* were used for the expression analysis of *PR* genes (*PR1, PR2, PR3, PDF1.2*).

Total RNA was extracted from the leaves of 5-weeks-old wild type (WT) and transgenic plants using the conventional SDS-phenol method, and the expression of *PR* genes were analyzed by northern hybridization described above. Each RNA probe of *PR* genes was prepared using the following plasmids

as templates and a DIG Northern Starter kit (Roche). DNA fragments were amplified with PCR using Riken Arabidopsis Full-Length (RAFL) cDNAs (*PR1*, cDNA clone number name: RAFL06-68-J19; *PR2*, RAFL09-66-C10; *PR3*, RAFL05-12-F22; and *PDF1.2*, RAFL06-82-G15) as templates and primer pairs (forward primer, cDNA clone specific common sequence: 5′-CCTCGAGTTAATTAAATTAATCC-3′; reverse primer, *PR1* gene specific primer: 5′-CATAATTCCCACGAGGATCA-3′, *PR2* gene specific primer: 5′-CTTCTCATACGTTGGTT-CCT-3′, *PR3* gene specific primer; 5′-TAATACTCTCGTTAC-TAAAT-3′, *PDF1.2* gene specific primer: 5′-TGTAAC-AACAACGGGAAA-3′), respectively. These PCR products were subcloned into pGEM-T vectors (Promega), and these plasmids were used as templates for probes.

Results

Cloning of SoWRKY1 cDNA gene

The yeast one-hybrid screening method was used to isolate cDNAs encoding WRKY transcription factors that interact with the TTGACT sequence in various gene promoters. From the screening of more than 1×10^6 yeast clones, 155 positive clones were isolated. Of these, 16 clones were analyzed. The nucleotide sequences of the inserted cDNA were determined and 9 clones were the same. The full-length cDNA was isolated by rapid amplification of cDNA ends. It contained a 465 bp 5′ untranslated region (UTR) and a 376 bp 3′ UTR with a poly(A) tail. The putative open reading frame encodes a protein of 362 amino acids (Figure 1). The deduced amino acid sequence of the transcription factor contained a WRKY DNA-binding domain and a zinc finger motif ($C\text{-}X_5\text{-}C\text{-}X_{23}\text{-}H\text{-}X_1\text{-}H$). According to the PSORT program (PSORT, prediction program of protein localization sites, http://psort.nibb.ac.jp), the SoWRKY1 sequence revealed the presence of a putative nuclear localization signal, KKRK, between positions 108 and 111 of the amino acid sequence. Comparison of the deduced amino acid sequence available in NCBI databases revealed that SoWRKY1 exhibited high sequence similarity to WRKY protein group IIa, including GmWRKY27, NtWIZZ, AtWRKY18, AtWRKY40, AtWRKY60, OsWRKY62 and OsWRKY71 (Figure 2).

SoWRKY1 is localized in nucleus

To analyze the subcellular localization of SoWRKY1 protein, we performed a transient expression analysis of the sGFP fusion protein in onion epidermal cells by particle delivery. SoWRKY1-sGFP fusion proteins were exclusively localized in the nucleus, whereas the control unfused sGFP protein was found throughout the cells (Figure 3). These results indicate that SoWRKY1 was a nuclear-localized protein, in consistent with its function as a transcriptional regulator.

SoWRKY1 binds to W-box elements

WRKY proteins have been identified as transcription factors to bind DNA sequences containing W-boxes (Ishiguro and Nakamura 1994; Ulker and Somssich 2004). In order to determine the DNA sequence that SoWRKY1 proteins bind to, random oligonucleotide selection was performed. *SoWRKY1* was expressed in *E.coli* as a fusion-protein with GST. Purified GST-SoWRKY1 fusion protein on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was a single band with an approximate molecular mass of 68 kDa which corresponded to GST-SoWRKY1 fusion protein (data not shown). This purified GST-SoWRKY1 fusion protein was used to select binding DNA sequences from a population of 4^{19} different molecules. The selected molecules were cloned and 77 individual colonies were analysed (Figure 4). All 77 clones contained TTGAC consensus sequence. To date, plant WRKY transcription factors have shown high binding preference to the DNA sequence element, 5′-TTGAC(C/T)-3′, termed the W-box (Eulgem et al. 2000). The invariant TGAC core sequence of the W-box is essential for binding of WRKY protein. 7 out of 77 clones had one TGAC W-box core sequence and the other 70 clones had two core sequences. These results suggest that GST-SoWRKY1 can bind single TTGAC(C/T) W-box and may bind more strongly to the tandem W-box core sequences.

DNA-binding affinity of SoWRKY1 protein in vivo

To confirm whether there is a difference in binding affinity for SoWRKY1 to selected sequence *in vivo*, we performed yeast one-hybrid system with 5 selected

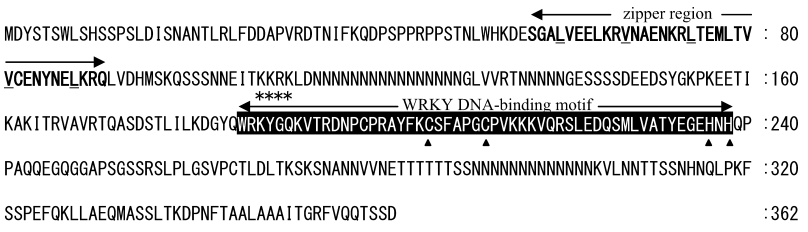

Figure 1. Deduced amino acid sequence of SoWRKY1 (362 amino acids). The putative leucine zipper motifs with five modules (7 amino acids) at the N terminus are shown in bold and the fourth residues (Leucine, Valine) of five modules are underlined. The putative nuclear localization signal is indicated by asterisks. The conserved WRKY domain is shown in black box, the two cysteines and the two histidines of the zinc-finger motif are indicated by arrow heads.

sequences indicated in Figure 4B as bait. β-galactosidase activity was higher than the control. In either direction, SoWRKY1 preferentially bound to the TTGAC×2 sequence (Figure 4, Figure 5).

Transactivation potential of SoWRKY1 protein

Based on the primary structure, SoWRKY1 contains a putative activator domain of the proline-rich type (PSPPRPPS, Triezenberg 1995). The transactivation potentials of SoWRKY1 protein were measured by a yeast GAL4 two-hybrid reporter assay. The coding region of SoWRKY1 was introduced into pBD-GAL4 Cam vector containing GAL4 DNA binding domain. The yeast transformant harboring *SoWRKY1* could grow on medium plates containing up to 25 mM 3-AT (Figure 6B). In contrast, the control transformant without *SoWRKY1* could not grow in the presence of 3-AT. These transformants were also used for the β-galactosidase assay. The transformant harboring *SoWRKY1* exhibited about 5 times higher β-galactosidase activity (0.269) than control transformants (0.057) (Figure 6C). These results indicated that SoWRKY1 contains an activation domain and suggest that SoWRKY1 acts as an activator in transcription.

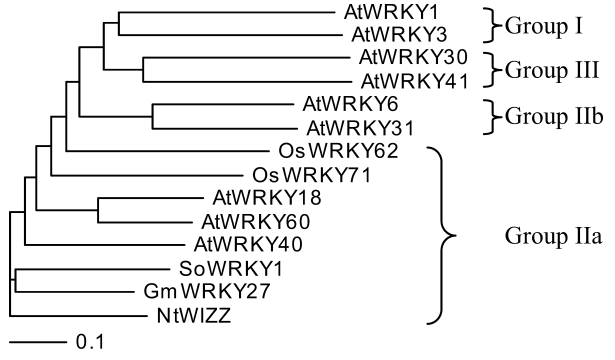

Figure 2. Phylogenetic tree of a selection of WRKY proteins. SoWRKY1 belongs to group IIa of the plant WRKY family. The deduced amino acid sequence of WRKY genes was aligned using CLUSTALW program. The phylogenetic tree was created using the TreeView program. Accession number: AtWRKY1, AB493546; AtWRKY3, AK229036; AtWRKY6, AB493514; AtWRKY18, AF428421; AtWRKY30, AK117885; AtWRKY31, AY052648; AtWRKY40, AK317045; AtWRKY41, AY133686; AtWRKY60, AK227987; OsWRKY62, AK067834; OsWRKY71, AB190817; GmWRKY27, DQ322695; NtWIZZ, AB028022; SoWRKY1, AB081952. The scale bar at the bottom left displays a distance corresponding to 0.1 amino acid substitutions per site. At, *Arabidopsis thaliana*; Os, *Oryza sativa*; So, *Spinacia oleracea*; Nt, *Nicotiana tabacum*; Gm, *Glycine max*.

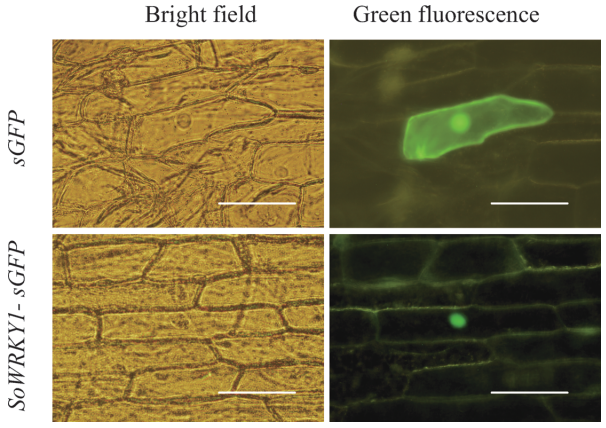

Figure 3. Nuclear localization of SoWRKY1. SoWRKY1-sGFP was transiently expressed in onion epidermal cells. Bright field images of the respective onion epidermal cells are shown on the left. SoWRKY1-sGFP is visible solely in the nucleus (lower right), whereas the control sGFP protein is present throughout the entire cell (top right). Scale bars, 100 μm.

A

5'-CGCGAATTCGGATCCTAGCNNNNNNNNNNNNNNNNNNNCGTCGTCGACTCGAGTCGA-3'
3'-GCGCTTAAGCCTAGGATCGNNNNNNNNNNNNNNNNNNNGCAGCAGCTGAGCTCAGCT-5'

B

DNA sequence	colonies	name
CGCGAATTCGGATCCTAGCATT**TGAC**T**TGAC**CAGCGACCGTCGTCGACTCGAGTCGA	1/77	W1
CGCGAATTCGGATCCTAGCTGGACCCT**TGAC**CTGGTCCCGTCGTCGACTCGAGTCGA	7/77	W2
CGCGAATTCGGATCCTAGCGCCACCT**TGAC**T**TGAC**TTCCGTCGTCGACTCGAGTCGA	9/77	W3
CGCGAATTCGGATCCTAGCGTCTCGGCT**TGAC**CT**TGAC**CGTCGTCGACTCGAGTCGA	10/77	W4
CGCGAATTCGGATCCTAGCCGCGACTT**TGAC**TG**TGAC**CCGTCGTCGACTCGAGTCGA	50/77	W5

Figure 4. Determination of SoWRKY1 binding site. (A) Doubled-stranded DNA oligonucleotide containing 19 bp random region flanked by 19 bp fixed sequence were used for random oligonucleotide selection. Arrows indicated position of primers used by PCR amplification. (B) The sequence of five selected SoWRKY1-binding element containing W-boxes. W-boxes are underlined and W-box core sequences are indicated in bold. Numbers are indicated the colony numbers the same sequence in 77 colonies.

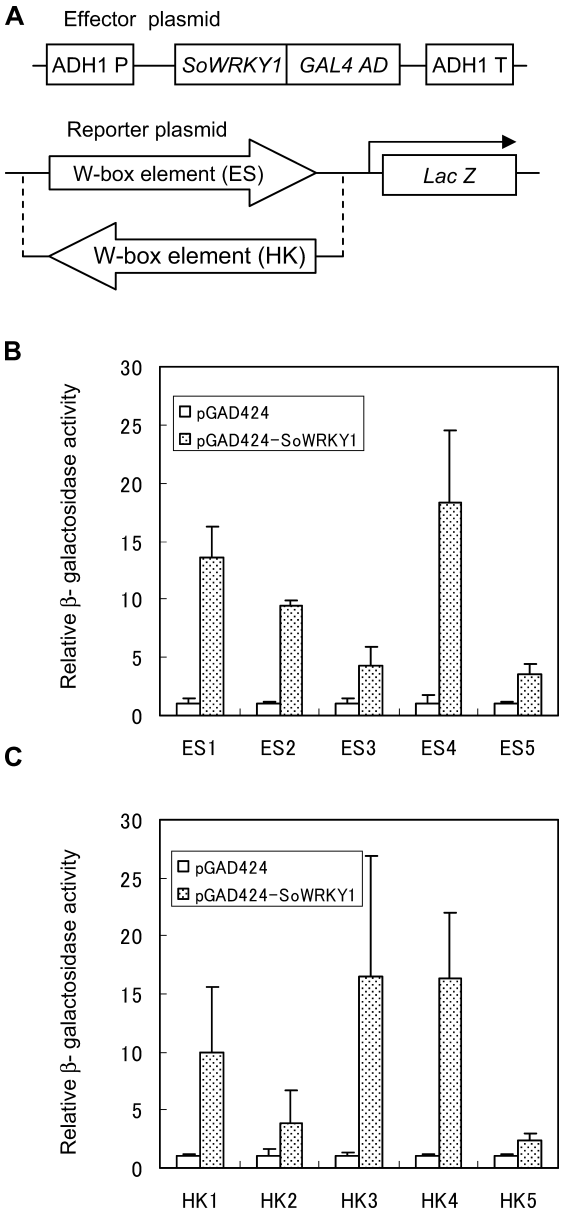

Figure 5. DNA binding affinity of SoWRKY1. (A) Srtucture of plasmids used for the one-hybrid system. (B) Binding activity of SoWRKY1 to five selected fragments containing W-boxes in one direction (B, ES1～5) and opposite direction (C, HK1～5) by the yeast one-hybrid system. Open and solid bars represent pGAD424 and pGAD424-*SoWRKY1*, respectively. Error bars represent ±SD of data derived from five or six independent experiments.

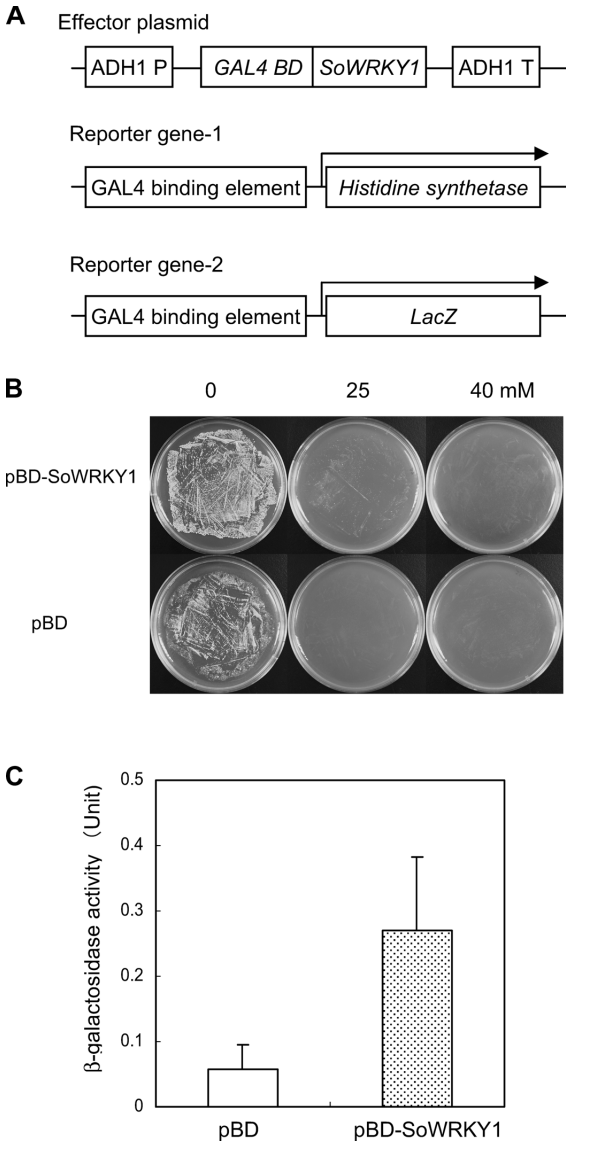

Figure 6. Transactivation analysis of SoWRKY1 in yeast YRG-2. (A) Structure of plasmid used for transactivation assay. (B) The SoWRKY1 transformants can grow on medium plates up to 25 mM 3-AT. In contrast, the control vectors only grow in medium plate without 3-AT. (C) Transcriptional activity of fusion vector of pBD-*SoWRKY1* and control vector pBD-GAL4 Cam. Error bars represent ±SD of data derived from six independent experiments.

Expression patterns of SoWRKY1 gene: SoWRKY1 is induced by wounding and SA

Different signaling pathways were involved in induction or suppression of WRKY family genes after infection with plant pathogens and wounding stress. Transcription of *SoWRKY1* was increased after wounding (Figure 7A). Transcript accumulation of *SoWRKY1* starts as early as 15 min after wounding, and reached a peak at 1 h post wounding, and then decreased dramatically. The expression of WRKY transcription factors has often been shown to be altered after treatments with compounds and signaling molecules involved in wounding stress like SA, JA, and ethylene, which are intensively investigated pathways (Oh et al. 2006; Park et al. 2006; Marchive et al. 2007). To examine what chemicals induce *SoWRKY1* transcripts, its expression patterns were analyzed after treatment of SA, MeJA (methyl jasmonate) and ACC (Figure 7B). Plants sprayed with SA accumulated higher levels of *SoWRKY1* transcripts than water-sprayed plants, whereas plants sprayed with MeJA and ACC (which releases ethylene after application) accumulated less. Interestingly, by treatment with protein synthesis

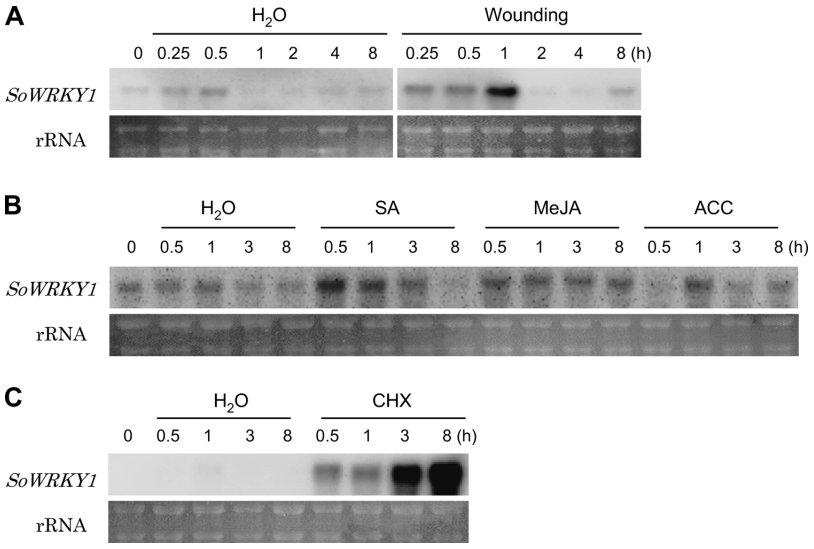

Figure 7. Expression patterns of *SoWRKY1* gene. (A) Expression of the *SoWRKY1* gene in response to wounding. (B) Expression of the *SoWRKY1* gene upon treatment with salicylic acid (SA), methyl jasmonate (MeJA), 1-aminocyclopropane-1-carboxylic acid (ACC). Spinach leaves were sprayed with 5 mM SA, 200 μM MeJA in 2% (v/v) ethanol, 200 μM ACC and water. (C) Expression of the *SoWRKY1* gene upon cycloheximide (CHX). h, hours after treatment. As controls for equal RNA loading, ribosomal RNA of the ethidium bromide-stained gel were used.

inhibitor CHX, the induction of the gene after wounding was conserved in 30 min, the maximum abundance was observed at 3 h and continued 8 h (Figure 7C). Similar accumulation of transcripts following CHX treatment was observed in *AtWRKY 33* (Lippok et al. 2007) and this accumulation of *SoWRKY1* may be the results of either transcriptional activation or increased mRNA stability.

Expression of target genes encoding PR proteins in transgenic plants overexpressing SoWRKY1

Ectopic overexpression of several *WRKY* genes often resulted in enhanced resistance against various pathogens (Chen and Chen 2002; Li et al. 2004). In most cases, this increase of resistance was correlated with an increase in expression of *PR* genes (Cao et al. 1998; Xu et al. 2006). The putative role of SoWRKY1 in plant was also determined by overexpressing the cDNA under the control of the CaMV35S promoter in *Arabidopsis* plants, since spinach transformation still present several difficulties. None of the generated transgenic plants showed any visible morphological changes compared with WT plants under normal growth condition.

The expression level of *PR* genes in independent transgenic lines was analyzed by using northern blot analysis. The *SoWRKY1* transcript levels were higher in transgenic lines than that in WT plants. Coordinated with *SoWRKY1* transcripts level, the expression level of *PR2* was enhanced in *SoWRKY1*-overexpressing transgenic plants, whereas no expression was found in WT plants. The expression levels of *PR1* were slightly higher in transgenic lines than that in WT plants. *PR3* and *PDF1.2* gene expression were not detected in both transgenic and

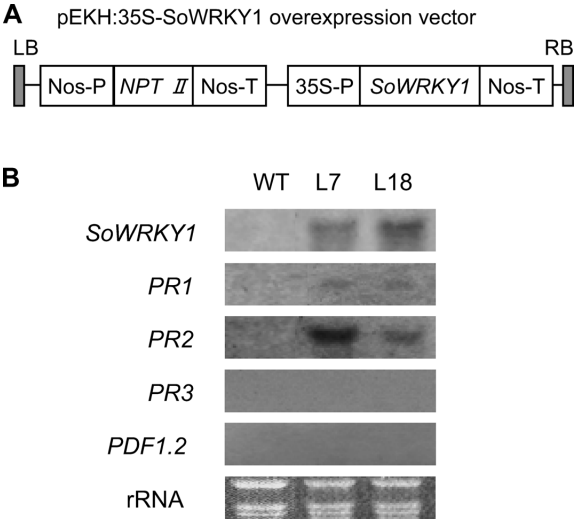

Figure 8. *PR* genes expression in transgenic *Arabidopsis* plants overexpressing *SoWRKY1*. (A) Schematic illustration of T-DNA region used in the transformation experiments. LB and RB, the left and right borders of the T-DNA. Nos-P, nopaline synthase promoter; NPT II, neomycin phosphotransferase II; Nos-T, nopaline synthase terminator; 35S-P, cauliflower mosaic virus 35S promoter. (B) Expression pattern of *PR1*, *PR2*, *PR3* and *PDF1.2* in transgenic *Arabidopsis* plants overexpressing *SoWRKY1*. AGI code; *PR1*, At2g14610; *PR2*, At3g57260; *PR3,* At3g12500; *PDF1.2*, At5g44420.

WT plants (Figure 8). These results suggest that in *Arabidopsis* exogenous SoWRKY1 as well as AtWRKY18 and 70 might function as a transcriptional activator upstream of *PR1* and *PR2* in defense response signaling pathways.

Discussion

Since the first WRKY cDNA (SPF1) was identified from sweet potato (Ishiguro and Nakamura 1994), many WRKY proteins have been cloned from various plants. Accumulating knowledge of WRKY proteins by analyzing mainly model plant help us to understand many plant processes including plant responses to biotic and abiotic stresses. However, it is still not enough that molecular characteristic of WRKY protein in plants excluding model plant. In this study, we isolated *SoWRKY1* from spinach using a one-hybrid system and characterized the functions as transcription factors.

SoWRKY1 has one zinc-finger domain composed of Cys2His2. Phylogenetic analysis showed that SoWRKY1 is most similar to GmWRKY27 and NtWIZZ. These data indicate that SoWRKY1 is a group IIa member of the WRKY superfamily. Subgroup IIa comprise three members in *Arabidopsis*, AtWRKY18, AtWRKY40, and AtWRKY60, playing important and partly redundant functions in regulating plant disease resistance (Xu et al. 2006). The N-terminal leucine zipper motifs of these members were shown to mediate homodimerization and heterodimerization of each other (Xu et al. 2006). SoWRKY1 also contains a leucine zipper motif in the N-terminal and might form a dimer in plants. Xu et al. reported that *Arabidopsis* overexpressing *AtWRKY18* are significantly smaller in size than WT (Xu et al. 2006). However, *Arabidopsis* overexpressing exogenous *SoWRKY1* showed no morphological change. These difference of phenotype suggested that it might be difficult to make a heterodimer with AtWRKY.

WRKY proteins were described as transcription factors which were capable of binding the W-box sequence elements (Eulgem et al. 2000) that are present in the promoters of a large number of defense genes including *PR* genes. SoWRKY1 also specifically bound to the TGAC W-box core sequence. The ratio of the number of clones isolated at the *in vitro* experiment to determine the binding sequence was not correlated with the ratio of binding affinity to each sequence in yeast one-hybrid system (Figure 4, Figure 5B, 5C). This discrepancy is likely to be cause by difference between *in vivo* and *in vitro*. In vivo, the binding affinity of SoWRKY1 to W-box was not related to the direction of W-box, but was influenced by the sequence in the neighborhood of W-box (Figure 4, Figure 5). As in the case of W1, W3, W4, the tandem W-box sequence (TTGAC(C/T)) which is not a spacer sequence, had a higher affinity for SoWRKY1 than single W-box. WRKY transcription factors up- or down-regulate gene expression. Transcriptional activation of SoWRKY1 assessed by measuring activation of the histidine synthetase and β-galactosidase dual reporter genes showed that this protein was a transcriptional activator. Thus, it is possible to predict that SoWRKY1 might induce the expression of genes containing W-box in their promoters. When *AtWRKY18* or *AtWRKY70* was overexpressed in *Arabidopsis*, transgenetic plants exhibited enhanced transcription level of *PR1*, *PR2* and *PR5* genes (Chen and Chen 2002; Li et al. 2004). The expression level of *PR1* and *PR2* were also upregulated in *Arabidopsis* overexpressing *SoWRKY1* (Figure 8B). This result suggests that SoWRKY1, like AtWRKY18 or AtWRKY70, can activate the expression of the *PR* genes and functions in plant defense-related signaling in *Arabidopsis*. Because many *PR* genes have been isolated from various higher plants including *Arabidopsis*, Tobacco, Cucumber, Tomato and Barley (Sels et al. 2008), some *PR* genes are likely present in the spinach genome. SoWRKY1 may also control the defense-related signaling through the transactivation of these *PR* genes in spinach. In *Arabidopsis*, both *PR1* and *PR2* genes induced by *SoWRKY1* contain W-box in their promoter sequence. *PDF1.2* gene, which does not contain W-box, was not induced in this transgenic plant. However, the *PR3* gene containing a W-box in its promoter sequence was not induced. Therefore, the regulation of these gene expressions may be more complicated.

Different signaling pathways are involved in activation of transcription of defense genes under biotic and abiotic stresses. The SA and JA/ET pathways, which are intensively investigated pathways, are able to differentially induce *PR* genes expression depending on the type of pathogen (Glazebrook et al. 2003). The expression of *SoWRKY1* in spinach leaves was investigated after treatment with these chemicals. As shown in Figure 7B, SA induced *SoWRKY1* gene expression; on the other hand, ACC and MeJA did not induce a visible change. Moreover, accumulation of the *PR1* gene, a reliable molecular marker of SAR, suggests that SoWRKY1 can participate under SA and induce the *PR1* gene.

Wounding stress immediately increased the expression of *SoWRKY1*. After 3 h, no mRNA encoding *SoWRKY1* could be detected. A fast and transient induction of immediate early transcription factor has been described in *OsWRKY71* and *CaWRKY2* (Liu et al. 2007; Oh et al. 2006), and is considered as a common feature of these genes. Interestingly, by treatment with cycloheximide, transcript of the gene was maintained at a high level even after 8 h. It appears that cycloheximide inhibited the synthesis of suppressor of *SoWRKY1* or protein involved in RNA degradation.

In this study, we characterized the general function of SoWRKY1 as a transcription factor. This study has also revealed a part of roles of SoWRKY1 upon wounding. Further analysis of this gene will help us understand the complex regulation mechanisms of gene expression in

response to wounding stress in spinach.

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