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Identification of a Pathogen-Induced *Glycine max* Transcription Factor *GmWRKY1*

Sang Gu Kang*, Euiho Park and Kum Sook Do

Department of Biotechnology, School of Biotechnology, Yeungnam University, Gyeongsan 712-749, Korea

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On screening pathogen-resistant soybean, we identified a WRKY type transcription factor named a *Glycine max* WRKY1 (*GmWRKY1*). Expression of *GmWRKY1* gene was induced in the soybean sprout by *Pseudomonas* infection. The *GmWRKY1* was expressed in all of the tissues with high levels in stems, leaves and developing seeds. The protein GmWRKY1 contains highly conserved two WRKY DNA-binding domains having two C_2-H_2 zinc-finger motif (C-X_{4-5}-C-X_{22-23}-H-X-H) in its N-terminal and C-terminal amino acid sequences. In electrophoresis mobility shift assay, the GmWRKY1 protein bound specifically to W-box elements in the promoters of defense related genes. These results demonstrated that *GmWRKY1* is one of the soybean WRKY family genes and the plant-specific transcription factors for defense processes.

Keywords : soybean, WRKY, W-box element, zinc-finger protein

The innate immune systems of plants acquired by the ways of the pathogen associated molecular pattern-triggered immunity (PTI) and the effector-triggered immunity (ETI) (Jones and Dangl, 2006). PTI is elicitated by pathogen microbe-associated molecular patterns which are activated by MAP-kinase cascades for getting Arbidopsis innate immunity (Asai et al., 2002). ETI, a major concept of gene-for-gene resistance, is triggered by plant disease resistant (R) proteins that initiate more efficient defense systems by specific recognition of effectors from certain pathogens (Jones and Dangl, 2006). When plants are exposed by PTI and ETI, plants initiate systematic acquired resistance (SAR) which is long-distance defense reaction against pathogens involving the specific hormones such as salicylic acid (SA) and jasmonic acid (JA) (Bostock, 2005). To conduct innate immunity systems in plants, members of transcription factor families specifically functioned as quantitative and temporal in defense transcriptome in response to effectors (Katagiri, 2004; Eulgem and Somssich, 2007). In plants, a group of zinc-finger type WRKY transcription factors are targeted to bind with specific promoters of defense genes containing W-box (C/TTGACC/T) and involve in regulating innate immunity and defenses.

WRKY proteins form a large transcription factor family with more than 70 in Arabidopsis and 100 in rice have been reported (Eulgem and Somssich, 2007). WRKY proteins are specifically named by containing the WRKY domain, which is a 60-amino acid stretch containing a conserved amino acid sequence of WRKYGQK together with a zinc-finger like motif (Eulgem et al., 2000). Some of WRKY proteins contain two WRKY domains in each protein (Eulgem et al., 1999; Cormack et al., 2002). The zinc-finger type WRKY proteins work as transcription factor target to bind to the W-box which is a cis-acting DNA element found frequently in the promoter of defense-related genes (Chen and Chen, 2000; Du and Chen, 2000; Rushton et al., 1996; Yang et al., 1999; Yu et al., 2001). Because of conserved of peptide motifs in WRKY proteins, WRKY transcription factors are classified based on phylogentic relationships (Eulgem et al., 2000; Eulgem and Somssich, 2007). Zinc-finger-containing WRKY DNA-binding domain of WRKY transcription factors is very unique and stereotypic binding preference to W-box. However, each WRKY transcription factor possesses specific binding affinity to its targeting W-box containing promoter of genes, resulting in distinct functions in defense signaling with gene to gene networking (Ciolkowski et al., 2008). Recently, in canola (*Brassica napus* L.), 13 *BnWRKY* genes were identified in responsive to fungal pathogens, *Sclerotinia sclerotiorum* and *Alternaria brassicae*, and hormone treatments, suggested that the selected BnWRKY proteins were involved in the transcriptional regulation of defense-related genes in response to fungal pathogens and hormone stimuli (Yang et al., 2007).

Many WRKY proteins involved in regulating plant defense responses. Recently, it has been discussed that the families of WRKY genes were shown to be functionally connected forming a transcriptional network in central positions for mediating fast and efficient activation of defense programs (Eulgem and Somssich, 2007). In the plant defense mechanisms, the roles of WRKY transcription

*Corresponding author.
Phone) +82-53-810-3025, FAX) +82-53-810-4769
E-mail) kangsg@yu.ac.kr

factors are slightly different among the groups of WRKY families. For example, *AtWRKY52* genes conferred resistance against bacterial pathogens acting as R protein (Deslandes et al., 2002). For example, *AtWRKY6* was involved in both senescence and defense-related processes (Robatzek and Somssich 2001). Parsley genes including *PcWRKY1* and *PcWRKY4* were involved in defense against pathogen infection and responded to elicitor treatment (Cormack et al., 2002; Rushton et al., 1996). Some of WRKY genes such as *AtWRKY70* controlled balance between signaling branches promoting salicylic acid dependent and suppressing jasmonic acid dependent responses (Chen and Chen 2000; Li et al., 2004; 2006). Loosing *AtWRKY70* gene function in plants showed susceptible to the bacteria *Erwinia carotovora* and *Pseudomonas syringea* and the fungi *Erysphe cichoracearum* and *Botrytis cinerea* (AbuQamar et al., 2006). In addition, *AtWRKY7* or CaWRKY1 genes act as transcriptional repressor (Kim et al., 2006; Oh et al., 2008). The subgroup IIa of WRKY genes, including *AtWRKY18*, *AtWRKY40*, and *AtWRKY60*, play redundant functions in regulating plant disease resistant. Mutation analysis using *Atwrky18/Atwrky40* and *Atwrky18/Atwrky60* double mutants showed resistant to *P. syinger* but susceptible to *B. cinerea* infection (Xu et al., 2006). Therefore, it has been suggested that these subgroup IIa members involved in both positive and negative roles in plant defense (Eulgem and Somssich, 2007). Some cases of WRKY genes showed as modulators of systematic acquired resistance such as *AtWRKY53* gene acting as a positive regulator and *AtWRKY58* gene as a negative regulator (Wang et al., 2005). The transcription factors *AtWRKY11* and *AtWRKY17* act as negative regulators of basal resistance in *Arabidopsis thaliana*. (Journot-Catalino et al., 2006).

Although many numbers of WRKY genes in rice, barley, canola, Arbidopsis and others have been characterized, but little is known for soybean WRKY genes. Here we found a WRKY-type gene, which coded for double WRKY domain of WRKY transcription factor, was induced by *Pseudomonas* infection. In this study, we demonstrated that the cloned soybean gene coded a newly identified soybean WRKY transcription factor to bind to W-box and discuss fundamental features of the gene.

Materials and Methods

Plant materials. The detail methods were previously reported in Kang et al. (2003). Briefly, a *Pseudomonas* sp. SN239 was isolated and cultured in NB broth (3.0 g/L beef extracts and 5.0 g/L pepton) at 28°C. For plant inoculation, bacteria were cultured until 0.5 of OD number at 600 nm. Soybean (*Glycine max*) cultivars including YNPCSS3-19 and Eunha were used in this experiment. Seeds were surface-sterilized with 3% sodium hypochlorite for 30 min and germinated in Petri dishes at 26°C in the dark for 2 days. Seedlings were imbibed for 2 hours with NB medium with 5×10^8 cfu/mL of bacteria for *Pseudomonas* infection or without bacteria as control for infection and then washed with sterilized water. The washed seedlings with/without pathogen treatments continued to grow at 26°C in the dark and harvested every 12 hours up to 48 hours. Upon harvest, samples were frozen in liquid nitrogen and stored at −80°C. Some of the treated seedlings after 48 hours were placed under light for 48 hours to develop chloroplasts to determine the survivability after *Pseudomonas* infection.

Cloning, DNA sequencing, and public databases for sequence analysis. A cDNA clone, GenBank accession number AY323128, encoding a putative WRKY was selected and isolated from a soybean cDNA library following the manufacturer's protocols (Stratagene La Jolla, CA, USA). Briefly, a radioactive labeled DNA fragment selected by differential display analysis was used as a probe (Kang et al., 2003). Nucleotide sequencing was performed with a BigDye™ Terminator Cycle Sequencing kit (PE Biosystems, Foster City, CA, USA) using an automated DNA sequencer (ABI 3100, Applied Biosystems, Rockville, MD, USA) at the Institute of Biotechnology, Yeungnam University, Gyeongsan, Korea. DNA sequences and their deduced amino acids were analyzed using the DNAsis (Hitachi, Japan), BLAST, and CLUSTAL W programs. To predict the structure of proteins, domain analysis programs, including Predict Protein (http://cubic.bioc.columbia.edu/), CLUSTAL W (http://www.ebi.ac.uk/clustalw), and PROSITE (http://www.expasy.ch/prosite) were used.

Northern blot analysis. RNA blot and genomic DNA blot analyses were performed. Briefly, 10 μg of total RNA was isolated and electrophoretically separated on 1.4% agarose gels using 1× formaldehyde gel-running buffer [0.02 M 3-(N-morpholino)-propanesulfonic acid (pH 7.0), 8 mM sodium acetate, 1 mM EDTA (pH 8.0)] buffer and transferred to Hybond N+ nylon membranes (Amersham, Piscataway, NJ) in 25 mM phosphate buffer (pH 7.0) for 12 h. Then the membranes were UV-cross linked. The α-[^{32}P] dCTP labeled DNA probe was generated by a random primer labeling system according to the manufacturer's protocol (Promega Co., Madison, WI). Pre-hybridization and hybridization were performed at 62°C in hybridization buffer. The membranes were washed in washing buffer once at room temperature and once at 65°C for 5 min. Hybridized membranes were exposed for 24 h to X-ray film (Fuji photo film Co., Tokyo, Japan) with intensifying screens.

Expression and purification of recombinant fusion protein. To express the recombinant *GmWRKY1*, a cDNA fragment containing open reading frame of *GmWRKY1* gene was amplified by PCR using the following two primers; Upper primer (U468) 5'-CACCATGCATGGTTCTA-TGCC-3' and lower primer (1943X) 5'-CGAATCTCGG-AGGTGGACCCATTAGTATTCTTCC-3'. The PCR amplified cDNA fragment encoding *GmWRKY1* was purified and ligated into Champion™, pET102 directional TOPO expression vector (Invitrogen, Life technologies, LaJolla, CA, USA) by manufacture's protocol. The plasmid with *GmWRKY1* was transformed into *E. coli* BL21star (DE3) and grown in LB plate containing 100 μg/mL ampicillin for selection. *E. coli* BL21star(DE3) transformed *GmWRKY* was grown in 3 mL of LB medium containing 50 μg/mL carbenicillin and 1% glucose for 12 hours at 37°C with continuous shaking. The initial culture was inoculated into 50 mL of the same LB medium and grown up to 0.5-0.8 in OD600. Protein expression was induced by the addition of 25 μM IPTG (isopropyl-β-D-thiogalactopyranoside) at the start of the culture. After incubation at 37°C for 4 h, cells were harvested by centrifugation, resuspended in 1×Ni-NTA bind buffer (50 mM sodium phosphate buffer, 300 mM NaCl, 10 mM imidazole, pH 8.0) and lysed by sonication. Crude bacterial proteins in 1×Ni-NTA bind buffer were mixed with Ni-NTA His-resin, applied column, washed with 4 mL of 1×Ni-NTA wash buffer (300 mM NaCl, 50 mM sodium phosphate buffer, 20 mM imidazole, pH 8.0), and eluted His tagged proteins with 1×Ni-NTA elution buffer (300 mM NaCl, 250 mM imidazole, 50 mM sodium phosphate buffer, pH 8.0). The protein contents were determined by the Bradford assay (Bio-Rad, Hercules, CA, USA).

Electrophoretic mobility shift assay (EMSA). Each 1 μg of complementary oligo nucleotides were annealed in 100 mM Tris-HCl pH 7.9, 50 mM NaCl, 10 mM EDTA starting at 95°C and cooled slowly to room temperature. Oligo nucleotides were PW1 (5'-TCGTTGACTTGACTTGGCT-CTGCTCGTCAATGGT), MPW1 (5'-TCGTTGAATTGA-ATTGGCTCTGCTCTTCAATGGT), and W1 (5'-GTTTG-ACCGAGTTATTTATTTGTGTTTGTTT). About 100 ng of annealed double-stranded DNA was used for end-labeling with 5 U of T4 polynucleotide kinase and 20 μCi [γ-^{32}P]ATP (Amersham) in a total volume of 50 μl. The radioactive end-labeled probes were purified on Sephadex G-25 columns and diluted to 5,000 cpm/μl. Radioactive end-labled probe and 5 μg of purified soluble bacterial protein extract were incubated in 1× binding buffer (20 mM Tris-HCl pH 8.0, 50 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 12% glycerol) for 1 h at 25°C. Gel electrophoresis was carried out in 5% polyacrylamide gel. After complete EMSA, the gel was exposed on X-ray film for autoradiography.

Results

Expression patterns of the *GmWRKY1* genes. We found that seedlings of a Korean indigenous soybean (*Glycine max*) cultivar YNPCSS3-19 showed a strong resistant against a sprout-rot pathogen *Pseudomonas* sp SN239 infection (Fig. 1). The lesion spots on the hypocotyls of the *Pseudomonas* treated soybean 'YNPCSS3-19' were developed but not expanded. However, the lesion spots of *Pseudomonas* on hypocotyls of soybean 'Eunha' were appeared in 24 hours and developed to necrotic lesions.

We examined how *GmWRKY1* gene expressed in seedlings of pathogen infected soybeans. The transcripts of *GmWRKY1* were increased after the *Pseudomonas* sp. SN239 infection compared with non-inoculated seedlings of soybean 'YNPCSS3-19' (Fig. 2). However, detection of transcripts of *GmWRKY1* in soybean 'Eunha' was failed due to degraded RNA because the soybeans were severely smashed after *P.* SN239 infection.

We also documented the expression patterns of the *GmWRKY1* gene in different soybean tissues. As shown in Fig. 2, *GmWRKY1* gene was expressed in all of the tissues tested; the expression levels of *GmWRKY1* transcripts were higher in stems, leaves and developing seeds compared with nodules and roots. We concluded that *GmWRKY1* is

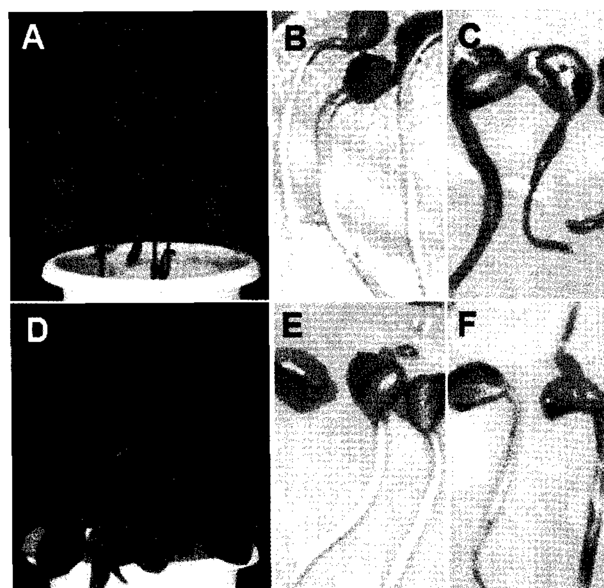

Fig. 1. Plants, *Glycine max* cv. Eunha and cv. YNPCSS3-19, and their seedlings with *Pseudomonas* infection. "Eunha" (A, B and C) and "YNPCSS3-19" (D, E, and F) are shown. Panels are shown for healthy mature plants (A and D), seedlings without infection (B an E) and seedlings infected *Pseudomonas* sp SN239 (C and F) on "Eunha" and "YNPCSS3-19", respectively.

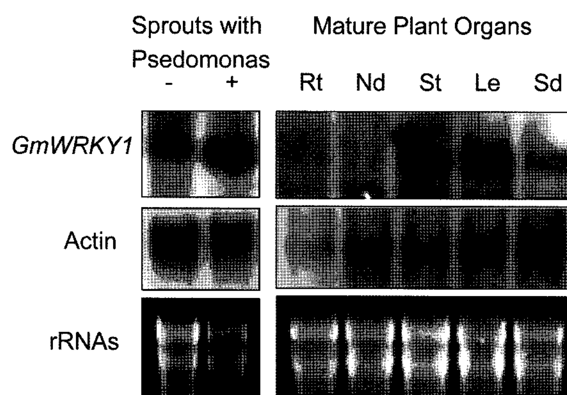

Fig. 2. Patterns of *GmWRKY1* gene expression of *Glycine max* cv YNPCSS3-19 by Northern blot analysis. Expression patterns of differentially induced cDNAs of sprout no treated as a control (–) and 12 hours after the *Pseudomonas* sp. SN239 infection (+). Total RNAs were isolated from seedlings placed for 24 hours under light after *Pseudomonas* infection (+) or not (–). Each radioactively labeled cDNA probe encoded for GmWRKY1 and actin for experimental control. Expression patterns of GmWRKY1 were examined by total RNAs isolated from the roots (Rt), nodules (Nd), stems (St), leaves (Le), and seeds (Sd) from mature health plants. In each lane 10 mg of total RNA were separated by electrophoresis and hybridized with appropriate probes. Ribosomal RNA is shown as a loading control.

constitutively expressed with active growth.

***GmWRKY1* gene is one of the WRKY gene families.** The deduced amino acids of the *GmWRKY1* cDNA comprised 493 amino acids and showed high identity with those of plant WRKY cDNA family reported in databases. Recently, almost 43 cDNA EST fragments showed homology to *GmWRKY1* in *Glycine max* have been identified in GenBank databases.

Since too many WRKY amino acid sequences have been aligned with GmWRKY1 in BLAST analysis, we selected high homologous and functional WRKY proteins in selected plants (Fig. 3). The results of alignments showed that deduced amino acid sequences of *GmWRKY1* (AY323128) of *Glycine max* was aligned in the functional domains with those of *AtWRKY20* (NM179119) of Arabidopsis, Susiba (AAQ63880) of *Hordeum vulgare*, and *PcWRKY1* (AF121353) of *Petroselinum crispum* (Fig. 3). Interestingly, these WRKY amino acids were highly shared with two repeats of a set of WRKY-domain and Zn finger-motif.

GmWRKY1 protein possesses two identical WRKY domains with zinc finger motifs. For clarifying the features of GmWRKY1 proteins, which is consisted with 493 amino acids, we aligned and characterized DNA-binding domains of the proteins as shown in Fig. 3. When amino acid sequences of GmWRKY1 protein was analyzed, the GmWRKY1 protein contains highly conserved two WRKY DNA-binding domains and a C_2-H_2 zinc-finger motif (C-$X_{4\text{-}5}$-C-$X_{22\text{-}23}$-H-X-H) in its N-terminal and C-terminal amino acid sequences. These WRKY DNA-binding domains of GmWRKY1 were consisted with 162-WRKYGQK in its N-terminus and 338-WRKYGQK in its C-terminus of amino acid sequences. As we predicted in topological analysis, even if data not shown here, the hydropath plot analysis showed that hydrophobic and hydrophilic chains in GmWRKY1 protein were distributed evenly in whole amino acid sequences indicating that the GmWRKY1 protein may possibly be soluble in nuclei.

The GmWRKY1 protein binds specifically to W-boxes. We tested whether GmWRKY is a transcription factor to bind with W-box containing sequence region. A cDNA fragment containing open reading frame of *GmWRKY1* gene was cloned into Champion™, pET102 directional TOPO expression vector. The recombinant GmWRKY1 protein with His tag was successfully induced after IPTG in *E. coli* BL21star (DE3). We isolated the crude extract of recombinant GmWRKY1 protein and applied into a His tagged column. With eluted His tagged proteins with 1×Ni-NTA elution buffer, the recombinant GmWRKY1 protein was eluted exactly as we designed expression analysis. Predicted molecular weight of GmWRKY1 is approximately 54 kDa. Therefore, the predicted recombinant GmWRKY1 protein is 70 kDa with 16 kDa of His-tag. As shown in the Fig. 5, we successfully eluted 70 kDa of recombinant GmWRKY1 protein.

In plants, a group of zinc-finger type WRKY transcription factors are targeted for binding with specific promoters of defense related genes containing the consensus motif W-box (C/TTGACC/T) or W-box palindromes (TTGACC/C-A/GTCA) (Eulgem et al., 1999; Yang et al., 1999). We tested whether GmWRKY1 protein target W-box element containing sequences in promoters in PR-genes. Briefly, we constructed two different types of nucleotides for one W-box and palindrome types of W-box (Fig 5). The gel mobility shift assay clearly demonstrated that GmWRKY1 protein with W1 oligo nucleotides complexes was shifted. Furthermore, GmWRKY1 protein with W-box palindromes also shifted in the EMS analysis. However, GmWRKY1 protein did not bind to a W-box mutant type oligo, which TTGAA was substituted instead of TTGAC (Fig. 5). Therefore, we understand that the GmWRKY1 protein is special to bind W-box elements in promoters of PR-like genes and functions for defense systems.

Discussions

In previous research, we found a wild type soybean cv. YNPCSS3-19 which was strongly resistant to a sprout-rot

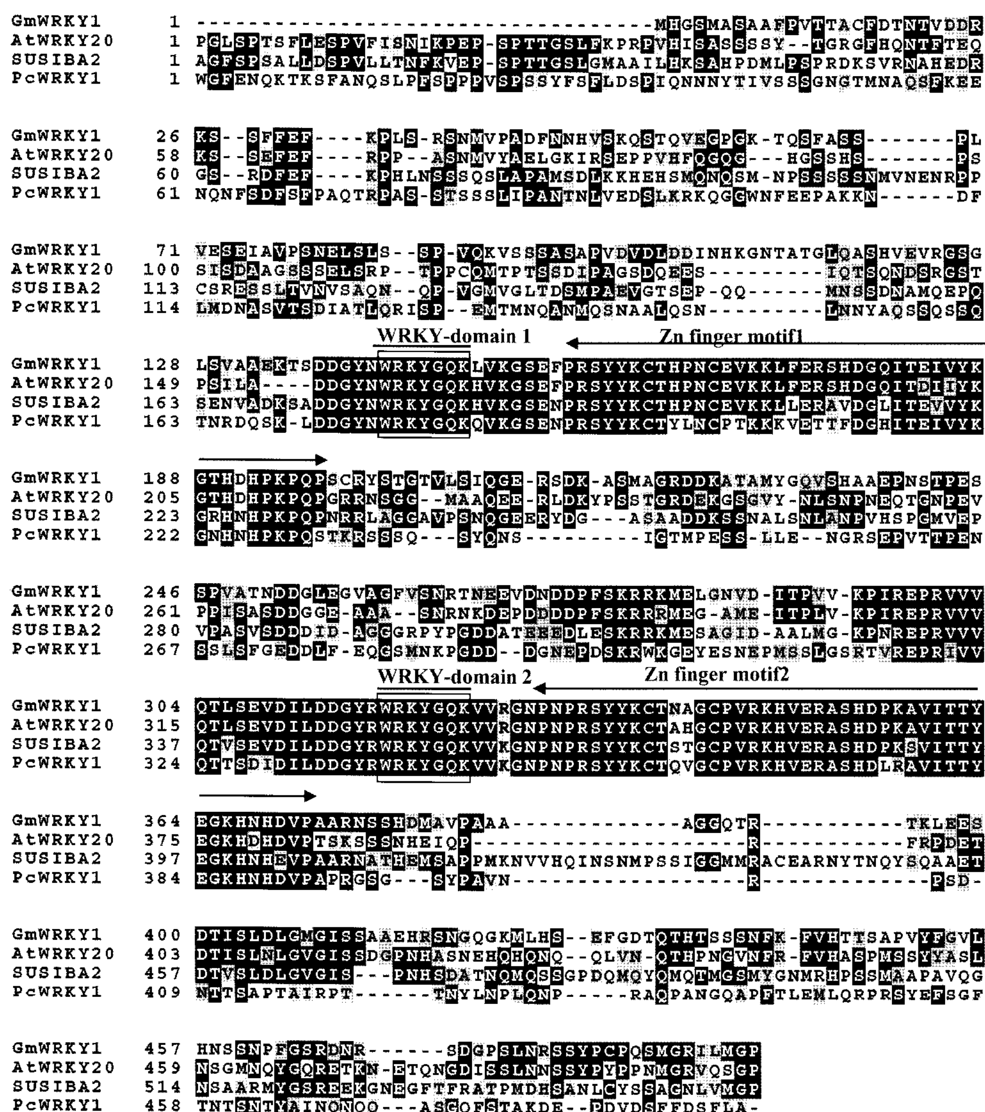

Fig. 3. Alignment of deduced amino acid sequences of WRKY proteins of selected plants. The deduced amino acid sequences of *GmWRKY1* (AY323128) of *Glycine max* was aligned with *AtWRKY20* (NM179119) of Arabidopsis, Susiba (AAQ63880) of *Hordeum vulgare*, and *PcWRKY1* (AF121353) of *Petroselinum crispum*. Identical and similar amino acids are shaded *black* and *gray*, respectively. Alignment was done using the CLUSTAL W program. Two WRKY-domians are *boxed*. Two WRKY-domains and two Zn finger motifs are marked *lines* or *arrows* on the amino acid sequence, respectively.

pathogen *Pseudomonas* sp. SN239 which is closely related with *Pseudomonas putida* in phylogenetic relationships using 16S ribosomal RNA sequences (Lim et al., 2008). Moreover, we identified specific genes involved in the resistance of the soybean and analyzed the pattern of gene expression against *Pseudomonas* sp. SN239 infection using differential-display reverse transcription PCR (Kang et al., 2003). More than 126 cDNA fragments resulted in DDRT-PCR were induced within 48 hours of the bacteria inoculation. Among them, 28 cDNA fragments were cloned and sequenced and some of the cDNAs were pathogenesis-related genes (PR genes) and PR-like genes, including an endo-1,3-1,4-β-D-glucanase, α-1,3-endoglucanase, α-1,3-exoglucanase and *WRKY*-like transcription, which named *GmWRKY1* in this report (Kang et al., 2003).

Northern blot experiment for the analysis of *GmWRKY1* gene expression pattern showed that the *GmWRKY1* gene was organ specific regulated and induced after pathogen infection (Fig. 2). Many previous reports demonstrated that gene expression patterns of *WRKY* family are relatively regulated in tissue specificity depending upon differential and developmental stages and responded to environmental stresses (Chen and Chen, 2002; Cormack et al., 2002; Li et al., 2004; Rushton et al., 1996; Robatzek and Somissich 2001). We also found that patterns of *GmWRKY1* gene expression are very similar to those of typical *WRKY* gene

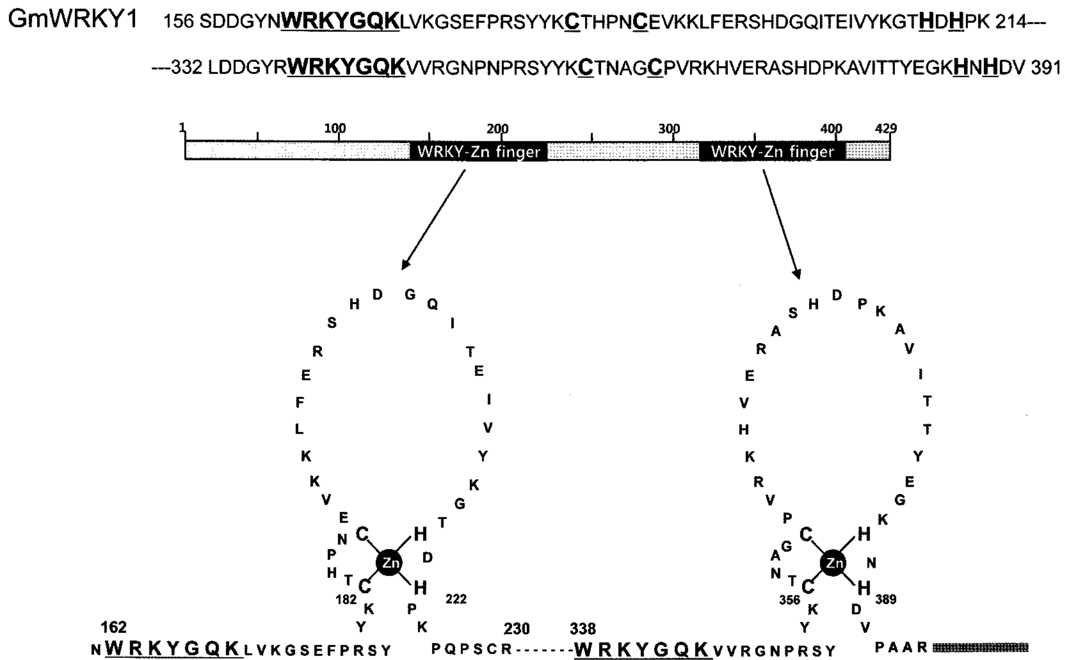

Fig. 4. Predictied structuture of the WRKY domains with zinc finger motifs in GmWRKY1 protein. Two Cys residues and two His residues coordinate to Zn ion in each zinc finger domain (C_2H_2/C_2H_2). The first zinc-finger DNA-binding domain is located amino acid residues from 162 to 230 and the second domain is located amino acid residue from 338 to 394 from the first metionine residue. Two WRKY amino acid sequences are placed 15-16 residues before zinc-finger domains. The numbers indicate amino acid from the first methionine residue.

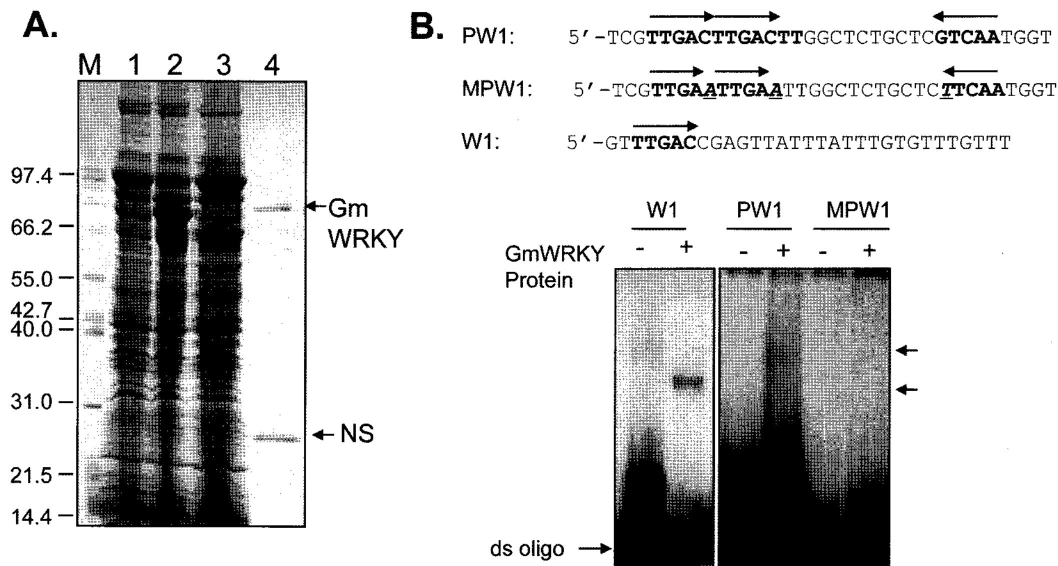

Fig. 5. DNA binding assay of GMWRKY1 protein. **A.** Expression of GmWRKY1 protein. GmWRKY cDNA was cloned in pET102 in BL21(DE3), expressed and purified using His column (M, Protein Mark; 1, BL21 extract; 2, Induced GmWRKY1 in BL21 extract with IPTG; 3, Flow-through; 4, 70 kDa of GmWRKY1 protein elute; NS, non-specific signal). **B.** DNA binding assay of GmWRKY1 as a transcription factor: WRKY domain specific to W-box (TTGAC) elements in PR-like gene promoters. PW1: TTGAC three repeats, MPW1: TTGAC element changed to TTGAA, W1: W box element (TTGAC). Gel shift assay were performed with double stranded oligos with or without the purified GmWRKY1. GmWRKY1 protein was bound to W box element, TTGAC, of oligos.

family responded to environmental stresses and pathogen. Therefore, we concluded that *GmWRKY1* gene is presumably one of the pathogen inducible genes for defense responses (Fig. 2).

More than 74 WRKY genes in the Arabidopsis genome and about 100 in rice genome have been identified using

nucleotide similarity comparison (Dong et al., 2003, Ulker and Somssich 2004). In *Glycine max*, we also identified more 43 cDNA EST fragments of WRKY genes. Therefore, it may expect that more the numbers of WRKY genes than identified 43 genes may distributed in *Glycine max* genome and also perform similar functions in defense system found in other plant kingdoms. Most WRKY domain in the proteins of WRKY genes contain a 60 amino acid long peptide region serves DNA binding function. The WRKY domain was named by conserved amino acid residues W, R, K and Y followed a zinc finger motif. The zinc finger motif is required for proper DNA binding function of WRKY protein (Ciolkowski et al., 2008; Eulgem et al., 1999; Maeo et al., 2001). Aligned motif showed GmWRKY1 protein also contained the typical conserved two WRKY DNA-binding domains and a C_2-H_2 zinc-finger motif, indicating that the DNA binding is a main function.

In previous research, WRKY proteins bind to W-box elements, TTGAC, were in Parsley WRKY proteins (Eulgem et al., 1999) and Arabidopsis WRKY proteins *AtWRKY6, 11, 26, 38* and *43* (Ciolkowski et al., 2008; Ulker and Somssich 2004). In this report, the EMS analysis in Fig. 5 demonstrated that GmWRKY1 protein bonded to not only a W-box elements but also W-box palindrome elements. However, GmWRKY protein failed to bind the TTGAA type element, which is only one nucleotide different in the fifth C to A in the TTGAC of W-box element, indicating that GmWRKY protein also specific W-box consensus, TTGAC.

In conclusion, with sequence analytical and protein functional comparisons with those characters of WRKY genes in other plants and binding to W-box element sequences in the promoters of defense related genes promoters, we first definite that *GmWRKY1* gene of *Glycine max* is presumably a transcription factor involved in defense-response system to regulate PR genes against pathogens.

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