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Capsicum annuum WRKYb transcription factor that binds to the *CaPR-10* promoter functions as a positive regulator in innate immunity upon TMV infection

Jee Hyuck Lim [a], Chang-Jin Park [b], Sung Un Huh [a], La Mee Choi [a], Gil Je Lee [a], Young Jin Kim [a], Kyung-Hee Paek [a,*]

[a] School of Life Sciences and Biotechnology, Korea University, 1, 5-ga, Anam-dong, Sungbuk-gu, Seoul 136-701, Republic of Korea
[b] Department of Plant Pathology, University of California, Davis, CA 95616, USA

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ABSTRACT

In plant, some WRKY transcription factors are known to play an important role in the transcriptional reprogramming associated with the immune response. By using WRKY-domain-specific differential display procedure, we isolated *CaWRKYb* gene, which is rapidly induced during an incompatible interaction between hot pepper and *Tobacco mosaic virus* (TMV) pathotype P_0 infection. The recombinant CaWRKYb bound to the *W* box-containing *CaPR-10* promoter probes efficiently and the specificity of binding was confirmed by mutant study and competition with cold oligonucleotides. Also, in GUS reporter activity assay using Arabidopsis protoplasts with the *CaPR-10* promoter, GUS activity was increased in the presence of CaWRKYb. And *CaWRKYb*-knockdown plant showed reduced number of hypersensitive response local lesions upon TMV-P_0 infection. Furthermore, *CaWRKYb*-knockdown plant exhibited compromised resistance to TMV-P_0 by accumulating more TMV, apparently through decreased expression of *CaPR-10*, *CaPR-1*, and *CaPR-5*. These results suggest that CaWRKYb is involved as a positive transcription factor in defense-related signal transduction pathways in hot pepper.

1. Introduction

Transcriptional regulation of gene expression is mediated largely by the level and/or activity of sequence-specific DNA-binding transcription factors [1]. And molecular and genomic analyses revealed several classes of *cis*-acting elements in plant stress-responsive promoters, including *GCC*, *W*, and *as-1* boxes [2]. The WRKY proteins are a family of plant-specific transcription factors that contain the WRKYGQK core sequence followed by a zinc-finger motif [3]. WRKY DNA-binding proteins recognize various *W* box elements with T/TGAC/C core sequences that are present in the promoters of many pathogenesis-related (*PR*) genes [2,4–7]. Thus, the WRKY DNA-binding proteins may function as common transcriptional regulators that regulate the expression of *PR* genes throughout the plant species. In addition to their DNA-binding ability, WRKY proteins share other features of transcription factors, such as nuclear localization and transcription activation/repression capability of target genes [8].

The Arabidopsis *AtWRKY23* is up-regulated almost immediately upon nematode infection and of which gene expression knockdown plants show increased resistance to the cyst nematode *Heterodera schachtii* infection [9]. Virus-induced gene silencing (VIGS) of three WRKY genes (*NbWRKY1*, *NbWRKY2*, and *NbWRKY3*) in tobacco led to compromised *N*-gene-mediated resistance to *Tobacco mosaic virus* [10]. Silencing of *NbWRKY8* decreased the expression of defense-related genes and increased disease susceptibility to the pathogens *Phytophthora infestans* and *Colletotrichum orbiculare* [11]. In rice it was revealed that the pair of allelic genes *OsWRKY45-1* and *OsWRKY45-2*, which encode proteins differing in ten amino acids, play opposite roles in rice–bacteria interactions [12]. *OsWRKY53*, a chitin oligosaccharide elicitor-responsive gene, has been found to be involved in defense responses in rice [13]. *CaWRKYa* isolated from hot pepper has a positive role for defense response against viral and bacterial pathogens [7]. VIGS of *CaWRKY1* in chili pepper leaves resulted in decreased growth of *Xanthomonas axonopodis* pv. *Vesicatoria race* 1 [14].

In this study, *CaWRKYb* was isolated from hot pepper encoding protein which can bind to the promoter of *CaPR-10* and investigated for its role in innate immune response. Also, the *CaWRKYb*-knockdown plant experiments indicated that *CaWRKYb* functions as a positive transcription regulator of several *PR* genes including *CaPR-10* upon TMV-P_0 infection.

2. Materials and methods

2.1. Isolation of CaPR-10 promoter

Using the *CaPR-10*-specific primer (GSP1 and GSP2; Supplementary Fig. 1), the promoter region was obtained by Universal

* Corresponding author. Fax: +82 2 928 1274.
E-mail address: khpaek95@korea.ac.kr (K.-H. Paek).

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Genome Walker™ Kit (CLONTECH, USA) according to the manufacturer's instructions. The *CaPR-10* GSP1 and GSP2 are 5′-CCCCATCAAGGCTATTCAAAGCT-3′, and 5′-CTTTACTGACAAGTCCACAGCCTCAGTTG-3′, respectively. The promoter was analyzed for its *cis*-acting elements using Web Signal Scan Program: PLACE (http://www.dna.affrc.go.jp/htdocs/PLACE/).

2.2. Biotin-labeled elution of CaPR-10 promoter with CaWRKYb

Biotin-labeled *CaPR-10* promoter was bound to the streptavidin magnesphere paramagnetic particles and then recombinant CaWRKYb protein was added along with the vector control. The bound protein with *CaPR-10* promoter was eluted and confirmed. Analysis was performed by using MagneSphere Magnetic Separation Products kit (Promega, USA).

2.3. Electrophoretic mobility shift assay (EMSA)

Mobility shift assays were performed essentially as described previously [7,15]. The binding reaction mixture contained 4 μl of buffer A (20 mM HEPS pH 7.8, 100 mM KCl, 2 mM DTT, 1 mM EDTA, 20% glycerol), 0.5 μg of poly (dI–dC), and 5 μg of BSA. Two complementary strands of the oligonucleotides were annealed and then labeled at the 5′-end using T4 polynucleotide kinase. Two hundreds nanogram of GST-CaWRKYb fusion protein and 2 ng of double stranded synthetic oligonucleotide labeled with ^{32}P-γ-ATP using T4 polynucleotide kinase were mixed (Sigma, USA). DNA–protein interaction complexes were allowed to form at room temperature for 30 min and then resolved on a 10% non-denaturing polyacrylamide gel in 0.5× TBE.

2.4. GUS promoter activity assay and protein gel blot analysis

Plasmids were introduced into Arabidopsis protoplasts by polyethylene glycol-mediated transformation [16]. GUS promoter activity analysis was carried out as described previously [17]. For protein gel blot analysis, 20 μg of total protein was prepared from the PEG-transfected protoplasts and separated by 12% SDS–PAGE. The proteins were then blotted onto a Hybond-P membrane using SemiPhor Semi-Dry Transfer Unit (Amersham Biosciences, UK). Protein gel blot analysis was carried out using the anti-GFP (Clontech, Japan). The protein blots were developed with an ECL detection kit (Amersham Phamacia Biotech, UK), and images were obtained using an LAS3000 image-capture system (Fujifilm, Japan).

2.5. RT-PCR, and quantitative real-time RT-PCR

The reverse transcriptase-polymerase chain reaction (RT-PCR) analyses were done as described previously [18]. Total RNAs were extracted from the leaves after each treatment and the reactions were conducted in accordance with the instructions provided in the manual for QuantumRNA™ Universal 18S Internal Standards (Ambion, USA) or oligo-dT. For *CaWRKYb* detection, a 112 bp fragment of *CaWRKYb* cDNA was amplified via PCR. *CaActin* was served as an internal control for the normalization.

Quantitative real-time PCR was performed to monitor quantitative levels of gene expression. Each reaction mix (20 μl) contained 10 μl of KAPA^{TM} SYBR® FAST qPCR Kit (KAPA Biosystems, USA) and 0.2 μM gene specific primers. Thermal cycling conditions consisted of 5 min at 95 °C, 40 cycles of 30 s at 95 °C, 30 s at 50 °C, and 30 s at 72 °C, and 10 min at 72 °C. Data acquisition and analysis were performed by using Roche LightCycler® 480 software (Roche, Swiss). Transcript levels were normalized to the expression of *CaActin* gene measured in the same samples.

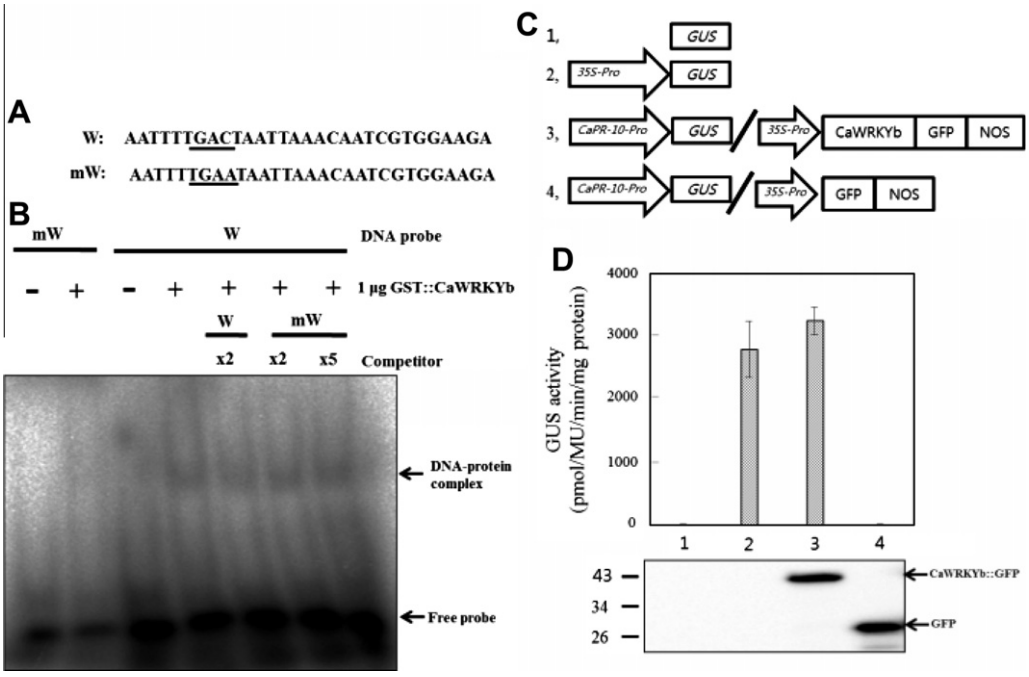

Fig. 1. *In vitro* binding assay of CaWRKYb to *W* box-containing *CaPR-10* promoter and transactivation of *CaPR-10* promoter by CaWRKYb. (A) Nucleotide sequences of *W* box-containing *CaPR-10* DNA probe; TGAC (W) and TGAA motifs in mutant (mW) are underlined. (B) EMSA was performed using freshly prepared recombinant CaWRKYb protein and ^{32}P-labeled *W* box probe. The specificity of *W* box binding activity was demonstrated by competition assay using excess unlabeled W and mW DNAs. DNA binding complex of CaWRKYb is indicated by the arrow (upper). (C) Schematic representation of *CaPR-10* promoter-GUS and *CaWRKYb*::*GFP* construct with control. (D) Transactivation of *CaPR-10* promoter::GUS gene expression by CaWRKYb in Arabidopsis protoplasts. A transient assay of GUS activities was carried out in protoplasts of Arabidopsis leaves 16 h after transfection with plasmid DNA. Western blot analysis was carried out to confirm expression of proteins using anti-GFP antibody (lower panel). Experiments were performed three times and similar results were obtained each time.