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In vivo binding of hot pepper bZIP transcription factor CabZIP1 to the G-box region of *pathogenesis-related protein 1* promoter

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Abstract

We find that salicylic acid and ethephon treatment in hot pepper increases the expression of a putative basic/leucine zipper (bZIP) transcription factor gene, *CabZIP1*. *CabZIP1* mRNA is expressed ubiquitously in various organs. The green fluorescent protein-fused transcription factor, CabZIP1::GFP, can be specifically localized to the nucleus, an action that is consistent with the presence of a nuclear localization signal in its protein sequence. Transient overexpression of the CabZIP1 transcription factor results in an increase in *PR-1* transcripts level in *Nicotiana benthamiana* leaves. Using chromatin immunoprecipitation, we demonstrate that CabZIP1 binds to the G-box elements in native promoter of the hot pepper *pathogenesis-related protein 1* (*CaPR-1*) gene in vivo. Taken together, our results suggest that CabZIP1 plays a role as a transcriptional regulator of the *CaPR-1* gene.

Keywords: *CabZIP1*; Hot pepper (*Capsicum annuum* L.); Nuclear localization; *CaPR-1* promoter; Chromatin immunoprecipitation (ChIP)

Transcriptional regulation is largely mediated through sequence-specific DNA-binding proteins that interact with *cis*-acting elements located in the promoter regions [1]. The interaction of transcription factors with relevant *cis*-acting elements alters the activity of the general transcription machinery and leads to the stimulation or suppression of the expression of a gene. In general, transcription factors recognizing the *cis*-acting elements can be classified on the basis of structural motifs [1]. One of the major family of transcription factors is the basic/leucine zipper (bZIP) protein [2]. For example, it is known that *Arabidopsis* contains approximately 100 bZIP-encoding genes [3]. The putative bZIP proteins have been clustered as 10 groups according to sequence similarities of their basic regions and additional conserved motifs [4]. It is currently known that bZIP transcription factors such as *Arabidopsis* ATBZIP11/ATB2 [5], maize OBF1 [6], tobacco (*Nicotiana tabacum*) TBZ17 [7], and BZI-2,3 [8] regulate gene transcription via binding to the hexameric G-box (CACGTG) within the promoter region of the target genes. A number of defense-related genes, including the well-studied *PR* genes, contain G-box elements in their promoter regions [9,10]. Previous studies have shown that these G-box sequences are specifically recognized by the bZIP proteins and are necessary for the induction of these genes.

However, the bZIP protein/G-box interaction has only been supported by in vitro assays, such as electrophoretic mobility shift assay and DNA-ligand binding. Although such studies have indirectly yielded important information, they do not reflect how bZIP factors modulate gene expression in vivo, nor can they detect specific interactions of bZIP protein with endogenous plant promoters. On the other hand, chromatin immunoprecipitation (ChIP) analysis after formaldehyde cross-linking provides an experimental tool for showing in vivo interactions under physiological conditions in a dynamic manner [11].

Here, we show that the expression of a gene encoding bZIP protein from hot pepper, *CabZIP1*, can be induced

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in its expression by salicylic acid (SA) and ethylene. The CabZIP1 protein was localized to the nucleus and induced the *PR-1* transcripts level. Finally, we show that CabZIP1 can bind to the *cis*-acting G-box elements of the native *PR-1* promoter region in hot pepper.

Materials and methods

Plant material and chemical treatments. Hot pepper (*Capsicum annuum* L.) cultivar Bugang plants were grown in a greenhouse with a 16 h light and 8 h dark photoperiod cycle at 25 °C. Healthy and well expanded leaves from two-month-old plants were sprayed with a solution of 10 mM SA and 10 mM ethephon, and were later used for nucleic acid extraction. Another set of control plants was similarly treated with distilled water. Leaves were harvested at the indicated time points after treatment, snap-frozen in liquid N_2, and stored at −80 °C before extraction of total RNA.

Isolation and characterization of a CabZIP1 cDNA clone and a PR-1 promoter. CabZIP1 cDNA was synthesized from total RNA isolated from hot pepper using a cDNA amplification kit (Clontech, USA) according to the instructions of the manufacturer. Two bZIP domain-specific primers (sense; 5′-GAGAGAAAACGAAAGAGAATGGAGTCCAAC-3′ and anti-sense; 5′-GAGTGAGCGAATTAAGGGAATCCAAGC-3′) were used for PCR amplification. To isolate full-length cDNA clones, rapid amplification of cDNA ends (RACE) was carried out using gene-specific primers (GSP1 5′-CAGTCAGTTCAGCAATTTGAGCCCTCAAG-3′ and GSP2 5′-CATTGTTCTCTGCTTCGACTGTGTGATAG-3′ for the 5′ end RACE, and GSP1 5′-GAATGGAGTCCAACCGTGAATCTGC AAGG-3′ and GSP2 5′-CACGGATGAGGAAACAGCAGCATTTGG AG-3′ for the 3′ end RACE) that corresponded to a previously isolated bZIP domain. PCR amplification was performed at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s for 30 cycles. The promoter of *CaPR-1* (GenBank Accession No. DQ201633) was amplified by gene-specific primers (GSP1 5′-GCCACCAGAGTGTTGCATCCTGCAGTCTC-3′ and GSP2 5′-GGCTGCTAGCCTATTGTCCCATGTCATAG-3′) from a hot pepper genomic DNA library constructed by following the manual of the Universal Genome Walker™ kit (Clontech, USA).

RNA gel blot analysis. Total RNA was isolated from the hot pepper leaves and Northern blot hybridizations were carried out with [^{32}P]-labeled probes. Blots were hybridized as described previously [12]. For the *CabZIP1* probe, a 400-bp fragment of pepper *CabZIP1* cDNA was amplified by PCR. Two specific primers, 5′-GATATGTTGCAGTTTTG AGCATCCTC-3′ and 5′-GACACTCAACTCGAGATTTATTTATT-3′, were designed based on the sequence. For the *CaPR-1* probe, a 450-bp fragment of pepper *CaPR-1* cDNA was amplified using two specific primers, 5′-GTACGTCTTGGTTGTGCTAGGGTTCG-3′ and 5′-CTC ATGAAGCTTTACACATTTTATTTG-3′.

Construction of green fluorescent protein (GFP) fusion plasmids and fluorescence microscopy observation. After PCR, the stop codon of the *CabZIP1* gene was removed by using two specific primers, 5′-GGATCC AATGGCTTCGACTCAGCAAC-3′ and 5′-CCTAGGAAAACTGCAA CATATCAGCAGA-3′. The PCR-amplified product was fused in-frame to the coding region of soluble modified green fluorescent protein (smGFP). Transient expression of green fluorescent protein (GFP) fusion constructs was performed by introduction of the constructs into the BY-2 tobacco protoplasts using the PEG-mediated transformation method [12]. Fluorescence photographs of the protoplasts were taken using a Zeiss Axioplan fluorescence microscope (Jena, Germany) fitted with fluorescein isothiocyanate filters (excitation filter, 450–490 nm; emission filter, 520 nm; dichroic mirror, 510 nm) and Fuji 400 color film. The optimal exposure time was 1 s.

Transient overexpression of CabZIP1 in tobacco plant. Four- to six-week-old wild-type tobacco plants (*N. benthamina*) were used for infiltration experiments. This involved leaf infiltration with a mixed culture of *Agrobacterium* strain EHA 105 containing the *CabZIP1* overexpression construct at an OD_{600} of 0.1 or an equal population of *Agrobacterium* containing the pCAMBIA2300 vector as a control. After 3 days, the infiltrated areas were cut from the leaf, frozen in liquid N_2, and stored at −80 °C until further analysis. For reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, total RNAs were extracted from leaves after each treatment and the RT-PCR was then performed by following the manual for Quantum RNA 18S as an internal standard (Ambion, USA). Using pairs of primers (5′-CACTCATCTCAAGCTC AAAACTCTC-3′ and 5′-CAATTACCAGGTGGATCATAATTG-3′) specific for *NbPR-1* (GenBank Accession No. CAA47374), PCR experiments were performed with the synthesized cDNAs as templates.

Antibody preparation and Western blot analysis. The *CabZIP1* cDNA fragment was prepared by PCR and cloned into the *Bam*HI site of pQE30 vector (Qiagen, Germany). The primer set used for the amplification was 5′-GGATCCATGGCTTCGACTTCAGCAAC-3′ and 5′-GGATCCTC AAAACTGCAACATATC-3′. The resulting in-frame fusion plasmid was transformed into *Escherichia coli* strain BL21 (DE3). The CabZIP1 protein, which was tagged with six His residues at the N-terminus, was overexpressed by 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 3 h at 30 °C. The *E. coli* transformants were collected after the induction, suspended in lysis buffer (50 mM Na_2HPO_4, pH 8.0, 300 mM NaCl), and subjected to sonication on ice for 1 min with a Vibracell sonifier (Sonics and Materials, USA). After the resulting lysate was centrifuged for 10 min at 13,000*g*, the supernatant was loaded on a Ni–NTA affinity resin (Qiagen, Germany). The recombinant protein was eluted at a gradient from pH 4.0 to pH 6.0 in eluent (50 mM Na_2HPO_4, 300 mM NaCl, and 10% glycerol).

The purified protein was injected into a rabbit to raise antibody levels [13]. For immunoblot analysis, 40 μg of protein extracted from tobacco leaves was separated by 12% SDS–polyacrylamide gel (SDS–PAGE) [14]. The proteins were then blotted onto a Hybond-P membrane (Amersham Biosciences, UK) using a SemiPhor Semi-Dry Transfer Unit (Amersham Biosciences, UK). Immunoblot analysis was carried out using the polyclonal antibody raised against the recombinant CabZIP1 as the primary antibody and horseradish peroxidase-conjugated anti-rabbit IgG antibody as the secondary antibody. The blots were developed with an ECL detection system (Amersham Biosciences, UK).

Nuclei extractions and chromatin preparations. Hot pepper leaves were cross-linked with 1% (w/v) formaldehyde for 7 min using a vacuum. Formaldehyde was quenched by the addition of a final 125 mM glycine, and the leaves were then ground to a fine powder in liquid N_2 [15]. Isolation of nuclei was performed by Wagner et al. [16]. The isolated nuclei that were cross-linked with formaldehyde were resuspended in 500 μl of nuclei lysis buffer (50 mM Tris–HCl, pH 8, 10 mM EDTA, 1% SDS, and 1 mM PMSF). Chromatin was sheared to an average size of 1.5 kb by repetitive sonication with a Vibracell sonifier (Sonics and Materials, USA) equipped with a microtip in an ice bath. The sonicated chromatin was cleared by centrifugation (13,000*g*, 10 min, 4 °C). The protein concentration was determined by the Bradford assay (Bio-Rad, CA) with BSA as the standard. Solubilized chromatin (180 μg) was incubated with 5–7.5 μl of pre-immune serum and anti-CabZIP1 antibody overnight at 4 °C with gentle mixing, and Protein A–Sepharose (Amersham Biosciences, UK; 40 μl, 50% slurry) was then added. The solution was incubated with gentle mixing for 2 h at 4 °C. Protein A–Sepharose beads and chromatin complex were washed twice with low salt wash buffer (150 mM NaCl, 0.2% SDS, 0.5% Triton X-100, 2 mM EDTA, and 20 mM Tris–HCl, pH 8.0), high salt wash buffer (500 mM NaCl, 0.2% SDS, 0.5% Triton X-100, 2 mM EDTA, and 20 mM Tris–HCl, pH 8.0), LiCl wash buffer (0.25 M LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, and 10 mM Tris–HCl, pH 8.0), and TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA), respectively. The immune complex was eluted by the addition of 250 μl eluent (1% SDS, 0.1 M $NaHCO_3$) and incubation at 65 °C for 15 min; this process was performed twice. The DNA cross-links were reversed by the addition of 20 μl of 5 M NaCl and eluted at 65 °C for overnight. After cooling, proteins were extracted by phenol:chloroform (1:1 v/v), and DNA was recovered by ethanol precipitation. The recovered DNA was used for amplification by PCR. One microliter of immunoprecipitated sample DNAs was amplified for 35 cycles of 94, 55, and 72 °C steps of 30 s each. In each experiment, four appropriate sequential additions in the corresponding input were run in parallel to ensure that PCRs were in a

semi-quantitative range. The following oligonucleotide sequences were used: CaPR1-1, 5′-CCATCTGCATCAACATCCATTATGTAC-3′ and 5′-GTTCGTACGATGAATTGGTAGAAAGCGT-3′; CaPR1-2, 5′-CCATCTGCATCAACATCCATTATGTAC-3′ and 5′-CAAAGCTTAATTCTAGGATGTCTATTG-3′; CaPR1-3, 5′-GACAAGCTRTATGATGTTTGTGGACTG-3′ and 5′-CAACTTCATCATCCACCATTATATATAC-3′; CaPR1-4, 5′-GTGCATTATTAGAAACTTGTGGGTG-3′ and 5′-GATACCAAGCAAGGTATTAACAAT-3′; CaPR1-5, 5′-CTAGAAATAACGTCAATAACACAA-3′ and 5′-CTCTTAGCTTTGTAATTCTATTTG-3′; CaPR1-6, 5′-GAGAGTGATTGAAGTGAAATAGAG-3′ and 5′-GTGCTTCACAGGATGGGTCGGCAT-3′; CaPR1-7, 5′-CACTGTCGGAGCTCACCGGAGCTC-3′ and 5′-ATACTGGTATTAGTTATGCACACT-3′; CaPR1-8, 5′-GTCAATGTAGTACTCCCCATCATC-3′ and 5′-GAAGATTTAATATTGATCAATGAT-3′; and CaPR1-9, 5′-GAGAAGAAGTCGATGATATCCAATCATC-3′ and 5′-GAGTTGATCTTGAATGAATGGGTTGCTTG-3′.

Results and discussion

Amino acid comparison of CabZIP1with other plant bZIP DNA-binding proteins

CabZIP1 encoding a putative bZIP transcription factor from hot pepper was identified by similarity analysis using standard BLAST searches against the GenBank and Swiss-Prot database [17]. The protein had 144 amino acids with a relative molecular mass of 16.5 kDa. As shown in Fig. 1, the CabZIP1 protein possessed a relatively high sequence similarity with other plant bZIP transcription factors from tomato (86%, GenBank Accession No. AF176641), tobacco (86%, GenBank Accession No. AB032478), and *Arabidopsis* (51%, GenBank Accession No. AY091421). These proteins are generally known to possess a basic/leucine zipper (bZIP) domain signature, a bipartite nuclear localization signal (NLS), a putative protein kinase II phosphorylation site, a protein kinase C phosphorylation site, and an N-myristylation site involved in signaling.

Cellular localization of CabZIP1

To investigate the cellular localization of CabZIP1, in vivo targeting experiments were performed with a CabZIP1 translationally fused with soluble modified green fluorescent protein (smGFP) as a fluorescent marker [18]. The introduced genes under the control of the cauliflower mosaic virus 35S (CaMV 35S) promoter were found to be expressed strongly in tobacco protoplasts. As shown in Fig. 2, the fusion protein was localized to the nucleus of BY-2 cells, whereas the control smGFP was uniformly distributed throughout the cell.

The import of proteins into the nucleus is mediated by NLS; commonly studied NLSs exist as a monopartite or bipartite type in higher plants [19]. From the computer predictions of CabZIP1, the typical bipartite type NLS could be found within the amino acid sequence of CabZIP1 (Fig. 1). This result suggested that nuclear targeting of CabZIP1–smGFP appeared constitutive, which indicates that external signals are not required for nuclear translocation of the protein.

Changes in CabZIP1 mRNA levels after SA and ethephon treatment

The signaling pathways involving SA and ethylene are well characterized in various plants, although some crosstalk occurs between them [20–22]. To determine whether those stimuli induce expression of *CabZIP1* gene when exogenously applied, SA and ethephon treatments were applied to the hot pepper plants. *CabZIP1* transcripts began to accumulate strongly 6 h after the plants were sprayed with 10 mM SA and decreased thereafter (Fig. 3A). In the case of the ethylene treatment, hot pepper plants were sprayed with 10 mM ethephon for the indicated period of time. Accumulation of *CabZIP1* transcripts

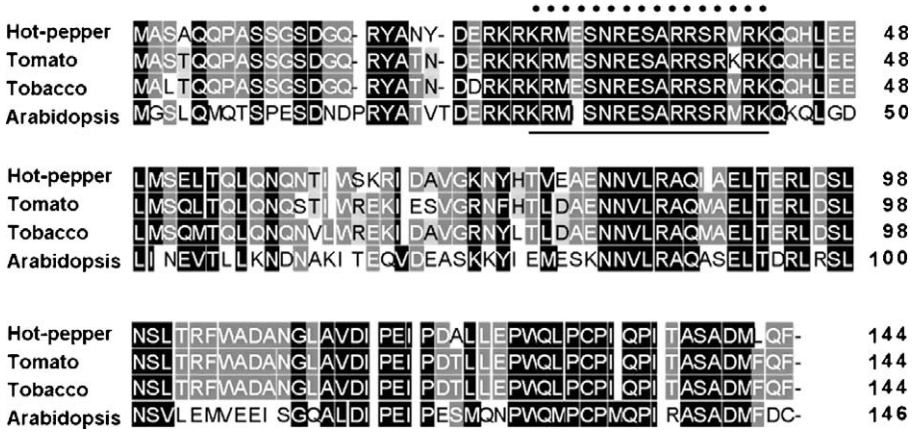

Fig. 1. Comparison of amino acid sequences deduced from *CabZIP1* (GenBank Accession No. DQ201634) with other *bZIPs* from other species, including tomato (*Lycopersicon esculentum*; GenBank Accession No. AF176641), tobacco (*Nicotiana tabacum*; GenBank Accession No. AB032478), and *Arabidopsis thaliana* (GenBank Accession No. AY091421). The predicted DNA-binding amino acid sequence of *CabZIP1* is underlined and the nuclear localization signal is designated by black dots. The black background indicates 100% identity. Similarity values between 50–100% and below 50% are printed in a gray and light-gray background, respectively. Alignment was facilitated by the Lasergene Megalign program (DNASTAR, USA).

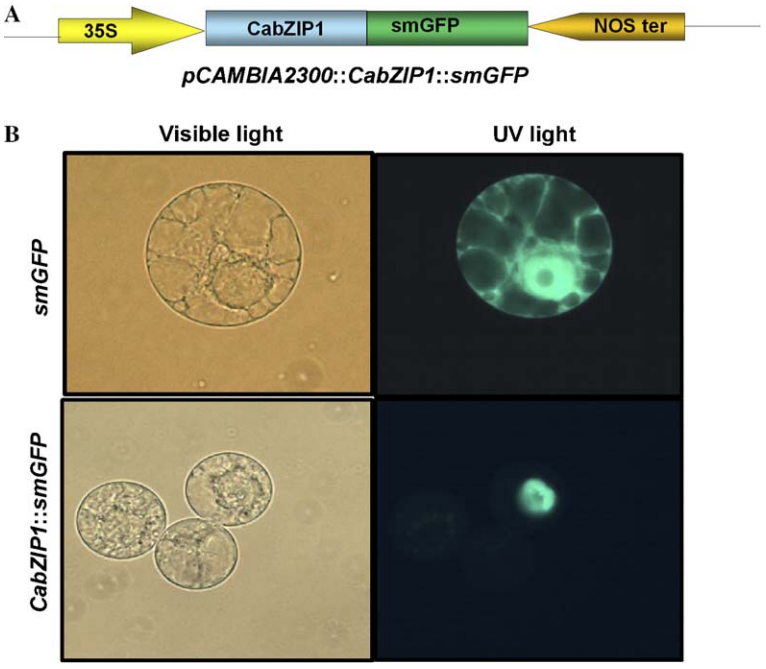

Fig. 2. Subcellular localization of *CabZIP1::smGFP* fusion protein. (A) Construction of *CabZIP1::smGFP* fusion gene in pCAMBIA2300 vector. (B) The *CabZIP1::smGFP* fusion and control *smGFP* constructs were introduced into tobacco BY-2 cells by PEG-mediated transformation. The *smGFP* was used as the cytosol localization marker (upper panel). The optimal exposure time was 1 s. The experiment was repeated at least three times and a representative result is shown.

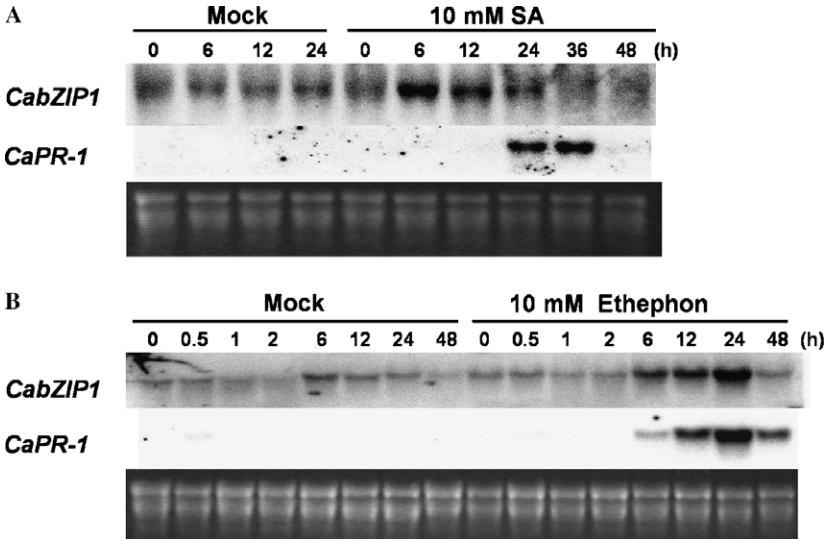

Fig. 3. Expression patterns of *CabZIP1* by SA and ethephon treatments. Hot pepper leaves were treated with 10 mM SA or 10 mM ethephon and detached at the time periods indicated for total RNA extraction. The gel blot of hot pepper leaf total RNA was hybridized with the [^{32}P]-labeled 3′ UTR of *CabZIP1* cDNA as a specific probe. rRNA bands in ethidium bromide-stained gels are shown as a loading control. The *CaPR1* gene was used as a positive control for the treatments.

was detected at 6 h, and the level was higher at 24 h (Fig. 3B). At 6 h after treatment, induction of the *CaPR-1* as a positive control of ethylene treatment was observed. To examine steady-state transcripts level of *CabZIP1* in various organs of pepper plants, total RNA was extracted from the roots, stems, leaves, flowers, green (unripe) fruits, and red (ripe) fruits. As shown in Fig. 4, *CabZIP1* gene transcripts were present in abundance in most organs. However, the level in the leaves was relatively low and was nearly undetectable in the flowers.

SA and ethylene (ET) are important components of signal transduction cascades that activate plant defense responses against pathogen attack [23]. Many *PR* genes, including *CaPR-1*, have been reported to be induced by SA treatment [24,25]. However, the induction of *CaPR-1* took a relatively long time, requiring about 24 h to induce

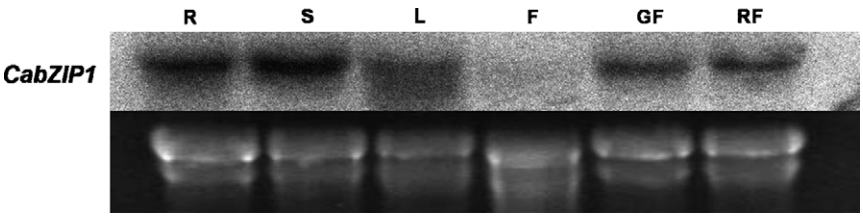

Fig. 4. Accumulation of *CabZIP1* transcripts in various organs. The RNA gel blot was hybridized with the [^{32}P]-labeled 3′ UTR of *CabZIP1* cDNA as a specific probe. rRNA bands in ethidium bromide-stained gel are shown as a loading control. Lanes: R, roots; S, stems; L, leaves; F, flowers; GF, green (unripe) fruits; RF, red (ripe) fruits.

expression. Thus, SA-induced expression of the *CabZIP1* gene seems to be preceded by that of the *CaPR-1* gene. Transcriptional activation of *CabZIP1* by SA treatment suggests that expression of the *CabZIP1* gene may be dependent upon SA signaling. As a replacement for ET, the ET-releasing compound, ethephon, can be used as an inducer of the defense reaction [25]. *CabZIP1* was also induced by ethylene treatment. Thus, *CabZIP1* induced by SA and ET treatment could function as an important transcription factor that is involved in crosstalk between these endogenous hormone signals in hot pepper.

Accumulation of PR-1 transcripts by transient overexpression of CabZIP1 in tobacco

To demonstrate whether this transcription factor could contribute to the expression of PR genes, including the *PR-1* gene, *Agrobacterium* harboring pCAMBIA 2300 binary vectors with a coding region of *CabZIP1* under the control of CaMV 35S promoter, or vector only, was infiltrated into *N. benthamiana* plant. After 3 days, total RNA from the infiltrated tobacco leaves was isolated for RT-PCR analysis using *NbPR-1* specific primers (Fig. 5). As a result, when CabZIP1 was overexpressed in tobacco leaves, the level of *NbPR-1* gene transcript increased compared with non-infiltrated and vector control plants. Total proteins were prepared from the same tobacco leaves, and the polyclonal antibody raised against CabZIP1 confirmed transient overexpression of CabZIP1 in the tobacco leaves. The specificity of the antiserum for CabZIP1 was confirmed by immunoblot analysis.

It is known that the bZIP transcription factors in *Arabidopsis* bind to promoter regions of pathogenesis-related protein genes and act as transcriptional regulators [26]. *CabZIP1* transcripts were accumulated at earlier time points compared to *CaPR-1* transcripts after SA treatment (Fig. 3A). In addition to nuclear localization (Figs. 1 and 2), CabZIP1 protein seems to share other features of transcription factors, such as transcription activation capability. A number of studies have shown that bZIP proteins have regulatory functions in transcription machinery. For instance, AtbZIP11/ATB2, which belongs to the same subgroup as CabZIP1, transactivated the *ProDH* gene in *Arabidopsis* [27]. Maize bZIP transcription factors, EmBP-2 and ZmBZ-1, are involved in the expression of the *rab28* gene, which is induced by abscisic acid [28]. In the case of vector control in experiments of transient overexpression, the *NbPR-1* transcripts level was slightly higher than that of the non-infiltrated plants. We believe that infiltration-associated wounding and/or compatible interaction with virulent vector bacteria probably contributed to the activation of the *NbPR-1* gene [29].

Binding of CabZIP1 to the promoter region of CaPR-1

Previously, when CabZIP1 was transiently overexpressed in the cell, increased level in *PR-1* gene transcripts was observed (Fig. 5). This could be mediated by CabZIP1

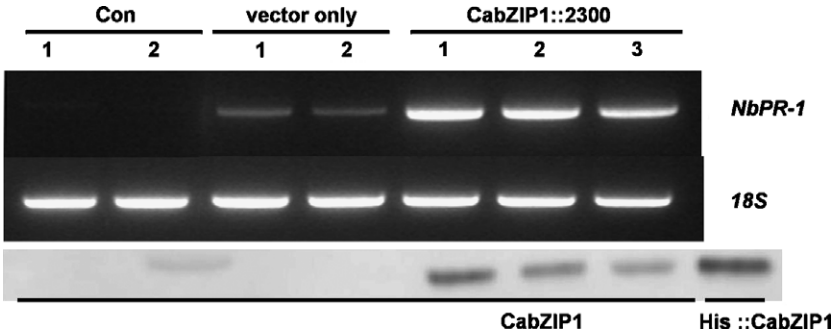

Fig. 5. *NbPR-1* expression activated by transient *CabZIP1* overexpression in tobacco leaves. Tobacco leaves that overexpressed *CabZIP1* or vector only by *Agrobacterium*-mediated transient transformation methods were harvested. cDNAs were synthesized from the total RNAs isolated from tobacco leaves, and RT-PCR was conducted with gene-specific primers. The level of 18S rRNA was estimated as an internal standard for cDNA quantity. Forty micrograms of total proteins was prepared from the same samples. The polyclonal antibody raised against the intact and recombinant CabZIP1 at 500-fold dilution was used as the primary antibody and horseradish peroxidase-conjugated anti-rabbit IgG antibody was used as the secondary antibody. As a positive control of Western blot analysis, 5 μg of purified CabZIP1 protein tagged with six His was also prepared.

binding to the *PR-1* promoter. Therefore, we established a formaldehyde-mediated cross-linking approach in hot pepper leaves to evaluate whether CabZIP1 protein/*CaPR-1* promoter interactions occur in the chromatin context in vivo. A *CaPR-1* promoter region amplified from the hot pepper genome was sequenced and searched for G-box elements using the PlantCare website (http://intra. psb. ugent.be:8080/PlantCARE/). Using anti-CabZIP1 antibody, we extracted the coimmunoprecipitated DNA and then subjected it to PCR with primer pairs corresponding to a G-box sequence in the *CaPR-1* promoter (Fig. 6A). Enrichments could be observed with primer combinations that covered the one or two G-boxes present in the *CaPR-1* promoter (PR1-1, PR1-2, and PR1-3 in Fig. 6B). Contrary to our expectation, however, G-boxes near the TATA box and transcription start site were not amplified by primer pairs (PR1-4, 5, 6, 7, 8, and 9 in Fig. 6B). Only a background level of PCR signaling was detected by pre-immune serum (pre-immune in Figs. 6B and C). An increased level of signaling was observed in the amplified PCR fragments when the precipitated DNA templates were added two or three times, indicating that PCRs were in a semi-quantitative range (lanes 1, 2, and 3 in Fig. 6C).

The chromatin immunoprecipitation technique allows the purification of an in vivo complex of DNA-binding protein and its associated DNA, and is one of the best approaches available for confirming the direct targets of a DNA-binding protein [30]. Antibodies against CabZIP1 were used for immunoprecipitating CabZIP1-DNA complexes. ACGT elements (ACEs) [31] have been shown to be associated with photosynthesis [32] and other light-inducible processes such as flavonoid biosynthesis [33] and responses to abscisic acid [34], auxin [35,36], wounding [37], and salicylic acid [38]. The proteins that bind to ACEs in plants almost universally belong to the bZIP class of transcription factors [39]. Different members of the plant bZIP family have different binding site preferences; ACEs are distinguished by the sequences flanking the ACGT core. One of the major groups of plant bZIP proteins binds preferentially to the CACGTG motif (known as the G-box) [40,41]. Our results provide that CabZIP1 binds to the *CaPR-1* promoter region. Because the binding site of CabZIP1 in the *CaPR-1* promoter is almost 2.5 kb upstream from the start codon, it could be argued that the binding is not relevant to the transcription regulation of the *CaPR-1* promoter. However, we think that an important *cis*-regulatory element can exist in the distal promoter regions, particularly in higher eukaryotes with relatively large genome size such as mammals and some crop plants. For instance, the promoter regions (−2090 to −1265) of rat selenoprotein W (SeW)-harboring metal response element (MRE), specificity protein 1 (Sp1), glucocorticoid response element (GRE), and liver-specific transcription factor (LFA-1) exhibited higher promoter activity than SV40

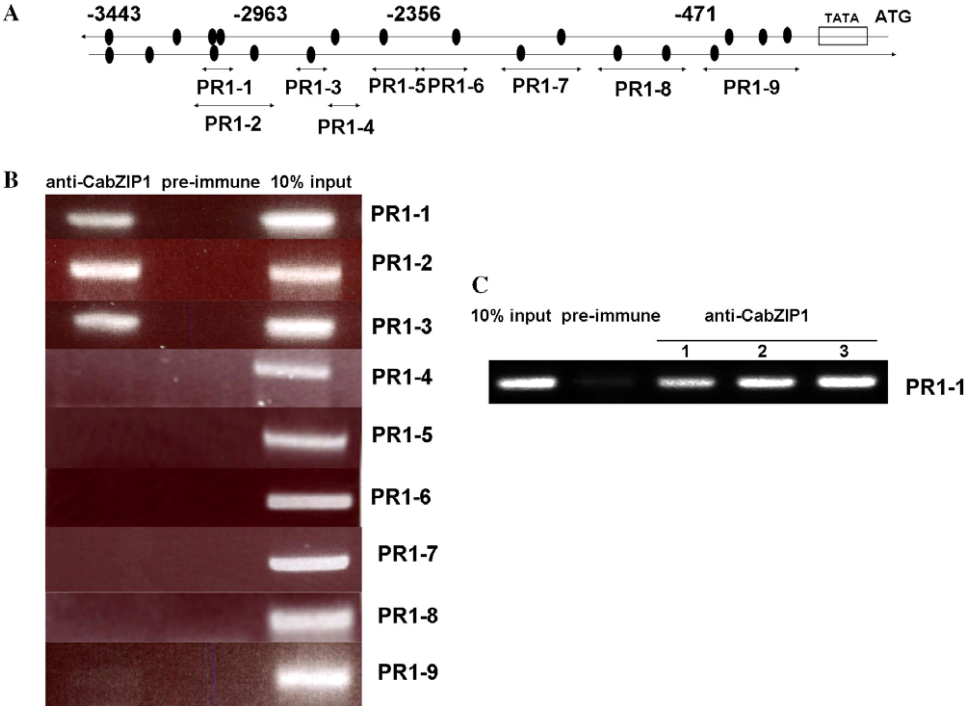

Fig. 6. G-box elements of *CaPR-1* promoter and analysis of DNA after anti-CabZIP1 immunoprecipitation. (A) G-box elements (black circles) in the *CaPR-1* promoter (GenBank Accession No. DQ201633) region and the locations of primer sets for putative CabZIP1 binding sites (↔) are marked. (B) Chromatin preparations from formaldehyde-fixed nuclei were immunoprecipitated with CabZIP1-specific antibody. The DNA was recovered after reversal of the cross-links and was analyzed for the enrichment of the *CaPR-1* promoter region by PCR. Immunoprecipitations were performed with pre-immune serum and anti-CabZIP1 antibodies. Formaldehyde-fixed nuclear extract was taken as a positive control (10% input) before immunoprecipitation. (C) Sequential twofold additions of input material from chromatin preparations were processed in parallel; lanes 1, 2, and 3.

promoter [42]. In parsley, ChIP resolution scanning of the WRKY protein family revealed that W-boxes at promoter sites are constitutively occupied by WRKY proteins. A proposed site occupation/site displacement model suggests that WRKY proteins act in a network of mutually competing participants [43]. G-boxes at *CaPR-1* promoter sites might be constantly occupied by CabZIP1, which make the interaction between CabZIP1 and *CaPR-1* promoter to be dynamical when proper stimuli are involved in the cell signaling pathway. Moreover, the *PR-1* gene is regulated by various stimuli such as pathogens, SA, ethylene, and wounds. Several transactivators also work for transcriptional regulation of the *PR-1* gene [22]. Similarly, other transcription factors might bind to this region when various stimuli are received in the cell due to substitution or interaction with other factors. A more extended analysis of CabZIP1 is necessary to understand the mode of action in the transcriptional regulatory network.

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