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Original Paper

Characterization of SBZ1, a soybean bZIP protein that binds to the chalcone synthase gene promoter

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Abstract Several plant basic leucine zipper (bZIP) proteins have been shown to play a role in chalcone synthase (*CHS*) gene expression, and some are regulated by phosphorylation/dephosphorylation. We isolated *SBZ1* (Soybean bZIP protein 1) and showed that the recombinant protein binds *in vitro* to the 5′ region of soybean *CHS1*, at a sequence that confers the elicitor-inducible expression of *CHS* genes. The deduced amino acid sequence of SBZ1 has features characteristic of bZIP transcription factors, including a highly basic putative DNA-binding domain containing a nuclear localization sequence, as well as four domains designated D1–D4 that are highly conserved among the subfamily of bZIP factors, which includes tobacco BZI-1 and parsley CPRF2. The presence of these regions indicates that SBZ1 is a CPRF2-related bZIP transcription factor. The protein kinase inhibitor K252a blocks *CHS* induction in elicited soybean cells, suggesting that protein phosphorylation is involved in induction of the *CHS* signal pathway. Phosphorylation assays indicated that SBZ1 is phosphorylated *in vitro* in a soybean cell extract, and that this phosphorylation depends on Ca^{2+}. Furthermore, recombinant soybean CDPK and the α subunit of CKII phosphorylate SBZ1 *in vitro*. However, unlike other related bZIP proteins, phosphorylation had no effect on either the DNA-binding activity of SBZ1. Therefore, we conclude that SBZ1 is regulated by phosphorylation, but in a different manner than are related bZIP factors.

Key words: bZIP protein, chalcone synthase, *Glycine max*, protein phosphorylation, transcription factor.

Plants exhibit a number of adaptive and protective responses to environmental stimuli. Of particular significance is the induction of the biosynthesis of secondary metabolites following environmental stresses, such as exposure to UV light, predation by insects, or microbial invasion. Among a diverse array of naturally occurring secondary metabolites, the flavonoids have been well characterized as defense substances. For example, the phytoalexins act as UV protectants and antimicrobial compounds (Dixon et al. 1983).

Chalcone synthase (CHS) catalyzes the first and key regulatory step of flavonoid biosynthesis, which involves the stepwise condensation of three acetyl units from malonyl-coenzyme A (CoA) with the coumaroyl moiety of 4-coumaloyl-CoA derived from the phenylpropanoid pathway, giving rise to the C15 flavonoid skeleton naringenin chalcone (Hahlbrock and Scheel 1989). Following the isomerization of naringenin chalcone, further substitutions lead to the formation of flavone, flavonol, anthocyanin, isoflavone, and the antimicrobial phytoalexin.

In most angiosperms, CHS is encoded by multiple gene copies, whereas *Arabidopsis*, parsley, and snapdragon each have a single copy of the gene. The expression of each member of the *CHS* multigene family is stimulated by developmental signals and a variety of environmental stimuli, such as light, pathogen attack, mechanical wounding, and nutrient stress (Dixon et al. 1995; Lawson et al. 1994; Leyva et al. 1995; Logemann et al. 2000; Loyall et al. 2000; Moriguchi et al. 1999; Sakuta et al. 2000; Seki et al. 1999; Shimizu et al. 1999; Qian et al. 2007; Zeier et al. 2004). The organization and differential regulation of individual genes in the *CHS* multigene family during development and in response to different environmental stimuli have been reported in detail (Christensen et al. 1998; Moriguchi et al. 1999; Ryder et al. 1987; Tuteja et al. 2004; Wingender et al. 1989). Analysis of upstream regions of individual genes

Abbreviations: BAP, 6-benzylamino purine; bZIP, basic leucine zipper; bHLH, basic helix-loop-helix; 2,4-D, 2,4-dichlorophenoxyacetic acid; CBB, coomassie brilliant blue; CDPK calcium dependent protein kinase (calmodulin-like domain protein kinase); CKII, casein kinase II; CHS, chalcone synthase; CPRF, common plant regulatory factor; DMSO, dimetyl sulfoxide; DTT, dithiothreitol; EGTA, ethylene glycol bis (β-aminoethylether)-*N*,*N*,*N*',*N*′,-tetraacetic acid; EST, expressed sequence tag; GFP, green fluorescent protein; G/HBF-1, G-box/H-box binding factor-1; GST, glutathione-S-transferase; IPTG, isopropyl-thio-β-D-galactopyranoside; NLS, nuclear localization signal; Nos, nopaline synthase; RT-PCR, reverse transcriptase-PCR; SBZ1, soybean bZIP protein 1; TC, tentative consensus; TIGR, The Institute of Gene Research

Note: The nucleotide sequence reported in this paper has been submitted to DDBJ under accession number AB246665 (SBZ1).

This article together with supplement materials can be found at http://www.jspcmb.jp/

in the *CHS* multigene family has led to the identification of *cis* elements that are involved in stimulus-responsive transcription. The sequence between bases −175 and −134 in the soybean *CHS1* promoter region is necessary for the elicitor inducibility of the gene. The soybean sequence is able to direct elicitor inducibility in parsley protoplasts, suggesting a conserved function of *cis* elements involved in plant defense (Wingender et al. 1990). Functional analysis of the promoter of the French bean *CHS15*, which is induced by pathogen attack, identified two types of *cis* elements, the G-box and H-box, within the TATA-proximal region of the *CHS15* promoter. The G-box and H-box motifs are essential for the transcriptional activation of *CHS15* in response to developmental and environmental cues (Arias et al. 1993; Loake et al. 1992). Delineation of *cis* elements and their *trans* factors underlining the rapid activation of *CHS* genes provides a basis for characterizing the terminal stages of a signal transduction pathway involved in the deployment of early transcription-dependent defenses.

G-box/H-box binding factor 1 (G/HBF-1), a basic leucine zipper (bZIP) protein that binds to the G-box and the adjacent H-box in the proximal region of the *CHS15* promoter, is rapidly phosphorylated in elicited soybean cells, an event that stimulates its binding to the *CHS15* promoter (Dröge-Laser et al. 1997). At first, G/HBF-1 was described to be isolated from soybean (Dröge-Laser et al. 1997), however it was revised that the isolated cDNA clone was originated from tobacco, and was found not to be full-length. Then the full-length cDNA for a tobacco bZIP protein was isolated and deginated BZI-1 (Heinekamp et al. 2002). BZI-1 is closely related to common plant regulatory factor 2 (CPRF2) from parsley. Members of the CPRF bZIP transcription factor family have been suggested as being involved in the regulation of *CHS* genes by light (Weisshaar et al. 1991; Wellmer et al. 1999; Wellmer et al. 2001). CPRF2 is a phosphoprotein *in vivo*, and its phosphorylation is rapidly increased in response to light. In contrast to G/HBF-1, the phosphorylation of CPRF2 does not alter its DNA-binding activity (Kircher et al. 1999; Wellmer et al. 1999).

Although the MYB and basic helix-loop-helix (bHLH) transcriptional activators appear to be responsible for the expression of tissue-specific genes involved in flavonoid biosynthesis, few transcription factors responsible for UV-, pathogen-, or stress-responsive activation of flavonoid biosynthesis genes have been identified unequivocally, despite considerable effort.

To elucidate the terminal events in the signaling processes involved in activating early transcription-dependent plant defense responses in leguminous plants, we are focusing on elicitor-inducible expression of soybean *CHS* genes and their *trans*-acting factors. In this study, we isolated the gene for soybean bZIP protein 1 (SBZ1), of which the recombinant protein was found to bind to the promoter regions of *CHS7*, and also that of *CHS1*, a region that has been shown to confer the elicitor-inducible expression of *CHS* genes, indicating that SBZ1 is involved in the activation of the plant defense response.

Materials and methods

Cell Cultures

Soybean (*Glycine max* L.) cells were grown in Murashige and Skoog medium (1962) supplemented with 0.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 2.2 μM 6-benzylamino purine (BAP), and 3% (w/v) sucrose. Suspension cells were elicited 3 days after transfer to fresh medium by treatment with 0.5 mM reduced glutathione (Dron et al. 1988) or distilled water.

Gene Isolation and RT-PCR

Total RNA was extracted from 100 mg of frozen cells with extraction buffer [4.2 M guanidine thiocyanate, 0.5% (w/v) *N*-lauroylsarcosine, 25 mM sodium citrate, 14 mM 2-mercaptoethanol] and phenol/chloroform, and precipitated with 2 M lithium chloride. Total RNA (2 μg) from soybean was used for first-strand cDNA synthesis at a concentration of 125 ng/μl with oligo(dT) primer and Superscript III (Invitrogen, Carlsbad, CA, USA). The samples were subject to denaturation at 65°C for 5 min followed by rapid cooling on ice, and then cDNA was synthesized by incubation for 50 minutes at 42°C and 15 minutes at 70°C. *SBZ1* was isolated by PCR using the primers SBG/H full-sp (CGGAATCCATGGATAAGGGTATTCTCA-GT) and SBG/H1-asp (CGGAATTCTTACTGCTCAGATG-GAGG), which were designed based on the TC (Tentative Consensus) sequence 122478 of the TIGR Soybean (*Glycine max*) Gene Index database (http://tigr.org/tdb/gmgi). PCR was performed for 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, using 1 μl of cDNA with *Pyrobest* DNA polymerase (Takara, Kyoto, Japan). The soybean *CDPK* cDNA was amplified by PCR using the primers p.CDPK1 (CGCGGATCCATGCAGAAGCATGGTTTTGC) and p.CDPK2 (GACGTCGACTAAGAAGAGTCTTTCATTC), which were designed based on the open reading frame of *Glycine* max calmodulin-like domain protein kinase isoenzyme beta (GenBank accession number U69173, Lee et al. 1997). PCR was performed for 30 cycles of 94°C for 15 s, 57°C for 30 s, and 68°C for 120 s, using 1 μl of cDNA synthesized from non-elicited cells with *Pyrobest* DNA polymerase (Takara).

The level of *CHS* mRNA was estimated by semi-quantitative RT-PCR. The degenerate PCR primers designed by conserved region of *CHS* from several plant spices, CHS-SP [GCG(C/G)ATGTG(C/T)GA(A/T/G)AAATTC(A/C/G)A] and CHS-ASP [GTGAT(C/T)C(A/T/C)GA(A/G)CA(A/G)AC(A/G/C)AC] were used to detect expressed soybean CHS gene family. PCR was performed for 26 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s, using 1 μl of a 2X dilution of cDNA with Takara *Ex Taq* polymerase. Following PCR, 5-μl aliquots of the PCR products were separated by electrophoresis

on 1% agarose gels. The expected sizes of RT-PCR-amplified fragments of *CHS* transcripts were about 0.4 kb.

DNA sequencing

Nucleotide sequences were determined using a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Bioscience, Piscataway, NJ, USA) and an ABI 373A sequencer (Applied Biosystems, Foster City, CA, USA), or a BigDye Terminator v. 3.1 Cycle Sequencing Kit and an ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems).

Transient Expression Assays Using Particle Bombardment

To construct cauliflower mosaic virus (CaMV) 35S-SBZ-sGFP(S65T)-Nos terminator, a C-terminal fusion of the *SBZ1* cDNA clone to sGFP(S65T) was inserted into pUC18. *SBZ1* was fused between the *Sal*I and *Nco*I sites of a CaMV35S-sGFP(S65T)-Nos terminator construct (kindly provided by Y. Niwa, Shizuoka University). Three constructions 35S-SBZ-sGFP(S65T)-Nos terminator, 35S-nuclear localization signal (NLS)-GFP(S65T)-Nos terminator and 35S-sGFP(S65T)-Nos terminator were bombarded to soybean cells to investigate the subcellular localization of SBZ1.

Soybean suspension cells were collected and resuspended in Murashige and Skoog medium supplemented with 0.5 μM 2,4-D, 2.2 μM BAP, 3% (w/v) sucrose, and 0.5 M mannitol. After incubation for 5 min, the cells were placed on filter paper and bombarded with DNA-coated tungsten particles using a PDS-1000/He particle gun (Bio-Rad Biolistics Particle Delivery System).

Preparation of tungsten microprojectiles (1.7 μm) were performed as previously described (Shimada et al. 2007). Bombardments were performed at a pressure of 1100 psi. After transformation, the cells were incubated for 24 hr at 25°C in darkness. Observations were made with a Leica DM IRBE microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) equipped with a mercury lamp and a GFP filter set (480/40 nm excitation and 525/50 nm emission).

Recombinant SBZ1 and CDPK

An *Eco*RI-*Sal*I fragment containing the *SBZ1* coding sequence was inserted into pET28a (Novagen, Madison, WI, USA) and pGEX-6p-1 (Promega, Madison, WI, USA) restricted with *Eco*RI and *Sal*I to give pET-SBZ1 and pGEX, respectively, which carry a translational fusion of the His6 epitope tag and a GST tag at the N-terminus of the *SBZ1* coding region. *E. coli* BL21 (pET-SBZ1 and pGEX-SBZ1) cells were grown in medium containing 0.8 mM isopropyl-thio-β-D-galactopyranoside (IPTG) for 3 h at 37°C to induce the expression of His_6-SBZ1 and GST-SBZ1. A *Bam*HI-*Sal*I fragment containing the *CDPK* coding sequence was inserted into pET28a restricted with *Bam*HI and *Sal*I to give pET-CDPK. *E. coli* BL21 (pET-CDPK) cells were grown in medium containing 0.8 mM IPTG for 4 h at 25 °C to induce expression. The extracted fusion proteins were purified under non-denaturing conditions using HisTrap columns (Amersham Biosciences) for His_6-SBZ1 and His_6-CDPK, and the MagneGST purification system (Promega) for GST-SBZ1. The purified proteins were checked by SDS-PAGE with staining of the proteins with Coomassie Brilliant Blue, and the protein concentrations were estimated using the Bradford assay (Bradford 1976).

In vitro phosphorylation of recombinant SBZ1

Crude protein extracts were prepared from elicited cells. Frozen cells were homogenized in extraction buffer [50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 1 mM Pefablock (Roche Diagnostics)]. The homogenate was clarified by centrifugation and used as a kinase solution in the following assays. Recombinant SBZ1 (500 ng) was incubated with cell extract (1 μg protein) in protein kinase buffer [250 mM HEPES (pH 7.5), 50 mM $MgCl_2$, 5 mM EGTA, 6 mM $CaCl_2$] supplemented with 0.1 mM ATP and 185 kBq of [γ-^{32}P]ATP in a total reaction volume of 10 μl for 10 min at room temperature. The reaction was stopped by adding SDS sample buffer. Phosphorylated SBZ1 was separated by SDS-PAGE and detected by autoradiography. For rephosphorylation of SBZ1, bacterially expressed recombinant proteins were dephosphorylated by treatment with calf intestinal alkaline phosphatase conjugated to agarose beads (Sigma) for 30 min at 37°C and used as substrates for phosphorylation as described above.

DNA-Binding Assay

Recombinant SBZ1 (300 ng) was incubated at room temperature for 30 min in 12.5 mM HEPES (pH 7.9), 25 mM KCl, 10% glycerol, 0.25 mM EDTA, 0.5 mM DTT, 0.5 mM Pefablock, 0.5 μg poly [d(I-C)][d(I-C)], and 0.1 ng ^{32}P-labeled nucleotide. The samples were electrophoresed in a 5% polyacrylamide gel for 2.5 hours at 7 watts in TGE buffer. The gels were dried under vacuum and exposed to an imaging plate (Fuji Film, Tokyo, Japan) overnight, and the autoradiographs were analyzed with a fluorescence image analyzer (FLA2000, Fuji Film).

Results

Isolation of SBZ1

The soybean *SBZ1* cDNA, which contains an open reading frame of 1254 bp and encodes 418 amino acids, was isolated by PCR using primers designed based on an expressed sequence tag (EST) present in a soybean gene database. The deduced amino acid sequence has features characteristic of a bZIP transcription factor, including a leucine zipper in which every seventh amino acid residue is leucine or another small hydrophobic residue, and a highly basic putative DNA-binding domain containing a nuclear localization sequence (Figure 1A). Four domains, designated D1–D4, which are highly conserved in tobacco BZI-1, parsley CPRF2, and maize OHP1 and OHP2 (Pysh et al. 1993), are also present in the SBZ1 amino acid sequence. The D1 domain is predicted to form a helix, and D2, which is relatively rich in acidic residues, is located at the N-terminus of the basic domain

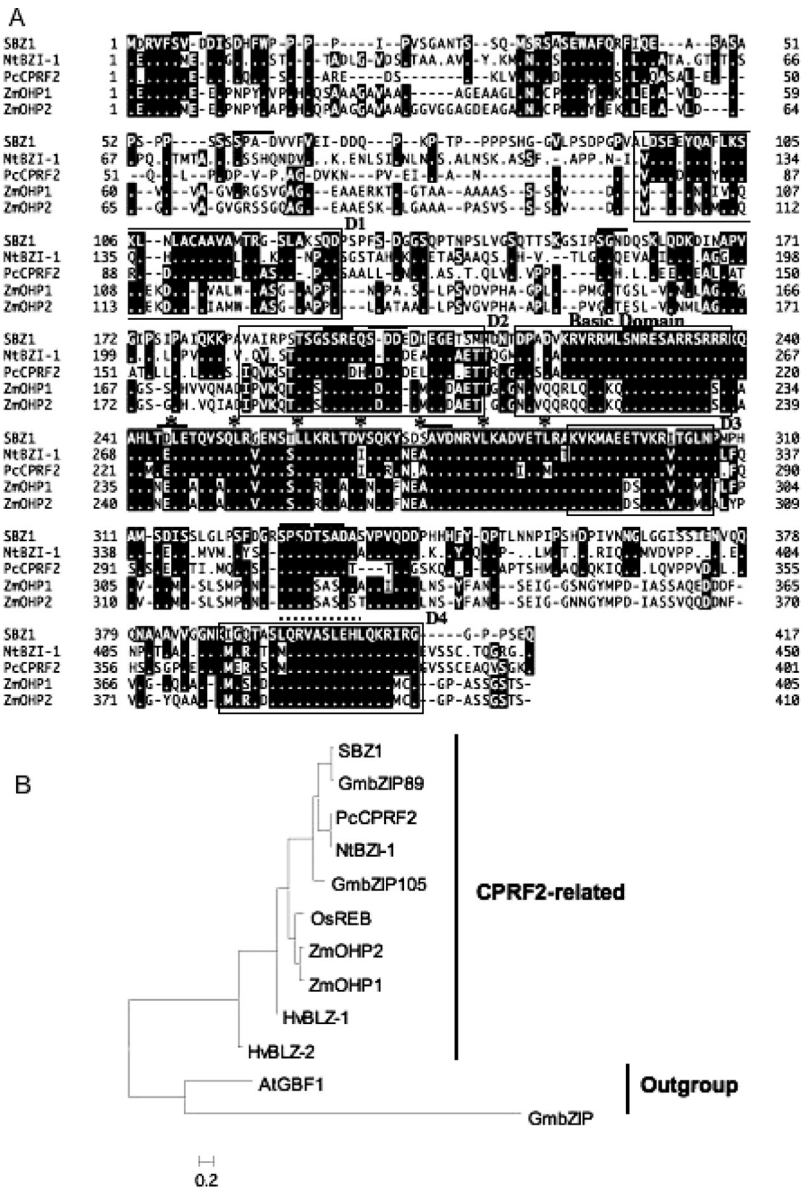

Figure 1. Alignment of the predicted amino acid sequence of SBZ1 with orthologous sequences. (A) SBZ1 is aligned with NtBZI-1 from tobacco, PcCPRF2 from parsley, and ZmOHP1/2 from maize. Conserved amino acid residues are shaded in black, and gaps maximizing the alignment are indicated by dashes. The conserved domains D1–D4 and basic regions are enclosed in boxes, residues of the putative leucine zipper are marked with asterisks, and putative phosphorylation sites are marked with dotted (CDPK) and solid (CKII) lines. (B) Phylogenetic analysis of relationships among the CPRF2-related proteins. To construct the tree, the full-length amino acid sequences of CPRF2-related bZIP transcription factors, GmbZIP89 from soybean (Accession no. ABI34655), GmbZIP105 from soybean (Accession no. Q0GPG9), BLZ-1 from barley (Accession no. P93667), BLZ-2 from barley (Accession no. CAA71795), REB from rice (Accession no. Q946H5) were acquired from the GenBank database. GmbZIP from soybean (Accession no. ABC47854) and GBF1 from Arabidopsis (Accession no. NP_849510) were used as outgroup proteins. The scale bar represents 0.2 substitutions per site.

(Dröge-Laser et al. 1997). D3 is adjacent to the bZIP region, and D4, which comprises the CDPK substrate motif (L-X-R-X-X-S/T-V-X-X-L), is located near the C-terminus. SBZ1 also contains a proline-rich region, predicted to be a transactivation domain, near the N-terminus (Heinekamp et al. 2002). Several putative CKII phosphorylation sites (S-X-X-D/E) are present, with one located in D2, and the basic domain is also conserved among this group of transcription factors. On the phylogenetic tree created using the neighbour-joining method in the CLUSTALW version 1.83 system (Thompson et al. 1994), CPRF2-related proteins were clearly distinguished from G-box binding factor-1 from Arabidopsis (Figure 1B). SBZ1 was more related to PcCPRF2 and NtBZI-1 than other CPRF2-related proteins from monocots, such as ZmOHP1/2 and OsREB.

Subcellular localization of SBZ1

To investigate the localization of SBZ1, transient

expression analysis was performed using a green fluorescent protein (GFP) fusion protein of SBZ1, with expression driven by the CaMV 35S promoter. Two constructs, containing either the entire *SBZ1* ORF or the nuclear localization signal fused in-frame to the GFP gene (SBZ1-GFP and NLS-GFP), were introduced into cultured soybean cells by particle bombardment. Transient expression of fusion proteins was monitored using confocal laser-scanning fluorescence microscopy. The fluorescent pattern of SBZ1-GFP was consistent with that of NLS-GFP (Supplement Figure 1). Comparison of the green fluorescent signals from fusion proteins indicated that SBZ1 accumulates predominantly in the nucleus.

DNA-binding activity of SBZ1

To elucidate the contribution of SBZ1 to the expression of the *CHS* genes, the binding of SBZ1 to several CHS promoters was examined with gel retardation assays using purified recombinant SBZ1 (Figure 2). The assays demonstrated that SBZ1 binds *in vitro* not only to the bean *CHS15* promoter region, but also to the soybean *CHS7* promoter region containing the G-box and H-box within the TATA-proximal region, which has a structure very similar to that of the bean *CHS15* promoter (Figure 2A, B). Furthermore, SBZ1 bound to the −175 to −134 region of the soybean *CHS1* 5′ region (Figure 2A, B), which has been shown to confer the elicitor-inducible expression of *CHS1* (Wingender et al. 1990). The binding activity of SBZ1 to the *CHS1* promoter was stronger than that to *CHS7* (Figure 2B). Furthermore, a competition assay using both the *CHS1* and *CHS7* promoters as probes revealed that the *CHS1* promoter competes more efficiently (Figure 2C). The 5′ (−175 to −134) region of the *CHS1* promoter contains the SBF-1 (a nuclear factor that binds to the silencer element; Lawton et al. 1991) binding domain-like sequence, between bases −170 to −159, and half of box I at −139 to −130 (Figure 3A). The sequence of box I is similar to that of parsley box II, which has been suggested as being involved in UV-light-responsive gene expression (Wingender et al. 1990). In gel retardation assays using unlabeled competitors, the −175 to −141 sequence, which contains the 5′-terminal half of the −170 to −134 region, bound SBZ1 more strongly than the −153 to

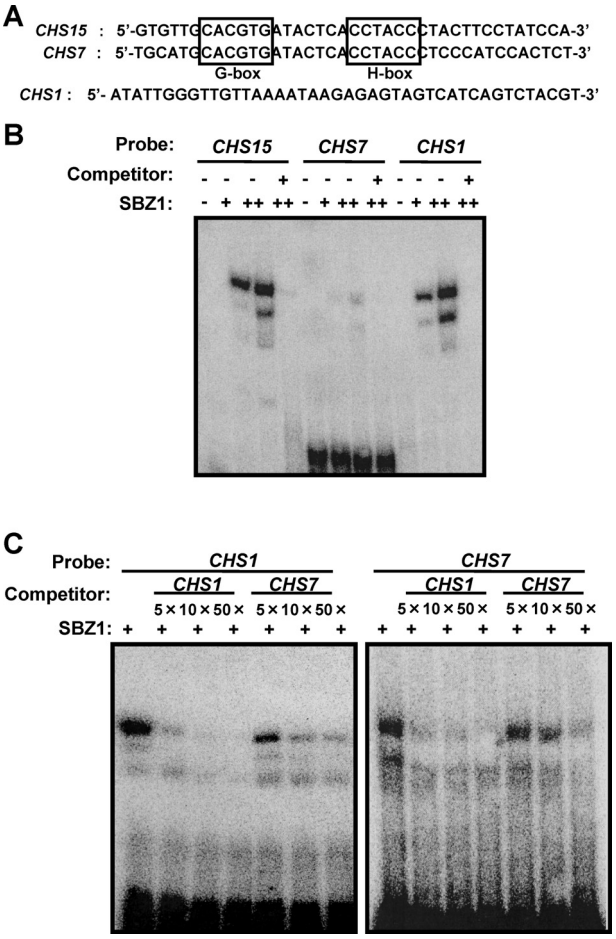

Figure 2. *In vitro* DNA-binding analysis of SBZ1. (A) Nucleotide sequences used in DNA-binding assays. (B) Gel retardation assay showing *in vitro* binding of recombinant His_6-SBZ1 to the region from −80 to −42 of *CHS15*, the promoter region of *CHS7* corresponding to the −80 to −42 region of *CHS15*, and the −175 to −134 region of *CHS1*. (C) Competition assays containing increasing excesses of unlabeled sequences of the *CHS1* and *CHS7* promoter regions.

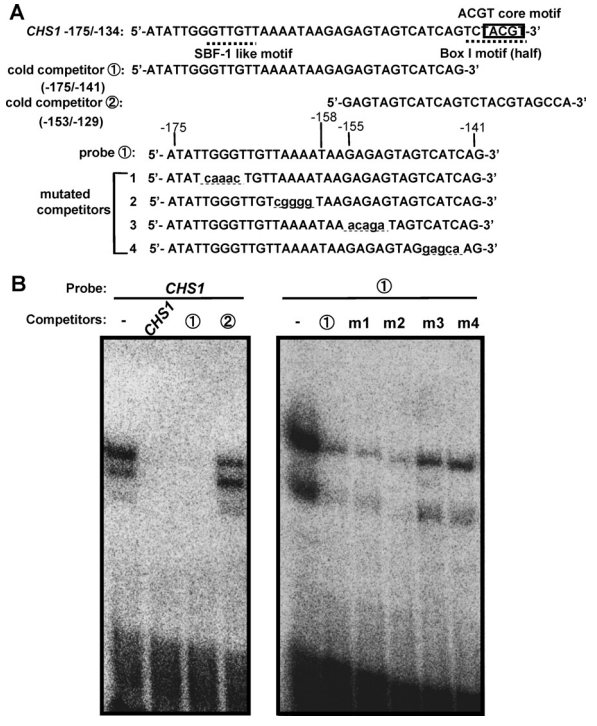

Figure 3. Competition analysis of SBZ1 binding activity. (A) Nucleotide sequences used as the probe and competitors. The mutated nucleotides are underlined and indicated by lower-case letters. (B) Analysis of DNA-binding specificity by including the *CHS1* sequence regions from −175 to −141, −153 to −129, and the mutated −175 to −141 as competing DNA targets in the binding reaction.

−129 sequence, which contains the box I motif in its 5′-terminus (Figure 3A, B). Detailed assays using mutated sequences as unlabeled competitors showed that the −141 to −158 region binds strongly to SBZ1 (Figure 3A, B).

In vitro phosphorylation of SBZ1

To monitor the expression of *CHS* following elicitation, semi-quantitative reverse transcriptase (RT)-PCR was performed (Figure 4). Total RNAs from suspension cells elicited 3 days after transfer to fresh medium by treatment with 0.5 mM reduced glutathione (Dron et al. 1988; Dröge-Laser et al. 1997; Wingate et al. 1988), 0.3 μM K252a, or 0.03% dimethyl sulfoxide (DMSO) were used in RT-PCR. *CHS* transcripts accumulated from low basal levels after elicitation with reduced glutathione. As *CHS* expression induced by elicitation was suppressed by K252a, a protein kinase inhibitor (Figure 4), phosphorylation appears to be involved in the elicitor-inducible expression of *CHS*. Phosphorylation of transcription factors is a common modification that can influence their cell biological properties (Hunter, 1995). The substrate motifs of some protein kinases present in the deduced amino acid sequence of SBZ1 suggest that SBZ1 is phosphorylated in a calcium-dependent manner. As a first step to investigating whether phosphorylation plays a role in the regulation of SBZ1, a bacterially expressed glutathione *S*-transferase (GST)-SBZ1 fusion protein (GST-SBZ1) was used as a substrate in an *in vitro* kinase assay in the presence of radioactively labeled ATP. GST-SBZ1 was phosphorylated by soybean cell extract, but GST alone was not (Figure 5A). Next, to examine the effect of Ca^{2+} on the phosphorylation of SBZ1 *in vitro*, ethylene glycol bis (β-aminoethylether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid (EGTA), a Ca^{2+} chelator, was added to the reaction mixture. EGTA inhibited the phosphorylation of SBZ1, an effect that was reversed by the addition of Ca^{2+} (Figure 5B). The CDPKs, a family of calcium- but not calmodulin-dependent protein kinases, have been reported to be involved in plant defense (Romeis et al. 2001). To investigate whether SBZ1 is phosphorylated by a CDPK, soybean CDPK beta was used in an *in vitro* kinase assay. Bacterially expressed histidine-tagged recombinant soybean CDPK (His_6-CDPK) was found to phosphorylate SBZ1 *in vitro* (Figure 5C). The SBZ1 amino acid sequence also contains a CKII substrate motif. Some transcription factors are regulated by CKII, a ubiquitous tetrameric

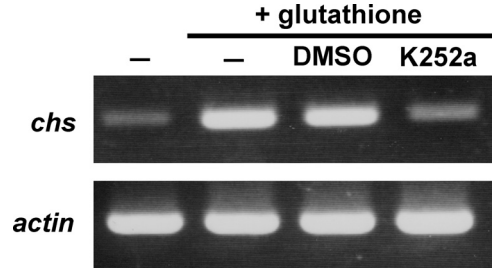

Figure 4. Elicitor-inducible and phosphorylation-dependent expression of soybean *CHS* gene. The levels of *CHS* mRNA after elicitation for 1 h of soybean cells with 0.5 mM glutathione in the presence of 0.3 μM K252a or DMSO were estimated by RT-PCR.

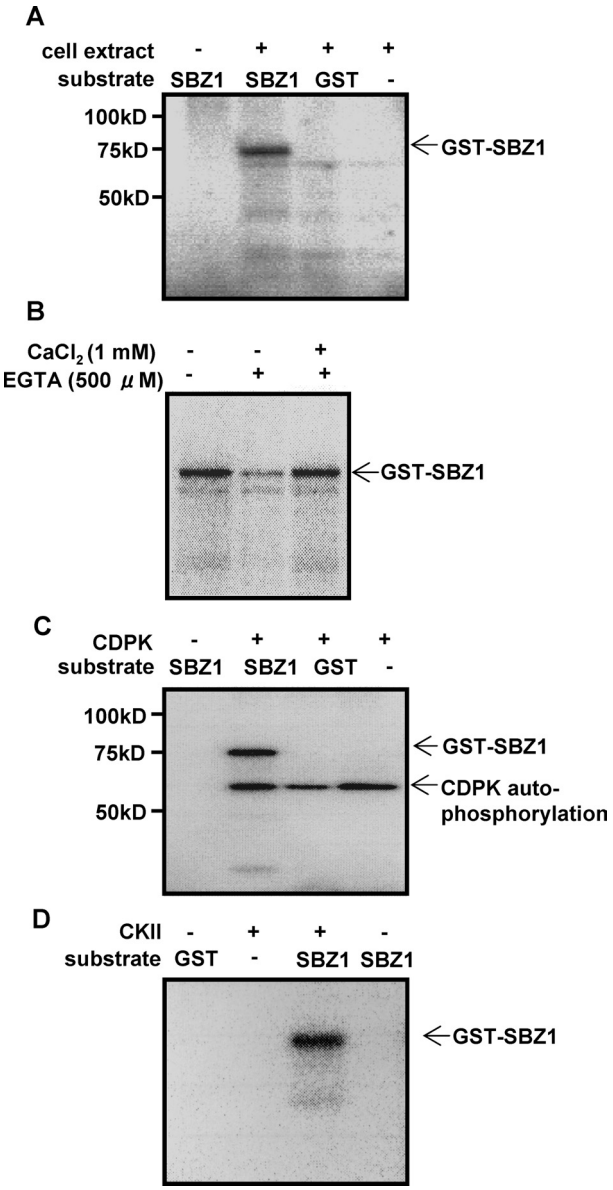

Figure 5. *In vitro* phosphorylation of SBZ1. (A) Bacterially expressed GST or GST-SBZ1 protein tested as substrates in *in vitro* kinase assays using equal amounts of protein extract from elicited soybean cells in the presence of radioactively labeled ATP. Autoradiographs show a signal at the expected size of GST-SBZ1, but no significant phosphorylation of GST, SBZ1, or soybean cell extract alone is visible. (B) Inhibition of SBZ1 phosphorylation by EGTA. GST-SBZ1 was incubated with extract from elicited cells in the presence of EGTA or both EGTA and Ca^{2+}. Ca^{2+} counteracted the inhibition of phosphorylation by EGTA. (C) Phosphorylation of SBZ1 by soybean CDPK. Bacterially expressed His_6-CDPK was purified and used for *in vitro* phosphorylation of GST-SBZ1. A signal corresponding to the autophosphorylation of CDPK was detected below the area of the expected size of GST-SBZ1. (D) Phosphorylation of GST-SBZ1 by the recombinant α subunit of CKII.

serine/threonine kinase composed of two catalytic (α/α') and two regulatory subunits. Interaction studies of CKII have shown that the basic domain is the main interaction site between bZIP proteins and CKII (Klimczak et al. 1995; Yamaguchi et al. 1998). *In vitro* kinase assays using the catalytic α subunit of recombinant CKII demonstrated that CKII also phosphorylates SBZ1 (Figure 5D).

Phosphorylation and dephosphorylation events are well-known mechanisms that regulate transcription factor activity in animals. Several reports have suggested that the activities of plant transcription factors can also be modulated by phosphorylation. Phosphorylation affects the affinities of some transcription factors for their DNA targets *in vitro* (Klimczak et al. 1995; Ciceri et al. 1997; Hardtke et al. 2000). We examined, therefore, whether phosphorylation has any influence on the affinity of SBZ1 for the binding sequence (Figure 6). First, His_6-SBZ1 was dephosphorylated by alkaline phosphatase treatment. Dephosphorylated His_6-SBZ1 was then phosphorylated by extracts from both elicited and non-elicited soybean cells, as well as both CDPK and *CKII*, and the dephosphorylated and rephosphorylated SBZ1 were tested for the ability to bind the *CHS1* promoter fragments using gel retardation assays. The binding activity of SBZ1 was not significantly affected by phosphorylation (Figure 6).

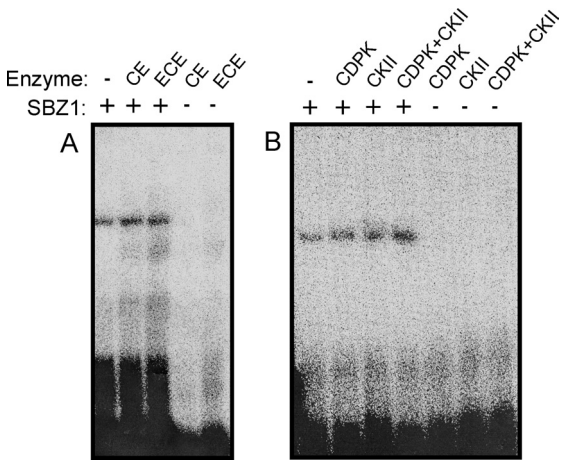

Figure 6. Effect of phosphorylation of recombinant SBZ1 on its binding to the −175 to −134 region of the *CHS1* promoter. (A) DNA-binding assay with phosphorylated His_6-SBZ1 in the presence of extracts from elicited (ECE) and non-elicited (CE) soybean cells. Alkaline-phosphatase-treated His_6-SBZ1 was rephosphorylated by incubation with cell extracts and subjected to DNA-binding assays. (B) DNA-binding assays using His_6-SBZ1 rephosphorylated by CDPK and/or the CKII α subunit.

Discussion

SBZ1 is a bZIP protein that contains a basic domain involved in DNA binding adjacent to a leucine zipper, with 63% amino acid sequence identity to G/HBF-1 and 60% identity to tobacco BZI-1. Parsley CPRF2, maize OHP1/2, rice REB, barley BLZ, and BZI-1 form a small group of transcription factors that are functionally homologous (Heinekamp et al. 2002). SBZ1 contains some features that are highly conserved in these transcription factors (Figure 1A), including the amino acid sequence, domain structures (D1–D4), DNA-binding domains (basic domain), leucine zipper domains, and N-terminal proline-rich putative activation domains, suggesting that SBZ1 belongs to this group.

In general, bZIP factors bind to *cis* elements that harbor an ACGT core motif (Foster et al. 1994; Jakoby et al. 2002). G/HBF-1, a bZIP transcription factor, binds within the bean *CHS15* promoter region not only to the G-box (CACGTG) but also to the H-box (CCTACC), which does not contain an ACGT sequence (Dröge-Laser et al. 1997). SBZ1 was also found to bind to the *CHS15* promoter. The G-box and H-box are essential for the transcriptional activation of *CHS15*, and *trans* factors that bind to the G- and H-boxes might contribute to the transcription of *CHS15* (Arias et al. 1993; Loake et al. 1992). Since SBZ1 binds to the soybean *CHS7* promoter, which has a similar G-box and H-box structure to the bean *CHS15* TATA proximal region (Figure 2A), SBZ1 may play a role in the transcription of *CHS7*. In addition, SBZ1 binds to the region from −175 to −134 in the soybean *CHS1* promoter more strongly than it binds to the *CHS7* promoter (Figure 2B, C). It has been shown that this 5′ region of *CHS1* is necessary for the expression of this gene that responds to a fungal elicitor (Wingender et al. 1990). Therefore, SBZ1, which binds to this sequence, is thought to take part in the elicitor-inducible expression of *CHS1*. This result, together with the binding of SBZ1 to the *CHS7* promoter region, suggests a broad range of binding specificity for SBZ1. However, the binding activity of SBZ1 to the *CHS1* promoter region is stronger than that to *CHS7*, suggesting that SBZ1 binds predominantly to the *CHS1* promoter region. In a detailed analysis using mutated competitors, the retarded band was not abolished by a competitor mutated from −143 to −155 in the *CHS1* promoter, indicating that the 18-bp sequence from −141 to −158 is important for SBZ1 binding (Figure 3). Since no G-box or other previously reported boxes were found in this sequence, the SBZ1 binding specificity appears different from those of other members of the bZIP transcription factor family, including CPRF2 and BZI-1.

The activity of transcriptional regulators can be controlled by several mechanisms. The most direct way to regulate the abundance of a factor is by adjusting the production of the encoding mRNA. However, SBZ1 activity is not controlled by mRNA abundance, as the level of *SBZ1* transcripts did not change following elicitation (data not shown).

The post-transcriptional modification of plant

transcription factors is another possible mechanism for regulating their activity. In particular, reversible phosphorylation is a common mechanism for the selective regulation of transcription factors.

In higher plants, the phosphorylation of transcription factors might be an important mechanism for the selective control of gene expression in response to environmental signals (Wellmer et al. 1999; Klimczak et al. 1995; Ciceri et al. 1997; Kircher et al. 1998; Zhou et al. 1997). In this study, SBZ1 was phosphorylated by a soybean cell extract *in vitro* (Figure 5A). Both elicited and non-elicited soybean cell extracts phosphorylated SBZ1, with no significant difference in the total phosphorylation level. However, because the phosphorylation of amino acid residues at specific positions may control the function of the protein (Montominy 1997), we searched for putative phosphorylation sites in the SBZ1 amino acid sequence. A scan for phosphorylation sites using the PROSITE database (Bairoch, 1991) revealed the presence of potential CKII phosphorylation sites, and a CDPK site was also found in the D4 domain. *In vitro* kinase assays using EGTA suggested the Ca^{2+}-dependent phosphorylation of SBZ1 (Figure 5B). Moreover, recombinant soybean CDPK phosphorylated SBZ1 *in vitro* (Figure 5C), suggesting that CDPK is related to the functional regulation of SBZ1. The importance of Ca^{2+} signaling in the transduction of environmental changes into plant responses has been demonstrated for a wide range of stimuli, including drought, cold, salinity, and pathogen attack. Many responses of plants to environmental stress depend on changes in the cytoplasmic concentration of free Ca2+. Changes in protein kinase activity have also been investigated during the early response of plant cells to fungal elicitors. The CDPKs, a family of calcium- but not calmodulin-dependent protein kinases found in a wide variety of plants and protists, which contain a catalytic domain and a calcium-binding domain, have been reported to be involved in the plant defense response (Sheen 1996; Romeis et al. 2001). Thus, CDPKs activated by calcium that respond to environmental stimuli are expected to control defense gene expression by phosphorylating transcription factors, and SBZ1 might be a target for these proteins.

CKII sites located in the D2 and basic domains are conserved in the CPRF2 group of transcription factors (Figure 1). Many transcription activators are phosphorylated, and their activities are either positively or negatively modulated by CKII. It has been demonstrated that the catalytic α and α' subunits of CKII directly interact with the bZIP DNA-binding domains in bZIP transcription factors (Yamaguchi et al. 1998). SBZ1 was phosphorylated by the α subunit of recombinant maize CKII (Figure 5D), suggesting that SBZ1 is a transcription factor whose activity is regulated by CKII.

Phosphorylation controls the functional activity of many animal transcription factors by modulating one or more of the following attributes: (1) DNA-binding affinity, (2) cellular localization, (3) interactions with other regulatory proteins, or (4) transactivation (Hunter and Karin 1992).

In most studies demonstrating the phosphorylation of a plant bZIP factor, this modification has been shown to be correlated with a change in its DNA-binding activity. For example, the affinity of G/HBF-1 for the CHS15 promoter is strongly stimulated following phosphorylation responding to fungal elicitor treatment *in vitro* (Dröge-Laser et al. 1997). However, phosphorylation of recombinant SBZ1 by either soybean cell extracts, CDPK, or CKII had no effect on its binding to the *CHS1* promoter *in vitro* (Figure 6). Therefore, we conclude that phosphorylation is not involved in the regulation of the DNA-binding activity of SBZ1.

It has been predicted that the phosphorylation of bZIP by CKII occurs in or N-terminal to the basic region, leading to a change in protein conformation and activation of the NLS, which causes the translocation of the transcription factor (Jans and Hubner 1996). The acidic D2 domain and the basic domain of SBZ1 contain CKII phosphorylation sites that are conserved among the small group of transcription factors that includes CPRF2. This protein is imported from the cytosol to the nucleus following light signaling, which might trigger a phosphorylation event that modulates NLS activity (Kircher et al. 1999), suggesting that SBZ1 is regulated in the same manner by elicitor treatment. However, investigation of the subcellular localization of SBZ1, studied using particle bombardment of soybean cells with constructs that constitutively overexpress a GFP-tagged SBZ1 gene, indicated that transiently expressed SBZ1 localizes to the nucleus. Therefore, we conclude that SBZ1 differs from CPRF2 in contribution of phosphorylation to the subcellular localization.

Phosphorylation may regulate other attributes of SBZ1. The phosphorylation of HY5, an *Arabidopsis* bZIP transcription factor that binds to the *CHS* promoter region, has been suggested as controlling the degradation of the protein following protein interaction (Hardtke et al. 2000). CKII phosphorylation controls both the regulation of degradation and the transactivation of HY5. It is possible that stability and activity of SBZ1 are affected by CKII phosphorylation.

The phosphorylation of DNA-binding proteins promotes the recruitment of coactivators that mediate target gene activation, in part, by destabilizing promoter-bound nucleosomes, thereby allowing the assembly of the transcriptional apparatus. It will be of great interest to identify components of the transcriptional machinery

that utilize SBZ1, and the effect of the phosphorylation of SBZ1 on its ability to engage the transcriptional machinery *via* its association with coactivators. Further experiments including yeast two-hybrid screening and GST pull-down assay provide a better picture of SBZ1 in the transcriptional machinery and effect of phosphorylation on it.

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