

# Characterization of a nuclear protein that binds to three elements within the silencer region of a bean chalcone synthase gene promoter

(cis elements/*Phaseolus vulgaris*/trans-acting factor/transcription)

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**ABSTRACT** The chalcone synthase (EC 2.3.1.74) gene promoter from the bean *Phaseolus vulgaris* L. contains a silencer element between positions –140 and –326 from the transcription start site that is functional in electroporated soybean protoplasts. This element contains three binding sites for a bean nuclear factor (SBF-1) with DNA sequence recognition properties that are very similar to those of nuclear factor GT-1. By using a synthetic tetramer of one of the binding sites as probe, we have purified sequence-specific SBF-1 activity approximately 1750-fold from suspension-cell nuclei, by using a combination of ammonium sulfate precipitation, gel filtration, heparin-agarose chromatography, and sequence-specific DNA affinity chromatography. The factor exhibited an apparent molecular weight of 160,000–200,000 on the basis of gel filtration. A subunit molecular weight of approximately 95,000 was determined from SDS/polyacrylamide gel electrophoretic analysis of purified fractions, followed by southwestern blot analysis (a protein blot probed with oligonucleotide probes), and from UV-cross-linking experiments. The factor lost DNA-binding activity on treatment with alkaline phosphatase. We discuss the properties of SBF-1 in relation to the functionality of GT-1 binding sequences in plant genes.

Selective transcriptional activation is a key feature underlying the plasticity of plant development and plants' adaptive and protective responses to environmental stress. Chalcone synthase (CHS; EC 2.3.1.74) catalyzes the first reaction specific for the biosynthesis of a diverse array of flavonoid natural products, which have important functions in the development of the plant and in interactions with its environment. The enzyme from the bean *Phaseolus vulgaris* L. is encoded by a family of seven genes (1) whose transcripts are highly regulated in a tissue- and cell-type-specific manner in both roots and aerial parts of the plant (2) and, furthermore, are markedly induced by wounding, fungal infection, or treatment with fungal elicitor macromolecules, associated with the biosynthesis of antimicrobial isoflavonoid phytoalexins (1, 3).

Analysis of bean CHS promoter- $\beta$ -glucuronidase gene fusions in transgenic tobacco has shown that elements responsible for tissue and cell-type specific expression and responsiveness to infection or elicitor treatments are localized within 1 kilobase upstream of the transcription initiation site (refs. 2 and 4; unpublished data). Functional analysis of the promoter of one bean CHS gene, CHS15, using reporter gene fusions in electroporated protoplasts (refs. 5 and 6; unpublished data), has revealed the presence of both positive and negative elements between positions –326 and –173. This region functions overall as a silencer in electroporated

soybean protoplasts (5). It also appears to be involved in regulating the organ-specific level of expression of CHS15 promoter activity in bean (unpublished data). Gel-retardation and DNase I-footprinting experiments have identified a nuclear factor, SBF-1, present in both bean and alfalfa suspension-culture cells, that binds to three sites (boxes I–III) within the region from position –326 to position –173 (ref. 7; unpublished data) (Fig. 1). On the basis of its recognition site sequence (core consensus GGTAA), SBF-1 may be related or even identical to GT-1, which is involved in the light-dependent expression of the *rbcS-3A* gene in green plant tissues (8, 9).

The most generally applicable strategy for isolating transcription factors is purification and subsequent generation of antibodies or oligonucleotide probes for gene isolation. Herein we report the purification of SBF-1 and describe some of the properties of this plant DNA-binding protein.

## MATERIALS AND METHODS

**Preparation of Nuclear Extracts.** Nuclear extracts were prepared from suspension-cultured bean (*P. vulgaris* cv. Canadian Wonder) cells according to Lawton *et al.* (7).

**DNA Probes and Gel-Retardation and DNase I-Footprinting Assays.** DNA probes and competitor DNA sequences (Fig. 1B) were synthesized on a Du Pont Generator oligonucleotide synthesizer. Probes for DNase I footprinting and gel-retardation assays were prepared by end-labeling with T4 polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP (NEN). Gel-retardation and DNase I-footprinting assays were performed as described (7). One unit of binding activity is defined as the amount of protein required to totally retard 0.1 ng of labeled box III tetramer probe in a gel-retardation assay.

**Purification of SBF-1.** All stages were carried out at 0–4°C. Glycerol was removed from crude nuclear extracts by gel filtration on PD-10 columns (Pharmacia), and box III binding activity was precipitated by saturation to 40% with ammonium sulfate followed by centrifugation. The pellet (10–15 mg of protein from 1.5 liters of cell suspension culture) was resuspended in Dignam's dialysis buffer (10) minus glycerol and subjected to gel filtration on a Sephacryl S-300 column (40 cm  $\times$  1 cm) at a flow rate of 0.25 ml/min with dialysis buffer minus glycerol. Active fractions were pooled and applied to a heparin-agarose column (1–4 ml) and eluted with a KCl step gradient in heparin-agarose buffer [20 mM Hepes, pH 7.9/10% (vol/vol) glycerol/100 mM KCl/0.2 mM EDTA/0.5 mM phenylmethylsulfonyl fluoride/0.5 mM dithiothreitol/0.1% Nonidet P-40]. Active fractions were dialyzed

Abbreviation: CHS, chalcone synthase.

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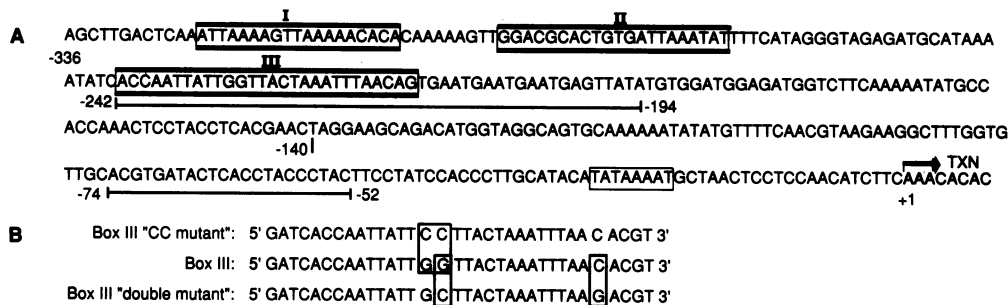


FIG. 1. (A) Bean CHS15 promoter upstream of the transcription start site. Boxes I–III are the three binding sites for SBF-1. The TATA box is boxed and regions of homology to the bean PAL2 promoter are underlined. (B) Box III probe and “mutant” sequences (“upper” strands of double-stranded oligonucleotides) used in competition binding experiments. The 5′ GA and 3′ CGT nucleotides were added to the CHS box III sequence to facilitate ligation of the oligomers.

(Pierce microdialyzer; molecular weight cut-off, 50,000) against heparin-agarose buffer containing 0.1 M KCl and applied to a DNA affinity column consisting of multimers (up to 50-mers) of the box III sequence linked to cyanogen bromide-activated Sepharose, prepared as described by Kadonaga and Tjian (11). Proteins were eluted with a KCl step gradient in heparin-agarose buffer and the combined active fractions were dialyzed against 0.1 M KCl in heparin-agarose buffer. The cycle of DNA affinity chromatography and dialysis was then repeated, and the active fractions were snap-frozen in 100- $\mu$ l samples and stored at  $-80^{\circ}\text{C}$ .

**SDS/PAGE.** SDS/polyacrylamide gels were electrophoresed under standard conditions (12) and fixed in 20% (wt/vol) trichloroacetic acid for 30 min, and proteins were visualized with silver staining (Bio-Rad kit).

**Southwestern Blot Analysis.** Protein blotting was performed essentially as described by Miskimins *et al.* (13). After transfer of proteins to Immobilon (Millipore) membranes and treatment with blocking solutions (13), the membranes were rinsed twice in TNE-50 (10 mM Tris-HCl, pH 7.5/50 mM NaCl/1 mM EDTA/1 mM dithiothreitol) and incubated with  $^{32}\text{P}$ -labeled probe (box III oligomers, 2 ng/ml) and a 500-fold molar excess of poly(dI-dC)-poly(dI-dC) in TNE-50 for a further 2 hr at room temperature with gentle agitation. Excess unbound probe was removed by three 10-min washes in TNE-50 at room temperature, and the membranes were dried and exposed to x-ray film (Kodak X-omat Ar) with intensifying screens for 12–24 hr at  $-80^{\circ}\text{C}$ . In competition Southwestern blots, the unlabeled competitor DNA was included in the probe/TNE-50 binding solution at 50-fold molar excess.

**Dephosphorylation of Nuclear Extracts.** Partially purified nuclear extracts (0.1–1.0  $\mu\text{g}$  of protein) were incubated with agarose-linked calf intestinal alkaline phosphatase (0.25–1.0 unit) (Sigma) in 20 mM Hepes, pH 8.5/0.1 M KCl/1 mM  $\text{MgCl}_2$ /0.1 mM  $\text{ZnCl}_2$ /10% glycerol for 15 min at room temperature. The immobilized enzyme was removed by centrifugation ( $10,000 \times g$ , 2 min,  $4^{\circ}\text{C}$ ) and then the supernatant was clarified by a similar centrifugation for 10 min. The binding activity of the supernatant was assayed by gel retardation. Modified buffers in which the phosphatase had a reduced activity (20 mM Hepes, pH 6.0/0.1 M KCl/1 mM  $\text{MgCl}_2$ /0.1 mM  $\text{ZnCl}_2$ /10% glycerol) or no activity (20 mM Hepes, pH 8.5/0.1 M KCl/10 mM EDTA/10% glycerol) were used in control preincubations. The activity of the alkaline phosphatase under the various conditions was assessed semiquantitatively by measuring the loss of label from a  $^{32}\text{P}$ -labeled box III tetramer probe, as visualized by autoradiography.

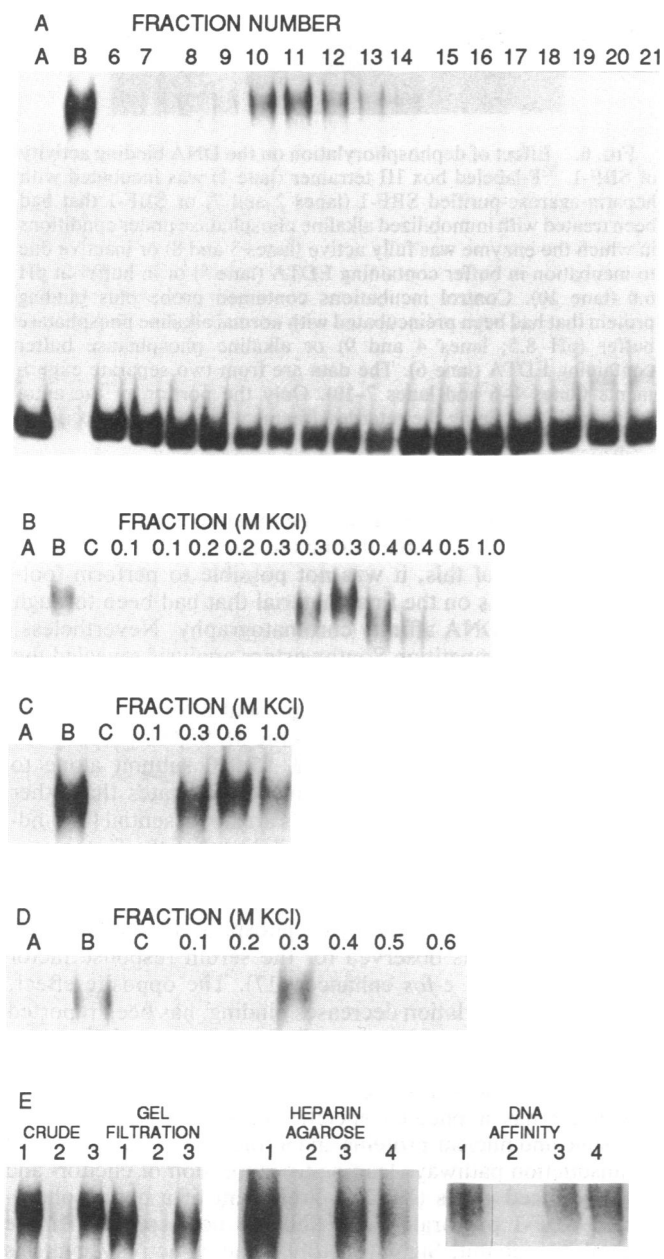
## RESULTS

The box III region of the CHS15 promoter defined by *in vitro* DNase I footprinting (Fig. 1A) has the highest affinity of the

three sites (boxes I–III) for the binding of SBF-1 from crude preparations (7), and a ligated tetramer of the box III sequence (Fig. 1B) was, therefore, used as probe in gel-retardation assays to monitor purification of the factor. A CHS promoter fragment (positions  $-326$  to  $-140$ ) containing all three binding sites was also used to monitor purification with identical results (data not shown). After salt extraction from isolated bean nuclei and precipitation with ammonium sulfate, the nuclear extract was subjected to gel filtration on Sephacryl S-300 (Fig. 2A). Binding activity was eluted in fractions 7–14, with maximum activity in fraction 11, corresponding to an apparent molecular weight for the native protein of 160,000–200,000. Binding activity was fully retained on a heparin-agarose column and was eluted with 0.3–0.4 M KCl (Fig. 2B). The activity was likewise fully retained on a DNA affinity column containing immobilized ligated box III multimers and was eluted with 0.3 M KCl (Fig. 2C); in a separate experiment, continued elution with 0.3 M KCl recovered all SBF-1 activity. On a second cycle through the affinity column, all activity was eluted in the 0.3 M KCl fraction (Fig. 2D). It should be noted that a single major gel-retarded complex was observed at all stages of purification. The mobility of this complex varied slightly due to the presence of residual salt after dialysis (e.g., Fig. 2B). The yield and fold purification of the factor at the various stages, computed from a number of independent purification runs, are given in Table 1. Competition gel retardation (Fig. 2E) indicated that SBF-1 retained DNA sequence specificity throughout purification, the binding being competed by excess unlabeled box III but not by the box III “double mutant” or “CC mutant” oligonucleotides shown in Fig. 1B.

In an *in vitro* DNase I footprinting experiment (Fig. 3), SBF-1 purified through the heparin-agarose stage protected boxes I, II, and III in a CHS promoter fragment consisting of the region between positions  $-326$  to  $-140$ . The footprint obtained with the purified factor was strongest in the box I and II regions, whereas box III was more strongly footprinted using crude extracts (7).

A major band of  $M_r \approx 95,000$  was observed on Southwestern blots of DNA-affinity-purified fractions (Fig. 4A). This band was present at each stage of purification. A minor band of  $M_r \approx 45,000$  was observed in highly purified fractions in some experiments (see Fig. 4B). This may be a degradation product of the  $M_r 95,000$  polypeptide; unlike the  $M_r 95,000$  polypeptide, its appearance did not correlate with binding activity. At earlier stages of purification, major bands of higher and lower molecular weights were also observed on Southwestern blots. However, this binding could not be competed with excess box III oligonucleotide and is, therefore, not sequence-specific. The  $M_r 95,000$  polypeptide retained sequence-specific binding after separation by SDS/PAGE, as hybridization of labeled box III oligomers to the blotted protein was competed by unlabeled box III but not by



**FIG. 2.** Gel-retardation analysis of fractions during purification of SBF-1. Probe minus nuclear extract is shown in lanes A. (A) Gel filtration on Sephacryl S-300. Lane B shows retardation by the 0–40% ammonium sulfate fraction applied to the column. Fraction volume was 1 ml. (B) Heparin-agarose chromatography. Lane B shows retardation by the gel-filtration fraction applied to the column, and lane C shows the flow-through. For each KCl concentration, one, two, or three fractions of 500  $\mu$ l were collected. (C) DNA affinity chromatography (first cycle). Lane B shows retardation by the 0.3 M KCl fraction from heparin-agarose applied to the column; lane C shows the flow-through. Fraction volume was 350  $\mu$ l. (D) DNA affinity chromatography (second cycle). Lane B shows retardation by the pooled 0.3, 0.6, and 1.0 M KCl fractions from the first DNA affinity step; lane C shows the flow-through. Fraction volume was 400  $\mu$ l. (E) Competition gel retardation at each stage of purification. Lanes 1 show the  $^{32}$ P-labeled box III probe retarded by crude or purified SBF-1; lanes 2–4 are as lanes 1 plus 100-fold molar excess of unlabeled box III (lanes 2), unlabeled box III “double mutant” (lanes 3), or box III “CC mutant” (lanes 4). Note that B–E show only the retarded fragment.

the box III “CC mutant” oligonucleotide (Fig. 4B). Cross-linking a  $^{32}$ P-labeled bromodeoxyuridine-substituted box III probe to components of a crude bean nuclear protein extract

**Table 1.** Purification of SBF-1 from bean nuclei

Fraction	Specific activity, units/ $\mu$ g of protein	Purification over previous step, fold	Recovery from previous step, %
Crude extract	0.07–0.10		
Ammonium sulfate + gel filtration	0.1–0.3	2–3.5	50–60
Heparin-agarose	0.6–1.0	3–5	50–80
First DNA affinity chromatography	10–20*	10–20*	10–50
Second DNA affinity chromatography	50–100*	5*	10

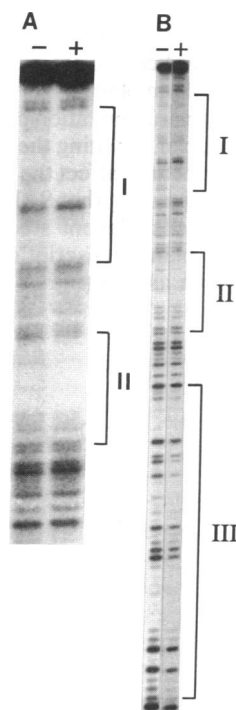
Data shown are the range of values obtained from a number of independent purifications (six times to the heparin-agarose stage, four times to the first DNA affinity stage, and one complete purification).

\*Estimated values for protein content were based on silver-staining intensity on SDS gels.

by UV irradiation, followed by SDS/PAGE analysis, revealed a major protein–DNA complex of  $M_r \approx 95,000$  with two minor components of  $M_r 45,000$  and  $M_r > 100,000$  (unpublished data).

Silver staining of SDS/PAGE gels (Fig. 5) revealed at least 30 polypeptide species in active fractions after heparin-agarose chromatography. After one round of DNA affinity chromatography, the active fraction contained the  $M_r 95,000$  polypeptide with three or four other polypeptides whose appearance varied from preparation to preparation and did not correlate with DNA binding activity. After two rounds of DNA affinity chromatography, SBF-1 preparations were extremely unstable and the  $M_r 95,000$  band was barely visible by silver staining (detection limit,  $\approx 10$  ng).

Pretreatment of heparin-agarose-purified SBF-1 with immobilized alkaline phosphatase eliminated the ability of the



**FIG. 3.** DNase I footprints on a lower-strand fragment from position –326 to position –140 of the CHS15 promoter obtained with 4  $\mu$ g of heparin-agarose-purified SBF-1 (lanes +). DNase I digestion pattern in the absence of SBF-1 is in lanes –. Results are from two experiments (A and B). Boxes I, II, and III are indicated.

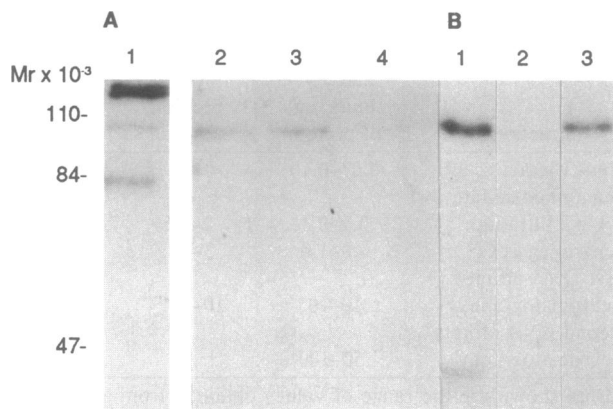


FIG. 4. Southwestern blot analysis of fractions during the purification of SBF-1. (A) SDS/PAGE blot probed with  $^{32}\text{P}$ -labeled box III oligomers. Each lane contained approximately 0.5 unit of binding activity after gel filtration (lane 1), heparin-agarose chromatography (lane 2), first-cycle DNA affinity chromatography (lane 3), and second-cycle DNA affinity chromatography (lane 4). (B) Blot of purified (gel filtration and heparin-agarose) SBF-1 probed with  $^{32}\text{P}$ -labeled box III oligomers alone (lane 1) or in the presence of a 50-fold molar excess of unlabeled box III (lane 2) or unlabeled box III "CC mutant" (lane 3).

preparation to retard the box III probe (Fig. 6) or the promoter fragment between positions  $-326$  and  $-140$  (data not shown). This effect was not observed if the pretreatment was carried out in the presence of buffers in which the phosphatase was inactive or in buffer minus the immobilized enzyme, indicating that dephosphorylation, rather than some other component in the preincubation, was causing the loss of activity.

## DISCUSSION

Apart from a recent paper describing the partial purification of a factor that binds to the *Arabidopsis* alcohol dehydrogenase gene (14), there have been no reports of the purification of transcription factors from plants. Herein we describe the purification and partial characterization of factor SBF-1 from bean suspension-cell nuclei. Treating the cell cultures with a fungal elicitor did not appear to affect the extractable activity of the binding factor, suggesting that SBF-1 is constitutively expressed in the bean cells. Recent studies have indicated that activity of some plant transcription factors may be inducible by stimuli such as ethylene or wounding (15, 16).

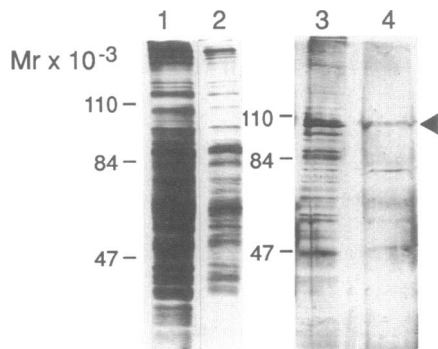


FIG. 5. Silver-stained SDS/polyacrylamide gels showing the polypeptide composition of active fractions during the purification of SBF-1. The 0–40% ammonium sulfate (lane 1) and gel-filtration (lane 2) lanes contain 2 units of binding activity per lane, whereas the heparin-agarose (lane 3) and first DNA affinity (lane 4) lanes contain 1 unit per lane. The 95-kDa polypeptide is indicated in the DNA affinity-purified fraction by an arrowhead.

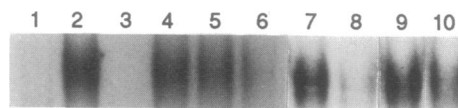


FIG. 6. Effect of dephosphorylation on the DNA binding activity of SBF-1.  $^{32}\text{P}$ -labeled box III tetramer (lane 1) was incubated with heparin-agarose-purified SBF-1 (lanes 2 and 7) or SBF-1 that had been treated with immobilized alkaline phosphatase under conditions in which the enzyme was fully active (lanes 3 and 8) or inactive due to incubation in buffer containing EDTA (lane 5) or in buffer at pH 6.0 (lane 10). Control incubations contained probe plus binding protein that had been preincubated with normal alkaline phosphatase buffer (pH 8.5; lanes 4 and 9) or alkaline phosphatase buffer containing EDTA (lane 6). The data are from two separate experiments (lanes 1–6 and lanes 7–10). Only the portion of the autoradiograph containing the retarded fragment is shown.

The final yield and fold purification of SBF-1 (at best 2.4% and 1750-fold, respectively) were within the range obtained for transcription factors from mammalian sources. SBF-1 activity is highly unstable during the later stages of purification. Because of this, it was not possible to perform footprinting analysis on the final material that had been through two cycles of DNA affinity chromatography. Nevertheless, the fact that competition Southwestern analysis revealed the presence of a single sequence-specific DNA-binding factor of  $M_r$  95,000 that was consistently present after DNA affinity chromatography clearly identifies the  $M_r$  95,000 polypeptide as SBF-1. The ability of the  $M_r$  95,000 subunit alone to specifically bind the box III sequence indicates that other potentially associated polypeptides are not essential for binding but does not rule out the possibility that the factor may bind as a dimer, consistent with the molecular weight of 160,000–200,000 that was determined by gel filtration.

An absolute requirement for phosphorylation for DNA binding activity is observed for the serum response factor that binds to the *c-fos* enhancer (17). The opposite effect, where phosphorylation decreases binding, has been reported for the AT-1 factor from pea (18). Inactivation of SBF-1 by dephosphorylation raises questions as to the regulatory function of this post-translational modification *in vivo*. Recent evidence has implicated specific phosphorylation of cytoplasmic and nuclear proteins and a role for  $\text{Ca}^{2+}$  in the signal transduction pathways leading to expression of elicitor- and light-induced genes (19, 20). Treatment of a dephosphorylated SBF-1 preparation with bovine heart protein kinase (catalytic subunit, Sigma) restores binding activity, but this activity then exists as two components that migrate faster than "native" SBF-1 in gel-retardation assays (unpublished data). This may be due to extra nonspecific phosphorylation. These data define an assay system for characterizing a protein kinase involved in gene regulation.

SBF-1 activity in crude bean nuclear extracts can bind to separate oligonucleotides containing the box I, II, or III sequences of the CHS15 promoter (7). Each box contains variants of the GT-1 consensus sequence GGTTAA surrounded by different flanking sequences. Herein we show that a partially purified SBF-1 preparation containing a single sequence-specific binding activity as revealed by Southwestern blot analysis binds to all three boxes in a CHS promoter fragment covering the region from position  $-326$  to position  $-140$ . GT-1 core sequences with a variety of flanking region sequences have been identified in a number of plant gene promoters, and GT-1 binding activities have been described in a range of tissues from at least five plant species (refs. 8, 21–23 and unpublished data). GT-1 and its corresponding cis element are involved in the induction by light of the *rbcS-3A* gene in green tissues (9), and GT-1 binding sites are also found in the promoter of the rice phytochrome gene (22), a gene that is down-regulated by light. Although CHS is

up-regulated by light, its tissue-specific expression is very different from that of the *rbcS-3A* gene (2). This therefore implies that the functional properties of GT-1 factors and their corresponding cis elements are dependent on both the structural context of the promoter in which the element resides and the cell-type nuclear environment. A family of GT-1 proteins may exist whose binding activities, as assayed *in vitro*, could be specific for any sequence with the basic GT-1 core but may exhibit slightly differing specificity *in vivo* (24). Alternatively, there may be a more limited number of GT-1-like factors that could act as core DNA-binding proteins, capable of binding to the GT-1 consensus sequence in its many different contexts (9).

Because of its very low abundance and lack of stability, it may not be easy to obtain sufficient SBF-1 for N-terminal sequence analysis. However, the molecular characterization of SBF-1 provides us with information to facilitate the generation of monoclonal antibodies. Preliminary data suggest that a polyclonal serum prepared from heparin-agarose/SDS-gel-purified nuclear extracts contains antibodies to SBF-1 that could provide probes for cloning and determining the structure of the factor, its potential interaction with other transcription factors, the tissue and cell-type distribution of its expression and activity, and whether its regulation is primarily at the transcriptional or post-transcriptional (phosphorylation) level. Answering these questions should further our understanding of the molecular networks that underlie the integrated expression program of a key developmentally and environmentally regulated plant gene.

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