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Silencer region of a chalcone synthase promoter contains multiple binding sites for a factor, SBF-1, closely related to GT-1

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Abstract

Bean nuclear extracts were used in gel retardation assays and DNase I footprinting experiments to identify a protein factor, designated SBF-1, that specifically interacts with regulatory sequences in the promoter of the bean defense gene CHS15, which encodes the flavonoid biosynthetic enzyme chalcone synthase. SBF-1 binds to three short sequences designated boxes 1, 2 and 3 in the region −326 to −173. This *cis*-element, which is involved in organ-specific expression in plant development, functions as a transcriptional silencer in electroporated protoplasts derived from undifferentiated suspension-cultured soybean cells. The silencer element activates *in trans* a co-electroporated CHS15-chloramphenicol acetyltransferase gene fusion, indicating that the factor acts as a repressor in these cells. SBF-1 binding *in vitro* is rapid, reversible and sensitive to prior heat or protease treatment. Competitive binding assays show that boxes 1, 2 and 3 interact cooperatively, but that each box can bind the factor independently, with box 3 showing the strongest binding and box 2 the weakest binding. GGTTAA(A/T)(A/T)(A/T), which forms a consensus sequence common to all three boxes, resembles the binding site for the GT-1 factor in light-responsive elements of the pea *rbcS-3A* gene, which encodes the small subunit of ribulose bisphosphate carboxylase. Binding to the CHS15 −326 to −173 element, and to boxes 1, 2 or 3 individually, is competed by the GT-1 binding sequence of *rbcS-3A*, but not by a functionally inactive form, and likewise the CHS sequences can compete with authentic GT-1 sites from the *rbcS-3A* promoter for binding. These data suggest that SBF-1 is identical to, or closely related to, GT-1.

Introduction

A striking feature of plant development is its environmental plasticity, and plants exhibit a number of adaptive and protective responses to environmental stresses. Of particular significance are flavonoid natural products, which have diverse functions in development and plant interactions with the environment. Chalcone synthase (CHS) catalyzes the condensation of three acetyl units from malonyl-CoA with 4-hydroxycinnamoyl-CoA to give naringenin chalcone, which

is the first committed step in the branch pathway of phenylpropanoid metabolism specific for flavonoid biosynthesis [9]. CHS mRNA and enzyme levels are highly regulated during the development of aerial organs associated with the tissue- and cell-type-specific accumulation of flavonoid pigments, and in response to light for the synthesis of flavonoids involved in UV protection. CHS genes are also expressed in roots, notably in the apical meristem and at the site of lateral root initiation [23], and this may be related to the functions of flavonoids as modulators of polar auxin transport [10] and in legumes as rhizosphere signals for the induction of *nod* genes in *Rhizobium* [21]. Moreover, in legumes CHS transcripts are markedly induced by wounding, fungal elicitor or infection associated with the synthesis of pterocarpan and isoflavonoid phytoalexins derived from flavonoid precursors [14].

Thus, CHS is a key metabolic control point, and provides an excellent system for analysis of the molecular mechanisms governing natural product biosynthesis. Run-on transcription assays in isolated nuclei and transient expression of CHS-reporter gene fusions in electroporated protoplasts have shown that UV irradiation of parsley cell cultures and elicitor treatment of bean or alfalfa cell cultures stimulate CHS transcription to initiate the synthesis of UV protectants and phytoalexins respectively [2, 5, 15, 20, 24]. Moreover, analysis of bean CHS-β-glucuronidase gene fusions in transgenic tobacco plants has demonstrated that these promoters are able to determine both tissue- and cell-type-specific control of flavonoid biosynthesis during development and also respond to externally imposed stimuli including light, wounding, fungal elicitor and infection [23, 27].

We are interested in dissecting the functional architecture of these promoters that confers the exquisite temporal and spatial control of CHS expression during development, while allowing flexible responses to diverse environmental stimuli, and analysis of CHS *cis*-elements and transcription factors provides an opportunity to examine the molecular mechanisms underlying key aspects of the unique developmental plasticity of higher plants.

Functional assays of the activities of mutated promoters in electroporated protoplasts have defined *cis*-acting elements involved in UV induction of parsley and *Antirrhinum* CHS genes [20, 24]. The TATA proximal region of the bean CHS15 promoter to -130 is sufficient for elicitor induction in electroporated protoplasts [2, 5]. In transgenic tobacco plants, CHS15 promoter sequences to -130 are sufficient for elicitor inducibility and for expression in roots and in the pigmented regions of petals (J. Kooter and C.J. Lamb., unpublished). In contrast, an upstream element of the CHS15 promoter from -326 to -173 functions as a silencer in electroporated soybean protoplasts [5]. Moreover, dissection of etiolated seedlings bearing CHS promoter-reporter gene fusions has shown that deletion of the silencer element severely reduces expression in the portion containing the cotyledons and apical meristem, but markedly enhances expression in hypocotyls, indicating that the silencer may have a key function in determining the organ-specific pattern of CHS15 promoter activity (J. Kooter and C.J. Lamb., unpublished).

In vivo and *in vitro* analysis has revealed that the induction of transcription by elicitor and by UV irradiation is accompanied by structural changes in the chromatin associated with CHS promoter regions [17, 24], probably reflecting the binding of transcription factors to *cis*-regulatory elements. The transcription factors interacting with CHS *cis*-acting regulatory elements represent the terminal steps of signal transduction pathways that allow flexible incorporation of environmental responses within a coherent developmental program of natural product biosynthesis. As a first step in the analysis of these signal pathways, we have identified, by gel retardation assays and DNase I footprinting, a nuclear factor, designated SBF-1, from bean cell suspension cultures that binds to 3 sites within the silencer (-326 to -173) element of the CHS15 promoter. We show that SBF-1 is closely related to or identical to GT-1, which is involved in the light-dependent expression of the ribulose bisphosphate car-

boxylase small subunit gene in green tissues [8, 13].

Materials and methods

General methods

The growth and elicitor treatment of bean (*Phaseolus vulgaris* L. cv. Canadian Wonder) cell suspension cultures were as described [16, 31]. Bovine serum albumin was Pentex Fraction V from Miles Laboratories, cellulase RS and pectolyase Y23 were obtained from Onozuka, protease inhibitors from Sigma.

Plasmids

CHS15p contains a 510 bp *Hin*d III-*Xho* II fragment of the CHS15 gene corresponding to the sequences −332 to +178 relative to the transcription start site. pCHC1 contains a 429 bp *Hin*f I fragment of the CHS15 gene corresponding to the sequences −326 to +107, fused to the coding region of the bacterial chloramphenicol acetyltransferase (CAT) gene [5]. pPAL2p contains a 1635 bp *Eco* RI fragment of the PAL2 gene corresponding to the sequences −1173 to +463 relative to the transcription start site [3]. Manipulation of DNA fragments was according to standard methods [22]. Oligonucleotides were synthesized on a Milligen/Biosearch Model 8750 DNA Synthesizer using phosphoramidite chemistry.

Isolation of nuclei

Nuclei were isolated using a modification of the method of Willmitzer and Wagner [30]. 7-day-old suspension-cultured bean cells (100–150 g fresh weight) were collected on Miracloth, and digestion medium (5 mg/ml Cellulase RS, 0.5 mg/ml Pectolyase Y23, 2 g/l bovine serum albumin, 0.7 M mannitol, 100 mM phenylmethanesulfonyl fluoride (PMSF), 10 mM 4-morpholineethanesulfonic acid, pH 5.8) vacuum-infiltrated, prior to incubation for 30 min in darkness at 28 °C with gentle agitation. Partially digested cells were collected on a 20 μm nylon mesh and washed extensively with digestion medium lacking the protoplasting enzymes. All subsequent manipulations were carried out at 0–4 °C, in solutions supplemented with the proteinase inhibitors leupeptin, pepstatin A, chymostatin and antipain (each at a concentration of 1 μg/ml), and PMSF (100 mM). Cells were washed in 200 ml of ice-cold homogenization buffer comprising 1.25 M sucrose, 50 mM NaCl, 25 mM EDTA, 0.75 mM spermine-HCl, 2.5 mM spermidine phosphate, 100 mM 2-mercaptoethanol, 0.04% (v/v) Triton X-100, 50 mM 4-morpholineethanesulfonic acid, pH 5.2. Washed cells were thoroughly homogenized in 1–1.5 L of homogenization buffer supplemented with 0.2% (v/v) Triton X-100 and 2% (w/v) Dextran T-40, using a Polytron on a low setting to avoid foaming. The slurry was passed through a series of nylon meshes (70, 41 and 20 μm) and centrifuged at 1500 × *g* for 5 min. The pellet was gently resuspended in homogenization buffer using a glass rod. 1.5 vol. of homogenization buffer, pH 5.8, containing 80% (w/w) Percoll, was added and the suspension centrifuged at 2700 × *g* for 15 min. The nuclear pellet was removed and washed twice in homogenization buffer.

Preparation of nuclear extracts

Soluble nuclear extracts were prepared according to the method of Dignam *et al.* [4]. Nuclei (packed volume ~0.5 ml) were resuspended in 1 ml lysis buffer (20 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid (Hepes), pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM dithiothreitol, 25% (v/v) glycerol) containing 0.1 M NaCl, and lysed by 10 strokes of a teflon/glass homogenizer. The slurry was centrifuged at 25000 × *g* for 20 min and the supernatant removed. The pellet was extracted either sequentially in lysis buffer containing 0.2 M, 0.3 M, 0.4 M and 1.0 M NaCl or in a single treatment with lysis buffer containing NaCl at a final

concentration of 0.42 M. Supernatants from the 25000 × *g* centrifugation were dialyzed for 5 h against two changes of 500 ml of dialysis buffer (20 mM Hepes pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM dithiothreitol, 20% (v/v) glycerol). The dialysate was clarified by centrifugation at 15000 × *g* for 5 min and the supernatant divided into aliquots of 50 μl, snap frozen in liquid N_2 and stored at − 80 °C. Protein determinations were performed by the method of Bradford [1].

Gel retardation

Assays were performed essentially as described by Singh *et al.* [25]. Fragments were 3′ end-labeled by filling-in overhanging ends with [α-^{32}P]dNTPs using the Klenow fragment of DNA polymerase, or by using [α-^{32}P]dideoxy ATP and terminal transferase [32]. Phosphatase-treated fragments were 5′ end-labeled using [γ-^{32}P]ATP and polynucleotide kinase. Binding reactions contained 0.1–1 ng end-labeled probe (300–1000 cpm), 10 mM Tris-HCl pH 7.9, 80 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 5% (v/v) glycerol, 5 μg poly(dI-dC)·poly(dI-dC) and 2 μl nuclear extract (equivalent to 10 μg of total protein for the 0.42 M crude extract or 1.5 μg total protein for the sequential 0.3 M fraction) in a final volume of 20 μl. For binding reactions with multimers of synthetic oligonucleotides, 0.25 pg (50–250 cpm) of labeled probe was used. Binding reactions were started by addition of nuclear extract and were incubated for 20 min on ice, before the addition of 2 μl loading dye and electrophoresis at 150 V through a 4% polyacrylamide gel in 1 × TBE (50 mM Tris-borate, pH 8.3, 1 mM EDTA) buffer at 4 °C. In some experiments the low ionic strength buffer system of Strauss and Varshavsky [28] was used for electrophoresis. Gels were dried under vacuum and exposed to X-Ray film.

DNase I footprinting

Incubation of nuclear extracts with DNA fragments was performed as in the gel retardation assays except that the reaction volume was increased to 50 μl and contained 2% polyvinyl alcohol. After incubation on ice for 20 min, samples were supplemented with 2.5 μl of 100 mM $MgCl_2$, 2.5 μl DNase I (0.5–1.0 mg/ml) and further incubated for 1 min at 4 °C. The digestion was stopped by the addition of 50 ml of stop buffer (20 mM EDTA, 200 mM NaCl, 1% SDS, 0.25 mg/ml tRNA). Samples were deproteinized by sequential extraction in phenol and chloroform, precipitated with ethanol and resuspended in 98% formamide, 0.02% bromophenol blue, 0.02% xylene cyanol. Samples were electrophoresed through 6% or 8% polyacrylamide gels containing 8 M urea and 2 × TBE. The gels were dried under vacuum and exposed to X-ray film.

Electroporation

Electroporation of plasmids into soybean protoplasts, treatment with glutathione and assay of CAT activity were as previously described [5]. Cells were electroporated in the presence of the indicated amounts of plasmid DNA, together with 50 μg of calf thymus DNA as a carrier.

Results

Trans-activation of the CHS15 promoter by Mae I-1

Functional analysis of CHS15 promoter deletions in electroporated soybean protoplasts defined the region extending from − 326 to − 173, relative to the site of transcription initiation, as a *cis*-acting transcriptional silencer [5]. We have tested whether the silencer can function *in trans* by co-electroporating plasmid pCHC1, which contains the chloramphenicol acetyltransferase (CAT) gene under the control of the full CHS15 promoter, and plasmid p*Mae* I-1, which contains only the silencer region (Fig. 1). The total amount

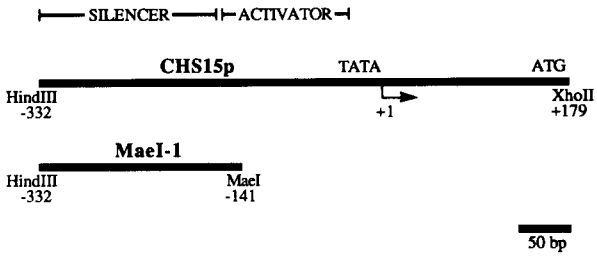

Fig. 1. Map of the promoter region of the CHS15 gene, indicating the fragments used in this study. The arrow labeled + 1 indicates the site of transcription initiation. The TATA box, the translation initiation codon, and the functionally defined silencer and activator regions are indicated.

of plasmid DNA was kept constant by the addition of compensatory amounts of a carrier plasmid, pCHS5-1, which contains CHS cDNA sequences. In the absence of co-electroporated p*Mae* I-1 plasmid, little expression of the CHS15 promoter-CAT gene fusion was observed in untreated protoplasts, while treatment of protoplasts with glutathione for 3 h markedly induced CAT activity (Fig. 2, lanes 1 and 3). Co-electroporation of pCHC1 with p*Mae* I-1 led to marked increases in the level of CAT activity in both control and elicitor-treated protoplasts (Fig. 2, lanes 2, 3 and 4). This response was enhanced as the ratio of p*Mae* I-1 to pCHC1 increased (Fig. 1, lane 5). In contrast, there was no effect of co-electroporated p*Mae* I-1 plasmid on the expression of a CAT gene under the control of the cauliflower mosaic virus 35S promoter (Fig. 2, lanes 6 and 7). Thus, the silencer region is able to act in *trans* to specifically stimulate CHS15 promoter activity.

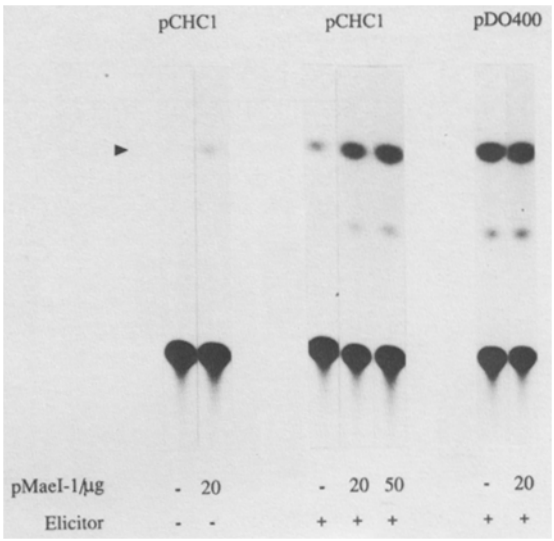

Fig. 2. Effect on CHS15 promoter activity of the *Mae* I-1 fragment *in trans*. 20 μg of the construct pCHC1, containing the CHS15 promoter-CAT gene fusion, was introduced into elicited or unelicited soybean protoplasts in the presence of the indicated amounts of p*Mae* I-1 competitor plasmid. Compensatory amounts of plasmid pCHS5-1 were added to give a total of 50 μg competitor plasmid in each reaction. Lanes 1 and 2: untreated protoplasts; lanes 3–7: protoplasts elicited with glutathione 24 h after electroporation. p*Mae* I-1 was also coelectroporated with the plasmid pDO400 [5], which contains the CAT gene under the control of the cauliflower mosaic virus 35S promoter and nopaline synthase terminator regions (lanes 6 and 7). CAT activity was determined 27 h after electroporation. Solid arrowhead denotes the major CAT product, 3-acetylchloramphenicol.

Sequence-specific binding of nuclear factors

Activation of the CHS15 promoter by increasing amounts of p*Mae* I-1 suggested that the two plasmids might compete *in vivo* for a soluble factor whose binding to the silencer element within the full promoter down-regulates transcription. Therefore, we tested whether CHS15 promoter fragments could specifically bind factors present in nuclear extracts prepared from suspension-cultured bean cells. Incubation of an end-labeled fragment containing the entire CHS15 promoter region (CHS15p, Fig. 1) with nuclear extracts from bean cell suspension cultures markedly reduced the electrophoretic mobility of the fragment in polyacrylamide gels (Fig. 3A, lanes 1 and 2). Formation of the binding complex between the CHS promoter and nuclear factors was not inhibited by a 5–10000-fold weight excess of the copolymer duplex poly(dI-dC)·poly(dI-dC) (Fig. 3A, lanes 2–6), or similar amounts of sheared salmon sperm DNA or *Hae* III-cut pUC19 plasmid (data not shown). All subsequent binding assays contained 5 μg poly(dI-dC)·poly(dI-dC) (average length 2400 bp) as non-specific competitor DNA.

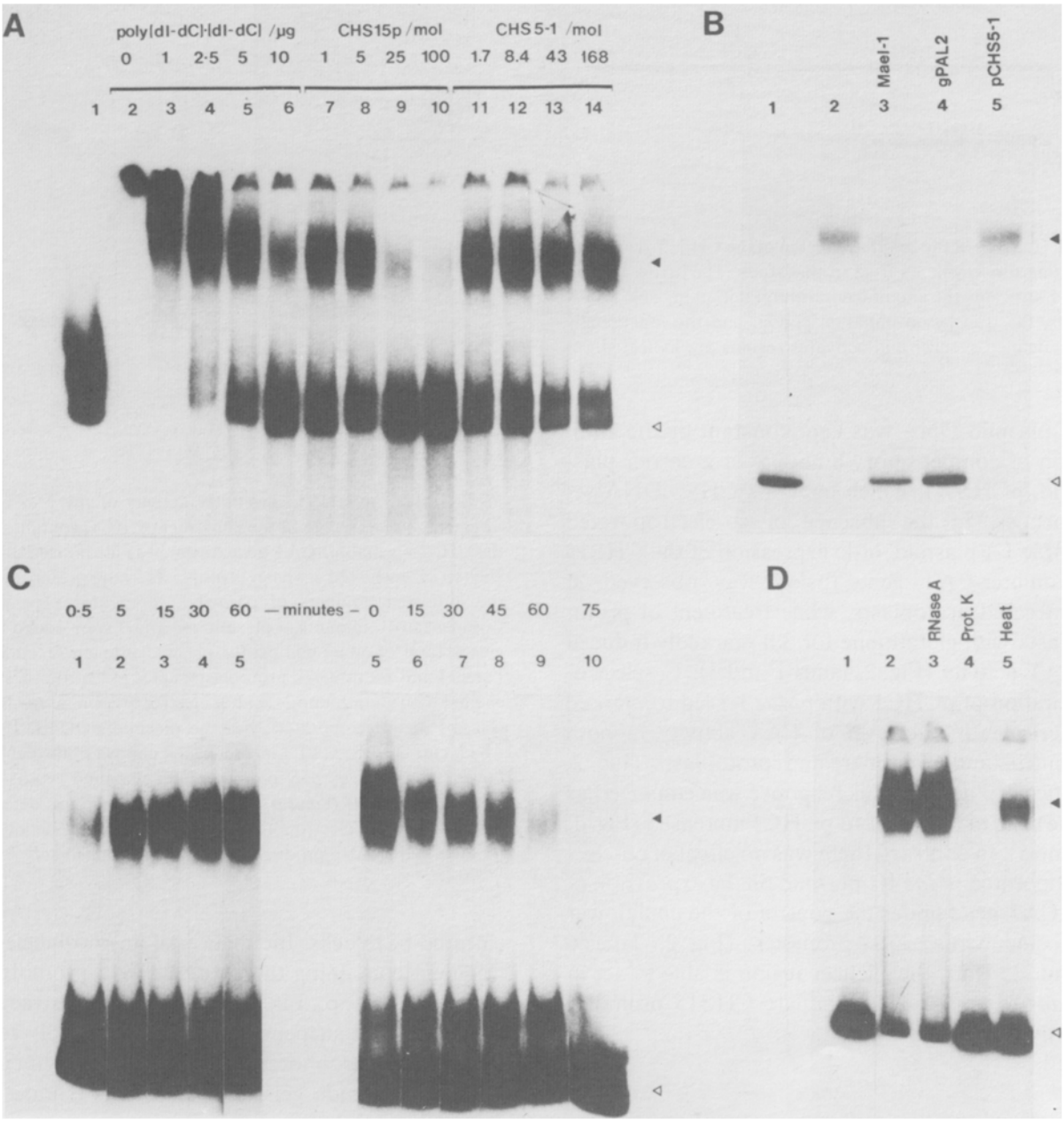

Fig. 3. Binding of a nuclear factor to the CHS15 promoter. The free and bound forms of the labeled fragments are indicated by the open and closed triangles, respectively. (A) Specificity of binding. End-labeled CHS15p fragment (1 ng) was incubated with 5 µg (total protein) of nuclear extract (lanes 2–14) in the presence of increasing amounts of poly(dI-dC)·poly(dI-dC) as non-specific competitor (lanes 2–6). Competition binding assays (lanes 7–14) were carried out in the presence of 5 µg poly(dI-dC)·poly(dI-dC) and the indicated amounts of unlabeled CHS15p fragment (lanes 7–10) or unlabeled CHS5-1 cDNA (lanes 11–14). Lane 1 shows the migration of the CHS15p fragment in the absence of nuclear extract. Samples were analysed by electrophoresis in a low ionic strength 4% polyacrylamide gel. (B) PAL2 promoter competition. Binding to the end-labeled 192 bp *Mae* I-1 fragment (0.5 ng) was performed in the presence of 5 µg poly(dI-dC)·poly(dI-dC). Unbound (lane 1) and bound (lane 2) forms of the fragment are indicated. Competition binding assays contained a 100-fold weight excess of unlabeled competitor: *Mae* I-1 (lane 3, 100-fold molar excess), a PAL2 promoter (lane 4, 12-fold molar excess); CHS5-1 cDNA (lane 5, 63-fold molar excess). (C) Kinetics of factor association and dissociation. Left panel: Association reaction. Labeled *Mae* I-1

Under these conditions, as little as a 25-fold molar excess of unlabeled CHS15p completely abolished binding to the labeled CHS15p fragment (Fig. 3A, lanes 7–10), whereas a 168-fold molar excess of the 303 bp CHS5-1 cDNA fragment had little effect (Fig. 3A, lanes 11–14). Thus, a factor present in nuclear extracts from bean cells binds in a sequence-specific manner to the CHS15 promoter region. Gel retardation assays using sub-fragments of CHS15p showed that binding was principally associated with a region encompassed by the 192 bp *Hin*d III-*Mae* I fragment, designated *Mae* I-1 (Fig. 1; Fig. 3B, lanes 1 and 2). This fragment extends from −332 to −141, encompassing the silencer region and an additional 32 nucleotides downstream. The mobility of the retarded *Mae* I-1 fragment was similar to that of the retarded CHS15p, suggesting that the same species bound to both *Mae* I-1 and CHS15p. In contrast only weak binding was observed using the 319 bp *Mae* I-*Xho* II fragment which spans the TATA-proximal activator region of the promoter, the transcription start site and untranslated leader sequence (data not shown).

Further studies were focused on factor binding to the silencer element contained in the *Mae* I-1 fragment. Some sequences homologous to the silencer are found in the promoter of the bean PAL2 gene encoding phenylalanine ammonia-lyase, which catalyzes the first step in phenylpropanoid biosynthesis [3, 5]. The PAL2 promoter could effectively compete with the *Mae* I-1 fragment for factor binding (Fig. 3B, lane 3). In this experiment, the specificity of binding was checked by competition with unlabeled *Mae* I-1 and pCHS5-1 as positive and negative controls respectively (Fig. 3B, lanes 3 and 5). Hence, the promoters of the co-ordinately regulated PAL2 and CHS15 genes contain binding sites for a common nuclear factor. The more complete abolition of binding by competition with PAL2 compared to *Mae* I-1 (cf. Fig. 3B, lanes 3 and 4) reflects the presence of several independent binding sites for the factor within the PAL2 fragment (S.M. Dean and D. Natt, unpublished results).

Binding kinetics

The kinetics of formation of the promoter DNA: *trans*-acting factor complex were followed by monitoring the appearance of the retarded *Mae* I-1 species at various times following the addition of nuclear extract to the labeled fragment (Fig. 3C, left panel). The binding complex could be detected within 0.5 min of addition of the extract and rapidly accumulated over the next 5 min. Thereafter, accumulation of the complex slowed, reaching a plateau after 30 min. To determine whether these saturation kinetics reflected the establishment of a reversible equilibrium between bound and unbound forms, labeled *Mae* I-1 was first allowed to form a complex and the ratio of bound to unbound labeled fragment then determined at various times after the addition of a 100-fold molar excess of unlabeled *Mae* I-1 (Fig. 3C). Following introduction of unlabeled *Mae* I-1, the labeled complex decayed with a half-life of about 15 min, whereas in the absence of unlabeled competitor fragment the proportion of *Mae* I-1 that was retarded remained constant with time (data not shown). Thus, the nuclear factor binds rapidly and reversibly to the silencer region.

Sensitivity of binding to proteinase K and heat

Heating of the nuclear extract to 65 °C greatly reduced binding (Fig. 3D, lane 5). Pre-treatment

fragment was incubated with nuclear extract for the indicated times prior to electrophoresis. Right panel: Dissociation reaction. Labeled *Mae* I-1 was incubated with nuclear extract for 15 min and then exposed to a 100-fold molar excess of unlabeled *Mae* I-1 for the indicated times, prior to electrophoresis. (D) Nature of binding factor. The effect of pre-treatment of nuclear extract with either 0.5 mg/ml RNase A (lane 3), 1 mg/ml proteinase K (lane 4) or by incubation at 65 °C for 5 min (lane 5) on the mobility of the labeled *Mae* I-1 fragment was compared with reference to the mobility of the fragment in the absence of nuclear extract (lane 1) or in the presence of untreated nuclear extract (lane 2).

of the extract with RNase A had no effect, whereas pre-treatment with proteinase K abolished binding (Fig. 3D, lanes 3 and 4), indicating that a nuclear protein is involved in the formation of the retarded complex.

Mapping of binding sites

The binding sites for the nuclear factor were delineated by gel retardation and DNase I-protection assays. Gel retardation assays using small fragments isolated from the *Mae* I-1 fragment indicated that only sequences extending from − 332 to − 177 contributed to the binding activity of the parent fragment (Fig. 4). This corresponds almost exactly to the region defined as a functional silencer *in vivo* (− 326 to − 173). Progressive removal of upstream sequences revealed that fragments 3 (− 326 to − 141) and 5 (− 273 to − 141) were retarded in the presence of nuclear extract, while fragments 7 (− 231 to − 141) and 9 (− 176 to − 141) were not retarded. Similarly, progressive removal of downstream sequences revealed that while fragments 8 (− 332 to − 177) and 6 (− 332 to − 232) were active in the gel retardation assay, fragments 4 (− 332 to − 274) and 2 (− 332 to − 327) were not. These data indicate that the region between − 273 and − 231 is critical for the efficient binding of either upstream or downstream fragments. However, this region alone showed no activity in a gel retardation assay (data not shown); rather, efficient binding occurred only when this region was adjoined to flanking upstream or downstream sequences. The two fragments generated from the 156 bp *Hin*d III-*Fok* I fragment by cleavage with *Hph* I (fragments 10 and 11), were each able to bind factor, albeit rather weakly. These results indicate that *Mae* I-1 contains at least two independent binding sites for nuclear factors extending either side of the *Hph* I site and that these sites are disrupted by cleavage with *Ssp* I or *Mae* III respectively.

The absolute amounts of bound and unbound forms of fragments 1 through 11 were determined by direct counting of dried gels on an AMBIS beta scanning system. This analysis revealed that

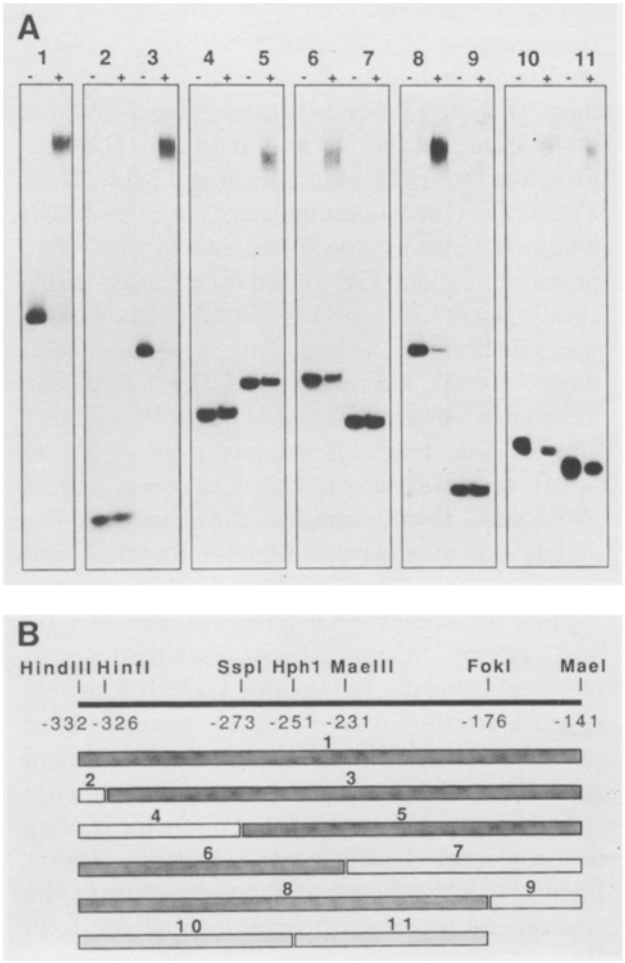

Fig. 4. Mapping of factor-binding sites by gel retardation assay. Fragments obtained by digestion of *Mae* I-1 with the indicated restriction enzymes were isolated on polyacrylamide gels and assayed for binding activity in gel retardation assays (A). The fragments tested are shown in B. The degree of shading indicates the relative binding of the radiolabeled fragments. Upstream and downstream fragments contain 55 and 14 nucleotides of polylinker sequence respectively which are not included in the figure.

while sequences extending from − 326 to − 273 and from − 231 to − 176 were not sufficient for factor binding (fragments 4 and 7, Fig. 4), they did contribute to efficient binding of the *Mae* I-1 fragment (binding to fragment 3 compared to 5, and binding to fragment 6 compared to 8, Fig. 4). In fact, extremely weak binding to fragment 4 could be detected upon considerable over-exposure of the gel to X-ray film (data not shown) or to the beta scanner, suggesting the presence of an

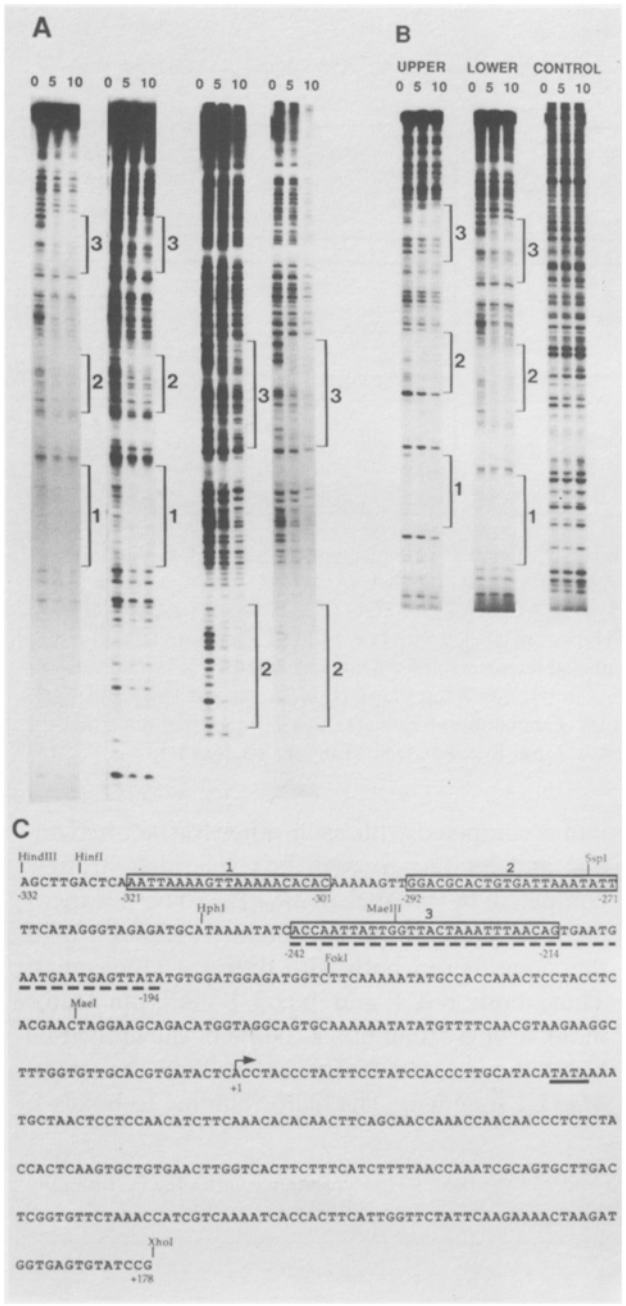

Fig. 5. DNase I protection assays. End-labeled *Mae* I-1 fragment was exposed to DNase I after incubation with 0, 5 and 10 μl of nuclear extract (1.8 mg/ml, initial concentration). The resulting DNA fragments were purified and electrophoresed on a 6% polyacrylamide sequencing gel. The solid bars indicate the DNase I-protected regions, defined as boxes 1, 2 and 3 respectively. (A) Mapping of binding sites. The *Mae* I-1 fragment was 3′ end-labeled at the *Hin*d III site using terminal transferase and [α-^{32}P]ddATP. Two electrophoretic separations and 2 exposures of the gel to X-ray film are shown to display footprints in both the proximal and distal regions of the fragment. (B) Strand comparison of DNase I-protected regions. The *Mae* I-1 fragment was labeled at the *Hin*d III site using either polynucleotide kinase and [α-^{32}P]ATP (5′-labeling; upper strand) or terminal transferase and [α-^{32}P]ddATP (3′-labeling; lower strand). An end-labeled pIBI24 fragment was included as a control for non-specific binding. (C) Location of DNase I-protected regions in the CHS15 promoter. DNase I-protected regions are denoted by the boxes labeled 1, 2 and 3. The arrow denotes the transcription start site (+1). Regions of homology with the co-induced PAL2 gene are underlined.

additional binding site in the upstream region of the silencer.

The existence of multiple binding sites in the *Mae* I-1 fragment was confirmed by DNase I footprinting. Nuclear extracts protected three distinct regions of *Mae* I-1, designated boxes 1, 2 and 3, from DNase I digestion (Fig. 5A). Identical protected regions were detected when either the upper or lower strand of the *Mae* I-1 fragment was examined (Fig. 5B). No difference was seen in the pattern of protection when nuclear extracts isolated from elicited or unelicited cells were used (data not shown). No specific DNase I-protected regions were observed in a control experiment with a fragment derived from the vector pIBI24.

The factor binding sites in *Mae* I-1 mapped by DNase I footprinting correlated with those defined by gel retardation assays (Fig. 5C). Thus, the *Ssp* I and *Mae* III sites, which defined separate binding sites by gel retardation assay (Fig. 4), lie within boxes 2 and 3, respectively. Furthermore, box 1 lies within fragment 4 and hence may account for the weak binding activity of this fragment in the gel retardation assay (Fig. 4). These data suggest that efficient binding of a factor (or factors) to the *Mae* I-1 fragment involves the recognition of several discrete sequences within the silencer.

Binding to oligonucleotides corresponding to boxes 1, 2 and 3

To determine if the protected regions represented binding sites for distinct or related nuclear proteins, oligonucleotides corresponding to boxes 1, 2 and 3 were synthesized and their binding properties studied in gel retardation assays. While

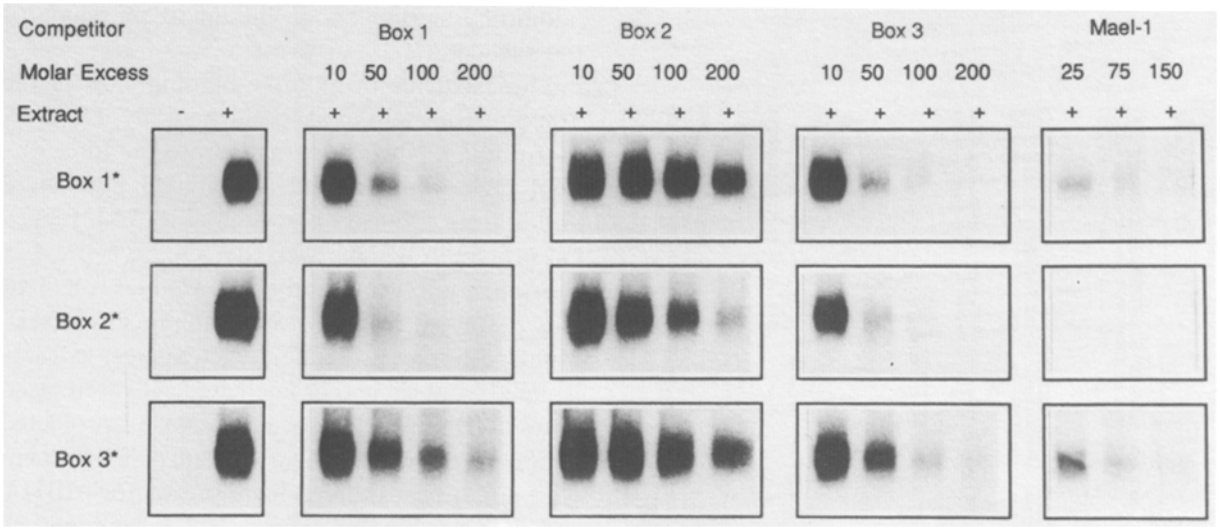

Fig. 6. Binding to synthetic oligonucleotides corresponding to boxes 1, 2 and 3. Complementary pairs of synthetic oligonucleotides corresponding to the nucleotide sequences 5′-CTCAAATTAAAAGTTAAAAACACACAAAA-3′ (Box 1), 5′-AGTTGGACGCACTGTGATTAAATATTTTC-3′ (Box 2), and 5′-ATCACCAATTATTGGTTACTAAATTTAACAGTGA-3′ (Box 3) were annealed and ligated. Gel-purified tetramers of each box were labeled with [α-^{32}P]ddATP, prior to factor binding in gel retardation assays. Only the retarded (bound) forms of the labeled tetramers are shown. The first two lanes indicate the formation of complexes with labeled box 1 tetramers (Box 1*, first row), box 2 tetramers (Box 2*, second row) and box 3 tetramers (Box 3*, third row) in the absence of unlabeled competitor. Competition binding assays were carried out using the indicated amounts of unlabeled tetramers of Box 1, Box 2 and Box 3 as well as unlabeled *Mae* I-1.

monomers of the binding sites were retarded in the presence of nuclear extract, factor binding to multimeric forms was much stronger (data not shown), and therefore for each box randomly ligated tetramers were used in these studies. Boxes 1, 2 and 3 were independently active in gel retardation assays, each giving rise to a retarded species with a mobility similar to that observed with the *Mae* I-1 fragment (Fig. 6). Boxes 1, 2, and 3 competed with each other for factor binding, and binding to each box was also strongly competed by unlabeled *Mae* I-1. These experiments demonstrated that boxes 1, 2 and 3 bind the same factor, buth with different efficiencies. Thus, both box 1 and box 3 bound the factor more strongly than box 2. None of the individual boxes bound the factor as well as the entire *Mae* I-1 fragment. The factor binding to boxes 1,

Table 1. Sequence comparison of SBF-1 binding sites in boxes 1, 2 and 3 of the bean CHS15 promoter with the GT-1 binding site of the pea *rbcS-3A* gene.

GT-1 Core consensus		GGTTAA	
rbcS-3A Box II (wild type)		GTGTGGTTAATATG	
rbcS-3A Box II (mutant)		GTGTCCTTAATATG	
CHS15 Box 1	−318	TAAAAGTTAAAAAC	−305
CHS15 Box 2	−285	CTGTGATTAAATAT	−272
CHS15 Box 3 (upper)	−236	TATTGGTTACTAAA	−223
CHS15 Box 3 (lower)	−212	TCACTGTTAAATTT	−225

Sequences in common with the GT-1 core consensus are underlined. The sequence of the inactive *rbcS-3A* box II mutant is also indicated.

2 and 3 of the silencer element was designated silencer binding factor-1 (SBF-1).

Competition by GT-1 binding sites

The ability of boxes 1, 2 and 3 to compete with each other for SBF-1 binding suggested the presence of a common binding site. Alignment of the sequences revealed no extensive homology apart from a conserved 9 bp element with the consensus sequence GGTTAA(A/T)(A/T)(A/T) present in the same orientation once in each box (Table 1). In addition, box 3 contained a second version of this element in the opposite orientation. The sequence common to all the boxes closely resembles the consensus sequence for the binding site of the transcription factor GT-1 (Table 1) implicated in the photoregulation of the pea *rbcS-3A* gene encoding the small subunit of ribulose bisphosphate carboxylase [7, 8, 13].

In order to test whether boxes 1, 2, and 3 of the CHS15 silencer region represent potential GT-1 binding sites, we performed competition binding assays with authentic GT-1 binding sites corresponding to box II of the pea *rbcS-3A* promoter [7]. A mutant version of the GT-1 binding site known to be functionally inactive *in vivo* and inactive in binding GT-1 in nuclear extracts from pea leaves [8, 12] was used as a control for binding (Table 1). In this mutant GT-1 binding site the two adjacent G residues, which contact the factor, are replaced by C residues. Tetramers of authentic GT-1 binding sites were able to compete with labeled *Mae* I-1 fragment for binding of SBF-1 from bean cell suspension cultures (Fig. 7A). In contrast, tetramers of the mutant GT-1 binding site did not compete with the *Mae* I-1 fragment. Since GT-1 is involved in the light-dependent expression of *rbcS-3A* in green tissues, we examined nuclear extracts prepared from tobacco leaves. Gel retardation assays showed that tobacco leaf nuclear extracts prepared according to Green *et al.* [7] contained a factor that bound to *Mae* I-1 and formed a complex with the same mobility as that seen using nuclear extracts from the suspension-cultured bean cells (data not shown). Competition experiments showed that, as for the bean cell culture SBF-1, wild-type but not mutant GT-1 binding sites could compete with the *Mae* I-1 fragment for binding the leaf nuclear factor (Fig. 7A).

Two further experiments extended the relationship between boxes 1, 2 and 3 of he CHS15 promoter and GT-1 binding sites in the *rbcS-3A* promoter. First, binding of the bean nuclear factor to labeled tetramers of the *rbcS-3A* box II GT-1 binding site was competed by unlabeled *Mae* I-1, or tetramers of CHS15 boxes 1, 2 and 3 (Fig. 7B). The effectiveness of individual CHS boxes as competitors of factor binding to the *rbcS-3A* box II was box 3 > box 1 ⩾ box 2, which was the same order as that observed in competitions between pairs of CHS boxes (Fig. 6). Second, substitution of C residues for the G residues at positions −271 and −231 within the two GT-1 core sequences of the CHS15 box 3 abolished binding of the bean SBF-1 (Fig. 7C). Only single C substitutions were made in order to avoid creating potential GT-1 core sequences on the opposite strand. The GT-1 core sequence in the inverse orientation in the 3′ region of the box appeared to be the major determinant of binding. Overall, these results indicate that the CHS silencer binding factor SBF-1, defined in the present study, is highly related to GT-1 and displays a similar specificity for sequences within the binding site as authentic GT-1.

Discussion

We have identified a factor SBF-1 present in nuclear extracts from bean cell suspension cultures that binds to four sites in three discrete boxes of the −326 to −173 *cis*-element of the promoter of the CHS15 gene, which encodes a key enzyme of flavonoid natural product biosynthesis. SBF-1 is proteinaceous and binds rapidly and reversibly to the CHS promoter *in vitro*. Competition experiments indicate that the three boxes bind either the same factor or highly related factors with overlapping specificities. Several lines of evidence suggest that SBF-1 is

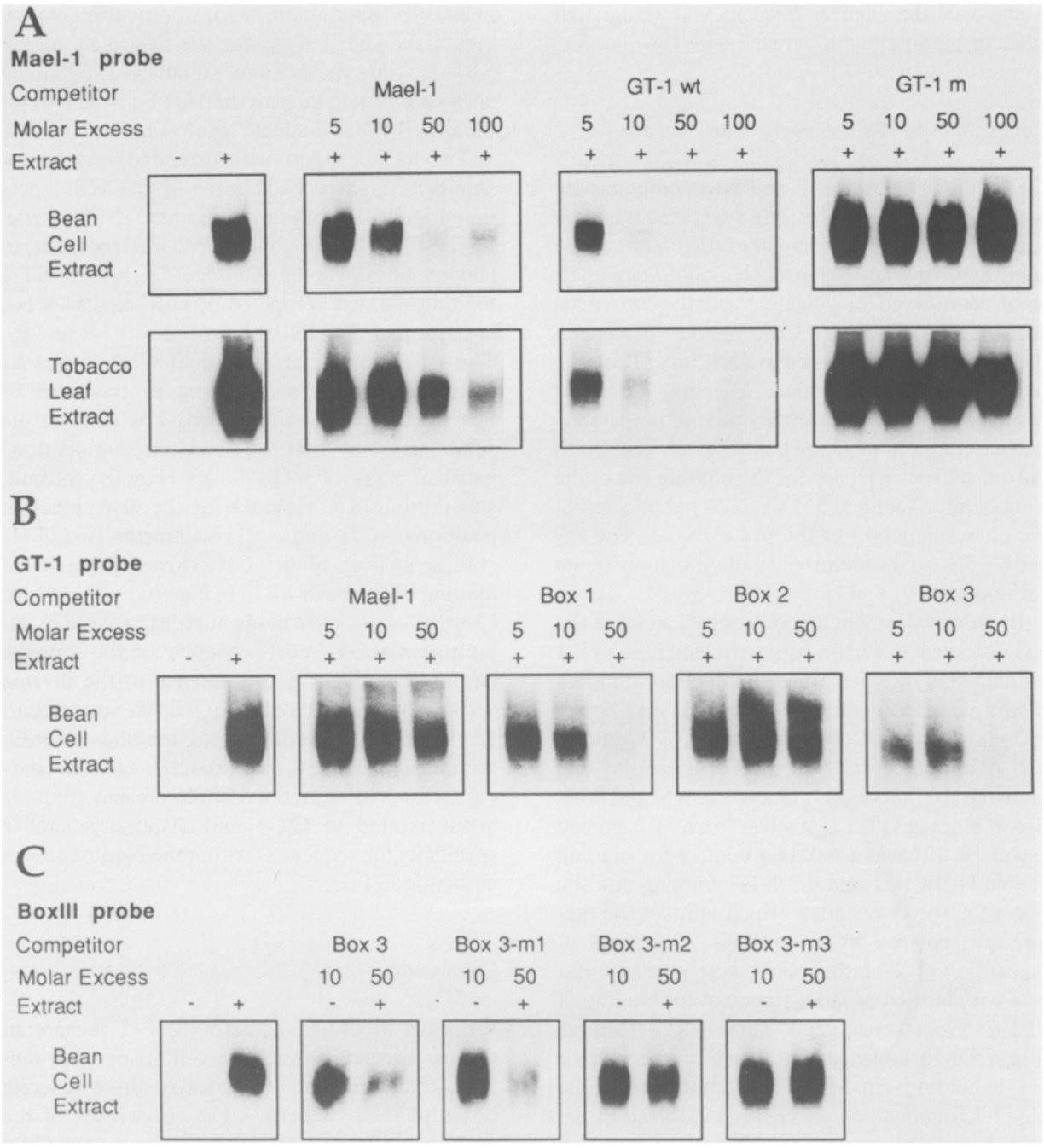

Fig. 7. Relationship between binding sites in the CHS15 silencer and GT-1 binding sites. In all cases only the bound form in a gel retardation assay is shown. (A) Labeled *Mae* I-1 was incubated with nuclear extract from bean cell nuclei (top row) or tobacco leaf nuclei (bottom row) in the absence of competitor or in the presence of the indicated molar excesses of unlabeled *Mae* I-1, unlabeled tetramers of wild-type GT-1 binding sites (GT-1 wt) or unlabeled tetramers of mutant GT-1 binding sites (GT-1 m). (B) Labeled tetramers of wild-type GT-1 binding sites were incubated with bean cell nuclear extract in the absence of competitor or in the presence of the indicated molar excesses of unlabeled *Mae* I-1, unlabeled tetramers of box 1, unlabeled tetramers of box 2, or unlabeled tetramers of box 3. (C) Mutational analysis of CHS15 box 3 binding site. Labeled tetramers of wild-type box 3 were incubated with bean cell nuclear extract in the absence of competitor or in the presence of unlabeled

closely related to, or identical to, GT-1 implicated in the regulation of the ribulose bisphosphate carboxylase small subunit gene [8, 13]. First, cross competition experiments have established that the CHS15 boxes and authentic GT-1 binding sites can bind the same factor. Second, both CHS15 boxes and authentic GT-1 binding sites form complexes with similar mobilities in gel retardation assays (data not shown). Third, mutations in GT-1 core sequences abolish binding to the CHS15 boxes as well as to authentic GT-1 sites. Fourth, an equivalent binding activity is present in both tobacco leaf nuclei and bean cell nuclei, consistent with the regulation of photosynthetic genes and natural product biosynthesis genes by a common factor. Thus, by these criteria SBF-1, defined by binding to the silencer region of the CHS15 promoter, is indistinguishable from GT-1. Although GT-1 and SBF-1 cannot be operationally distinguished at present, we retain the designation SBF-1 for the bean cell factor until purification, molecular cloning and manipulation by gene transfer reveal the molecular and genetic relationships between the factor(s) defined by binding to the *rbcS-3A* and CHS15 promoters.

The consensus sequence of GGTTAA-(A/T)(A/T)(A/T) most likely represents a core sequence for the binding of SBF-1 to the CHS15 promoter. G to C transitions inhibit binding indicating that these residues are essential, as previously observed for GT-1 binding to *cis*-elements in the *rbcS-3A* promoter [8]. However, the sequences flanking the core binding site appear to influence the affinity and possibly the specificity of binding. Thus, a fragment containing a CHS15 box 3 half-site includes a core binding sequence but is unable to bind SBF-1, while the length of the DNase I-protected regions suggests that sequences flanking this motif may also make contact with the factor and thus contribute to binding. Hence differences in flanking sequences may be responsible for the differences in binding affinities of the three boxes, as is the case with the ubiquitous Oct-2 factor, where the affinity of binding to a range of octamers containing an AT-rich box in the 3′ half of the recognition site is dependent upon the sequences flanking the octamer motif [6].

Successive deletion of individual boxes within the *Mae* I-1 fragment progressively reduced the amount of binding more than expected on a stoichiometric basis for independent, non-interacting sites. Moreover, individual sites only bound SBF-1 efficiently when ligated into multimers, suggesting that binding of the factor is cooperative. Similar effects have been noted in the binding of factors to oligonucleotide sequences corresponding to DNase I-protected regions of the promoter of the pea *rbcS-3A* gene [8] and a number of other eukaryotic promoters [26, 29]. All species of randomly ligated multimers of individual CHS15 boxes were effectively bound in gel retardation assays, suggesting that this effect is not dependent on the relative orientations of individual sites. These data, coupled with the observed rates of complex association and dissociation, indicate that enhanced binding to multimers or to the natural silencer element may result from the increased local concentration of binding sites, which promotes the reassociation of dissociated factors and hence lowers the effective rate of dissociation, rather than from direct cooperative interactions between factors.

SBF-1 and the corresponding *cis*-elements are very likely to play a major role in the functional properties established for the -326 to -173 regulatory region of the CHS15 promoter. Thus, SBF-1 represents the major binding activity of the CHS15 promoter *in vitro*, and there are four sites for binding of the factor which span 69 bp of the 153 bp region. Moreover, the rapid and reversible binding of the factor *in vitro* is consistent with the

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tetramers of wild-type box 3 (Box 3), unlabeled tetramers of box 3 mutated in the upper GT-1 binding site by replacing G_{-231} with a C residue (Box 3-m1); unlabeled tetramers of box 3 mutated in the lower GT-1 binding site by substituting G_{-217} with a C residue (Box 3-m2); unlabeled tetramers of box 3 mutated in both upper (5′) and lower (3′) GT-1 binding sites (Box 3-m3).

ability of the − 326 to − 173 element to activate the CHS15 promoter in *trans* when co-electroporated into protoplasts. Recent *trans* competition experiments with a tetrameric version of the box 3 sequence have directly demonstrated that inhibition of factor binding to this specific *cis*-element modulates promoter activity (M.J. Harrison and A. Choudhary, unpublished results). Thus, SBF-1 is likely to function as a repressor of CHS15 expression in cell culture protoplasts and hypocotyls, corresponding to the silencer activity of the − 326 to − 173 region. In contrast, this region appears to enhance the activity of the CHS15 promoter in cotyledons, and hence SBF-1 may play a critical role in determining the organ specificity of CHS15 expression in young seedlings.

No differences were observed in SBF-1 activity in nuclear extracts isolated from elicited or control cells, consistent with the functional analysis of the − 326 to − 173 region, which is effective in both unelicited and elicited cells. Although the − 326 to − 173 region is not essential for induction by elicitor in bean protoplasts or transgenic tobacco leaves, we have observed that deletion of the silencer region leads to a more prolonged induction in transient assays and no subsequent down turn in expression (M. Dron, unpublished observations). Hence SBF-1 may play a role in down regulation during a late phase of the characteristically transient induction of the CHS15 gene and possibly other phenylpropanoid biosynthetic genes following stimulation by developmental or environmental cues [14]. Competition assays have shown that the coordinately regulated PAL2 promoter also binds SBF-1 (S.M. Dean and E. Natt, unpublished). The CHS15 box 3 sequence, which displays the highest affinity for the factor, lies within a region of the CHS15 promoter (− 242 to − 194) that is homologous to a 48 bp region of the PAL2 promoter [3]. Thus SBF-1 and the corresponding *cis*-elements may be involved in the coordinate regulation of the CHS15 and PAL2 genes.

GT-1 and the corresponding *cis*-element have been implicated in the light-dependent expression of *rbcS-3A* in green tissues [13], and GT-1 binding sites have also been found in the promoter of a gene encoding the photoreceptor phytochrome, which is down-regulated by light [11]. While CHS and PAL genes are also light-regulated, they display quite different patterns of organ and tissue specificity, e.g. PAL2 and CHS15 are only very weakly expressed in maturing leaves [18, 19, 23], whereas *rbcS-3A* is strongly expressed in this organ [8]. This would imply that the functional properties of the GT-1/SBF-1 *cis*-element and the corresponding factor(s) are highly dependent on the context of the promoter in which the *cis*-element resides, and the particular combination of factors present in the nuclei of different cell types. Contextual modification of the functions of GT-1/SBF-1 and combinatorial interactions with other transcription factors would allow a relatively small number of *cis*-elements and cognate *trans*-factors to specify an array of different gene expression patterns and at the same time provide a mechanism for flexible integration of diverse environmental responses within a coherent developmental program.

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