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Rapid stimulation of a soybean protein-serine kinase that phosphorylates a novel bZIP DNA-binding protein, G/HBF-1, during the induction of early transcription-dependent defenses

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The G-box (CACGTG) and H-box (CCTACC) *cis* elements function in the activation of phenylpropanoid biosynthetic genes involved in the elaboration of lignin precursors, phytoalexins and the secondary signal salicylic acid as early responses to pathogen attack. We have isolated a soybean cDNA encoding a novel bZIP protein, G/HBF-1, which binds to both the G-box and adjacent H-box in the proximal region of the chalcone synthase *chs15* promoter. While G/HBF-1 transcript and protein levels do not increase during the induction of phenylpropanoid biosynthetic genes, G/HBF-1 is phosphorylated rapidly in elicited soybean cells, almost exclusively on serine residues. Using recombinant G/HBF-1 as a substrate, we identified a cytosolic protein-serine kinase that is rapidly and transiently stimulated in cells elicited with either glutathione or an avirulent strain of the soybean pathogen *Pseudomonas syringae* pv. *glycinea*. Phosphorylation of G/HBF-1 *in vitro* enhances binding to the *chs15* promoter and we conclude that stimulation of G/HBF-1 kinase activity and G/HBF-1 phosphorylation are terminal events in a signal pathway for activation of early transcription-dependent plant defense responses.

Keywords: bZIP transcription factors/plant disease resistance/plant gene expression/protein phosphorylation/protein-serine kinase

Introduction

Plants respond to pathogen avirulence signals by the elaboration of inducible defenses including oxidants, phytoalexins, cell wall modifications and deployment of pathogenesis-related (PR) proteins, such as chitinase, with antimicrobial activities (Briggs, 1995). Defense induction associated with the expression of localized hypersensitive resistance is observed in the early stages of incompatible interactions following attempted infection by a non-pathogen or avirulent strain of a pathogen (Lamb *et al.*, 1989). In some cases, these responses are also observed in distant tissue, associated with the expression of systemic acquired resistance in which immunity to a broad range of normally virulent pathogens gradually develops throughout the plant (Ryals *et al.*, 1994). Defense responses can also be induced by defined molecules including microbial glycan and peptide elicitors, or metabolites such as arachidonic acid, glutathione and salicylic acid (Ebel and Cosio, 1994).

With the exception of callose production and oxidative responses including cross-linking of cell wall structural proteins, induction of this battery of defenses involves a massive switch in host gene expression (Dixon *et al.*, 1994), and there is a temporal and spatial hierarchy of defense gene activation with some genes exhibiting rapid, localized activation, whereas many, but not all, PR protein genes undergo slower activation, both locally and then systemically throughout the plant (Ryals *et al.*, 1994; Hahlbrock *et al.*, 1995). Prominent among the class of rapidly induced defense genes are those encoding enzymes of phenylpropanoid biosynthesis involved in the synthesis of lignin precursors and a number of phytoalexins (Dixon and Paiva, 1995). In addition, the phenylpropanoid-derived metabolite salicylic acid is a signal potentiating the expression of both localized hypersensitive resistance and systemic acquired resistance (Gaffney *et al.*, 1993; Delaney *et al.*, 1994; Mauch-Mani and Slusarenko, 1996). The early activation of phenylpropanoid biosynthetic genes is correlated with the expression of hypersensitive resistance in tissues challenged with an avirulent pathogen or non-pathogen (Dixon and Paiva, 1995; Hahlbrock *et al.*, 1995), and in parsley and bean cell suspension cultures transcription of these genes is transiently activated within 10–20 min of elicitor treatment, with maximum rates of transcription after ~1 h and maximum accumulation of transcripts after 3–4 h (Chappell *et al.*, 1984; Cramer *et al.*, 1985; Lawton and Lamb, 1987).

Delineation of the *cis* elements and cognate *trans* factors underlying the rapid activation of phenylpropanoid biosynthetic genes provides the basis for characterizing the terminal stages of a signal transduction pathway involved in the deployment of early transcription-dependent defenses and the generation of salicylic acid as a secondary signal. Two *cis* elements, the G-box (CACGTG) and H-box (CCTACC), are found in the proximal region of the promoters of a number of genes encoding phenylpropanoid biosynthetic enzymes including phenylalanine ammonia-lyase (*pal*) and 4-coumarate:CoA ligase, which catalyze the first and third steps respectively in the central, common pathway of phenylpropanoid biosynthesis, and chalcone synthase (*chs*), which catalyzes the first step in a branch pathway specific for the synthesis of flavonoids

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and isoflavonoid-derived pterocarpan phytoalexins (Lois *et al.*, 1989; Ohl *et al.*, 1990). These *cis* elements are the sites of elicitor-inducible *in vivo* footprints in the parsley *pal1* promoter (Lois *et al.*, 1989) and likewise an H-box in the *chs15* promoter is the location of an elicitor-inducible DNase I-hypersensitive site in chromatin from bean cells (Lawton *et al.*, 1990). G- and H-box functions in *chs15* expression have been confirmed by functional analysis of the effects of promoter mutations on reporter gene expression in electroporated protoplasts (Dron *et al.*, 1988; Loake *et al.*, 1992), stably transformed cells and transgenic plants (O.Faktor, R.A.Dixon and C.Lamb, unpublished data), as well as by *in vitro* transcription assays (Arias *et al.*, 1992; W.P.Lindsay, R.A.Dixon and C.Lamb, unpublished data).

The G-box functions in the regulation of diverse genes by developmental cues, abscisic acid, light, UV irradiation and wounding as well as pathogen signals, and a family of bZIP proteins that bind G-box sequences has been described (Foster *et al.*, 1994; Menkens *et al.*, 1995). Functional specificity appears to be determined by DNA-binding affinities governed by nucleotides immediately flanking the G-box ACGT core and by combinatorial interactions with other *cis* element–*trans* factor systems (Williams *et al.*, 1992; Izawa *et al.*, 1993; Menkens *et al.*, 1995). However, only the *in vivo* function of *opaque2*, as a regulator of zein expression, has been fully established (Schmidt *et al.*, 1992), and while transgenic manipulation of CPRF-1 disrupts light induction of parsley *chs* (Feldbrügge *et al.*, 1994), there is no information on the function of G-box factors in elicitor or pathogen activation of phenylpropanoid biosynthetic genes. Likewise, a flower-specific Myb transcription factor, which stimulates *pal2* transcription through binding to the H-box, appears to function in developmental regulation and there is no report of its involvement in elicitor or pathogen induction (Sablowski *et al.*, 1994, 1995). A novel factor from parsley, BFP-1, binds to an AC-rich element related to the H-box that is also *in vivo* footprinted in elicited parsley cells (da Costa e Silva *et al.*, 1993). BFP-1 transcripts are induced in elicited cells and infected tissues, consistent with a function in defense gene regulation, although it is not clear whether the activity of this factor is directly modulated by elicitor or pathogen signals for the rapid initial stimulation of early defense genes. Polypeptides of 97 and 56 kDa, designated KAP-1 and KAP-2 respectively, which bind to H-boxes in the bean *chs15* promoter, have been purified by DNA affinity chromatography (Yu *et al.*, 1992). Elicitation with glutathione does not affect the total cellular activities of KAP-1 or KAP-2, but causes a rapid increase in the specific activities of both factors in the nuclear fraction, consistent with a role in the induction of *chs15* and related defense genes.

While these studies have identified a number of *trans* factors which might function in the elicitor or pathogen induction of phenylpropanoid biosynthetic genes, no picture has yet emerged of how the functional activities of factors interacting with the G-box and H-box *cis* elements are regulated in the initial stimulation of early defense gene transcription. In the present study, we describe a novel bZIP protein, G/HBF-1, which binds to the G-box and the adjacent H-box in the proximal region of the *chs15* promoter. While G/HBF-1 transcript and protein levels do not increase during the induction of *pal* and *chs* transcription, G/HBF-1 is phosphorylated rapidly in elicited cells. Using recombinant G/HBF-1 as a substrate, we identify a cytosolic protein-serine kinase activity that is rapidly and transiently stimulated in elicited cells and demonstrate that G/HBF-1 phosphorylation enhances binding to the *chs15* promoter. These observations delineate a terminal event in a signal pathway for activation of early transcription-dependent defense responses and indicate that in plants, as in animals, stimulus-dependent transcription factor phosphorylation contributes to the selective regulation of gene expression.

Results

Isolation of a soybean cDNA encoding G/HBF-1, a novel bZIP DNA-binding protein

Two soybean (*Glycine max*) cDNA libraries were constructed in λgt11 and λZAP-II vectors using respectively poly(A)$^+$ RNA from control soybean cell suspension cultures and a mixture of poly(A)$^+$ RNA from control cells and cells 4 h after elicitor treatment. A total of 3×10^5 plaques from each library were probed with the –80 to –42 fragment of the bean *chs15* elicitor-inducible promoter. This fragment contains both the G-box and adjacent, TATA-proximal H-box (H-box III). A clone, designated λG/HBF-1, expressing a protein that strongly bound this promoter fragment, was isolated from the λgt11 library. λG/HBF-1 contained a 1.4 kb insert, and repeated screening of the λgt11 and λZAP-II cDNA libraries with this insert as a probe failed to identify hybridizing clones with larger inserts.

The λG/HBF-1 cDNA contains a single long open reading frame of 1134 bp encoding a protein of 41 kDa (Figure 1). The putative ATG start codon is flanked by a nucleotide sequence optimal for translation initiation in plants (Lütke *et al.*, 1987). The deduced protein product shows characteristic features of a bZIP transcription factor including a highly basic, putative DNA-binding domain and a leucine zipper domain in which every seventh amino acid residue is leucine or another small hydrophobic residue (Figure 1). G/HBF-1 also contains two domains rich in proline and acidic amino acids respectively.

Protein sequence alignments reveal substantial similarities in the bZIP region to the equivalent regions of other plant bZIP proteins such as RITA-1 (Izawa *et al.*, 1994) or *opaque2* (Schmidt *et al.*, 1992). However, while the C-terminal half of the basic region is highly conserved among G/HBF-1 and many other plant bZIP factors binding G-box or related motifs, the N-terminal part of the basic region of G/HBF-1 only exhibits a high degree of similarity to the common plant regulatory factor 2 (CPRF-2) from parsley (Weisshaar *et al.*, 1991). Four other domains, designated D1–D4, are highly conserved among G/HBF-1, CPRF-2 (Weisshaar *et al.*, 1991) and the maize *opaque2* heterodimerizing proteins OHP1 and OHP2 (Pysh *et al.*, 1993). D1, which is located half way between the N-terminus and the basic domain, comprises a peptide predicted to form a helix, and D2, which is located just N-terminal of the basic domain, is relatively rich in acidic residues. D3, which is adjacent to the bZIP region, is similar to a domain also present in some animal transcription factors including the helix–loop–helix protein

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CCTCTTCCGAATGGGCTTTCCAGCGCTTCCTCCAAGAAGCCACCGCCGCCGGTACCTCCACTTCATCTCCCCCTCAGCCACCTACAATGA    90
                                                                                        M     1

CGGCGTCTTCCTCGTCATCTTCGCACCAAAACGACGTCGTGGAGATCAAGGATGAGAATCTTTCTATTCCTAATTTAAATCCCAGTACGG   180
T  A  S  S  S  S  S  S  H  Q  N  D  V  V  E  I  K  D  E  N  L  S  I  P  N  L  N  P  S  T      31

CGTTGAATTCGAAGCCGGCGTCGTCGTTTGGCCTCGCACCTCCGCCGAATATTGCCGTTGATTCCGAAGAGTATCAAGCATTCCTCAAAA   270
A  L  N  S  K  P  A  S  S  F  G  L  A  P  P  P  N  I  A  V  D  S  E  E  Y  Q  A  F  L  K      61

GCCAACTCCATTTGGCTTGCGCCGCCGTTGCTTTGACTCGCGGCAAGAGTTTAAACCCTCAGGATTCAGGCTCTACAGCTCACGACAAAG   360
S  Q  L  H  L  A  C  A  A  V  A  L  T  R  G  K  S  L  N  P  Q  D  S  G  S  T  A  H  D  K      91

GATCAGAGACTGCTAGTGCAGCTCAATCAGGATCTCACGTCTCCACTTTAGGATCTGGTCAGGAAGTGGCAAAAATTCAAGATAAGGATG   450
G  S  E  T  A  S  A  A  Q  S  G  S  H  V  S  T  L  G  S  G  Q  E  V  A  K  I  Q  D  K  D     121

CTGGTGGACCAGTTGGAATTCCCTCCTTGCCCCCGGTGCAAAAGAAACCTGTGGTGCAGGTGAGGTCAACAACCAGTGGTTCATCTAGAG   540
A  G  G  P  V  G  I  P  S  L  P  P  V  Q  K  K  P  V  V  Q  V  R  S  T  T  S  G  S  S  R     151

AGCAATCTGATGATGATGAAGCTGAAGGAGAAGCAGAAACAACTCAAGGCATGGATCCAGCTGATGCAAAACGTGTAAGGAGAATGCTTT   630
E  Q  S  D  D  D  E  A  E  G  E  A  E  T  T  Q  G  M  D  P  A  D  A  K  R  V  R  R  M  L     181

CAAATAGAGAATCAGCCAGACGTTCAAGGAGAAGAAAGCAGGCCCATCTGACAGAACTCGAGACACAGGTTTCTCAACTCAGAGTAGAAA   720
S  N  R  E  S  A  R  R  S  R  R  R  K  Q  A  H  L  T  E  L  E  T  Q  V  S  Q  L  R  V  E     211

ACTCCTCTCTACTGAAACGTCTGACTGACATAAGCCAGAAATACAATGAAGCAGCGGTTGATAATCGAGTCTTGAAAGCAGATGTTGAGA   810
N  S  S  L  L  K  R  L  T  D  I  S  Q  K  Y  N  E  A  A  V  D  N  R  V  L  K  A  D  V  E     241

CATTAAGAAGGAAGGTAAAGATGGCTGAAGAAACTGTTAAAAGAGTTACTGGGTTAAATCCGTTATTCCAAGCTATGTCAGAGATTTCCT   900
T  L  R  T  K  V  K  M  A  E  E  T  V  K  R  V  T  G  L  N  P  L  F  Q  A  M  S  E  I  S     271

CAATGGTAATGCCATCCTACTCTGGTAGTCCTTCAGACACATCGGCAGACGCTGCTGTACCTGTGCAAGATGATCCAAAACATCACTACT   990
S  M  V  M  P  S  Y  S  G  S  P  S  D  T  S  A  D  A  A  V  P  V  Q  D  D  P  K  H  H  Y     301

ACCAACAGCCGCCAAATAATCTTATGCCAACCCATGATCCTAGAATCCAAAATGGTATGGTAGATGTTCCTCCAATAGAAAATGTAGAGC  1080
Y  Q  Q  P  P  N  N  L  M  P  T  H  D  P  R  I  Q  N  G  M  V  D  V  P  P  I  E  N  V  E     331

AGAATCCTGCAACGGCAGCAGTTGGGGGGAACAAGATGGGTAGAACAACTTCAATGCAGCGGGTTGCTAGCTTGGAGCATCTGCAGAAGC  1170
Q  N  P  A  T  A  A  V  G  G  N  K  M  G  R  T  T  S  M  Q  R  V  A  S  L  E  H  L  Q  K     361

GCATCCGTGGTGAAGTGAGTTCCTGTGGAACTCAAGGCAGGGGAGAGCAATAAACACGGTTAAAGCCACCAGCTGCACCGAAGATTTTGC  1260
R  I  R  G  E  V  S  S  C  G  T  Q  G  R  G  E  Q  *                                         378

ACAGCATGTTGATAAAATCATACTGTTCCAATTCCAACCGTGGCAGGAATTGCCCATTCTTTGTATGACAATGCCTTCTTGGATGAGACT  1350

AATGCATTGACATCTCTTTTCT                                                                      1372
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Fig. 1. Nucleotide sequence of the λG/HBF-1 cDNA. The deduced amino acid sequence of the longest open reading frame is given below. The basic domain is shown in bold and the heptameric repeats of the leucine zipper are underlined.

MyoD (Scales *et al.*, 1990). D4, located near the C-terminus, shows no distinctive structural features. Overall, G/HBF-1 showed a high degree of similarity to CPRF-2 (67% identity in 361 amino acids) and OHP1 and OHP2 (48% identity in 339 amino acids), and these sequence comparisons define a sub-family of plant G-box-binding bZIP proteins. Southern blots of soybean genomic DNA probed with G/HBF-1 sequences at high stringency showed only one or two hybridizing bands in a range of restriction endonuclease digestions (data not shown), indicating that G/HBF-1 is likely to be encoded by a single copy gene.

G/HBF-1 binding to the chs15 G-box and H-box III

DNA binding by G/HBF-1 was examined in experiments using radiolabeled oligonucleotides to probe plaque lawns of λG/HBF-1 (Table I) and by gel retardation assays with purified recombinant G/HBF-1 (Table I and Figure 3). G/HBF-1 cDNA was fused to the T7 promoter in the vector pET-28a (Novagene), and the recombinant G/HBF-1 carrying an N-terminal hexameric histidine peptide tag, G/HBF-1(His_6), was purified from *Escherichia coli* extracts by immobilized Ni affinity chromatography. Gel retardation assays with the recombinant factor demonstrated binding to the –80 to –42 *chs15* promoter sequence containing both the G-box (–72 to –67) and TATA-proximal H-box (H box III, –59 to –53) and to each of these two *cis* elements when tested separately (Figure 3A). The major binding complex formed with the –80 to –42 sequence had a similar electrophoretic mobility to the complexes formed with either the *chs15* G-box sequence CACGTG (–74 to –69) or the extended H-box III sequence TCA**CCTACC**CTA (–65 to –53) when tested separately, suggesting that G/HBF-1 binding was mainly at one location when both *cis* elements were in close proximity. Incubation of G/HBF-1 with the –80 to –42 sequence also generated a second, low abundance complex with increased electrophoretic mobility not observed with either *cis* element alone.

Nucleotide sequence requirements for G/HBF-1 binding were analyzed by direct binding of test oligonucleotides to λG/HBF-1 plaque lawns and by competition with the –80 to –42 *chs15* promoter fragment for binding to recombinant G/HBF-1(His_6) in gel retardation assays. Mutation of the G-box sequence GCACGTGA to GCgtacGA abolished binding when tested in isolation from H-box III. However, the core ACGT was not sufficient for G/HBF-1 binding since the *as-1* sequence TGACGTT was not recognized. Likewise, the parsley *chs* G-box, CCACGTGG, involved in light regulation (Weisshaar *et al.*, 1991), was not recognized, indicating that not only the ACGT core but also immediately flanking nucleotides appear to be important for G/HBF-1 binding. Interestingly, G/HBF-1 bound to a larger fragment of the

Table I. DNA-binding activity of the G/HBF-1 protein

Box	Oligonucleotides	Binding	Pizza
–80/–42	GTG TTG CAC GTG ATA CTC ACC TAC CCT ACT TCC TAT CCA	++	+
–80/–42 (MI)	GTG TTG Cgt acG ATA CTC ACC TAC CCT ACT TCC TAT CCA	+	n.d.
–80/–42 (MII)	GTG TTG CAC GTG ATA CTC Aaa TAa aCT ACT TCC TAT CCA	++	n.d.
–80/–42 (MIII)	GTG TTG Cgt acG ATA CTC Aaa TAa aCT ACT TCC TAT CCA	–	n.d.
HI	AGA AAC TCC TAC CTC ACG AAC TAG GA	–	–
HII	T TGC ACT GCC TAC CAT GTC TGC TTC CT	–	–
HIII	AGA CTC ACC TAC CCT ACT TCC TAT CC	++	+
HIII (MI)	AGA CTC ACC atC CCT ACT TCC TAT CC	+	–
HIII (MII)	AGA CTC Aaa TAa aCT ACT TCC TAT CC	–	–
HIII (MIII)	AGA CTC ACg TAC CCT ACT TCC TAT CC	n.d.	+
HIII (MIV)	AGA CTC AgC TAC CCT ACT TCC TAT CC	–	–
HIII (MV)	AGA CTC ACC TAt tCT ACT TCC TAT CC	+	+
H consensus	CGA CTC ACC TAC CTG ACA TGC TAC GCA G	–	n.d.
G	GCT TTG GTG TTG CAC GTG ATA CT	++	+
G (MI)	GCT TTG GTG TTG Cgt acG ATA CT	–	n.d.
boxII-*s*	C TTA TTC CAC GTG GCA G	–	n.d.
boxII-*1*	GAT CTC TTA TTC CAC GTG GCC ATC CGG ATC C	+	n.d.
as-1	GAT ATC TCC ACT GAC GTA AGG GAT GAC GTT AAC	–	n.d.

Summary of 'Pizza blot' and gel retardation experiments monitoring the binding of recombinant G/HBF-1 to specific oligonucleotides. The H-box core sequence is single underlined and the G-box is double underlined. Mutated bases are printed in lower case. The H-box consensus sequence was derived by Yu *et al.* (1992).

parsley *chs* promoter containing not only the G-box but an immediately downstream sequence with an AC-rich *cis* element resembling the H-box.

Mutation of the H-box core motif CCTACC to CCatCC or aaTAaa severely impaired binding to the H-box III *cis* element when tested in isolation from the G-box, whereas binding was observed with the sequence CCTAtt. However, no binding was observed with upstream fragments of the *chs15* promoter-containing H-box I (–159 to –135) or H-box II (–139 to –113) in which the CCTACC motif is embedded in different flanking sequences compared with the TATA-proximal H-box III (Yu *et al.*, 1992). Thus, sequences in addition to the core H-box motif are important for G/HBF-1 binding to H-box III and, when immediately flanking sequences are taken into account, a single mutation in H-box III, CACCTACC to CACgTACC, generates an almost perfect second version of the *chs15* G-box (CACGTG). The CACGTACC sequence, intermediate between the G-box and H-box III, was also bound by G/HBF-1.

G/HBF-1 regulation during chs induction

G/HBF-1 sequences hybridized at high stringency to a single transcript in total cellular RNA isolated from uninduced soybean cells (Figure 4). The size of the transcript was 1.4 kb, consistent with the size of the full-length G/HBF-1 cDNA. Elicitation of soybean cells with reduced glutathione caused little change in the level of G/HBF-1 transcripts, whereas *chs* transcripts rapidly accumulated from low basal levels (Figure 4A).

To monitor G/HBF-1 regulation at the protein level, we generated a panel of polyclonal antisera, including two peptide antibodies, α-G/HBF-1(P2) and α-G/HBF-1(P4), to two internal peptides showing little similarity to the peptide sequences found in the corresponding regions of otherwise closely related bZIP transcription factors such as CPRF-2 (Figure 2). α-G/HBF-1(P2) bound to a single protein in Western blots of total cellular protein (Figures 4 and 5A), whereas α-G/HBF-1(P4) and a cross-reacting antibody, α-OHP, raised against the maize OHP C-terminal region, which shows very strong peptide sequence identity to the corresponding region of G/HBF-1, bound to two protein species exhibiting almost identical electrophoretic mobilities (Figure 5A). The protein species recognized by these antibodies were found in both cytosolic and nuclear fractions and neither their overall abundance nor distribution between cytosol and nucleus changed appreciably during glutathione induction of *chs* transcription. However, antibody supershift gel retardation experiments indicated that G/HBF-1 was involved in a *chs15* promoter–nuclear protein binding complex induced in elicited cells (Figure 5B). The major binding complex formed by incubation of soybean nuclear extracts with the –80 to –42 region of the *chs15* promoter was neither elicitor regulated nor supershifted by incubation with α-G/HBF-1(P4) prior to gel retardation analysis. In contrast, the other prominent complex was observed only with nuclear extracts from elicited cells, and pre-incubation with α-G/HBF-1(P4) caused a marked reduction in the electrophoretic mobility of this complex. This supershifted DNA-binding complex co-migrated with the DNA-binding complex formed with recombinant G/HBF-1 in the presence of α-G/HBF-1(P4).

Phosphorylation of G/HBF-1

Incorporation of G/HBF-1 into a *chs15* promoter-binding complex in elicited cells implied post-translational regulation of G/HBF-1, and we next investigated whether G/HBF-1 was phosphorylated *in vivo* by labeling soybean cells with [^{32}P]phosphate. Immunoprecipitation of G/HBF-1 from extracts prepared after exposure of cells to [^{32}P]phosphate resulted in the incorporation of radioactivity into a single protein of the appropriate electrophoretic mobility (Figure 6B). Moreover, several fold greater incorporation of ^{32}P into immunoprecipitable G/HBF-1 was observed in [^{32}P]phosphate-labeled cells after treatment with 0.5 mM glutathione than in equivalent pulse-labeled control cells. Phosphoamino acid analysis of ^{32}P-labeled, immunoprecipitable G/HBF-1 demonstrated that phosphorylation was almost exclusively on serine

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G/HBF-1   MTASSSSSSHQND-----------------------VVEIKDENLSIPN-----------LNPS-TALNS
 CPRF-2   --------EFAAAFWSPPARE-----DSSKL--------VMNRSDSEWAFQSFLQQA---SA•ES•-QPLP•
  OHP-1   MERVF•VEEIP•PYWVPPHPQSAAAGAVAAP-----AGEAAGLM•RCPSEWYFQKFLEEAVLDS•VPV•GV•
  OHP-2   MERVF•MEEIP•PYWAPPHPQPAAGGAVAAPGGVGGAGD•AGAM•RCPSEWYFEKFLEEAVLDS•GPV•GVG

                                            D1                      P2
                                                             ••••••••••••
G/HBF-1   KPASSFGLAP-------------PPNIAVDSEEYQAFLKSQLHLACAAVALTRGKSLNPQDSGSTAHDKGSE
 CPRF-2   D•VPVA•DVKNPVE--------I•A•VP••••D•••Y•••R•D••••••••••AS••K••••-AALL•N••Q
  OHP-1   RGSVGA•VEAAERKTPGTAAAAAASSSV••PV••N•IV•QK•EKDL•••••W•ASGAA•P•N-----SPAGS
  OHP-2   RSSGQA•VEAAESKPLGAAAPASVSSSV••PV••N•M••QK•EKDL••I•MW•ASGAA•P•L-----SATAA

                                                                  D2
G/HBF-1   TASAAQSGSHVSTLGSGQEVAKIQDKDAGGPVGIPSLPPVQKKPVVQVRSTTSGSSREQSDDD-EAEGEAET
 CPRF-2   ASNTS•LVSQ•PPK•••HDLS•EE••E•LAATAT•L••AL•••SAI••K••••••••DH••••D•L•••T••
  OHP-1   SLPSVDVPHAGPLKPM•GTGSLV•N•L••A•G•G-•S•H•VQNADIP•KQ•••S•••••••••D-M••D•••
  OHP-2   SLPSVGVPHAAPLKPV•GTESLV•NML••A•••G-•G•HIVQIADIP•KQ•••S•••••••••D-M••D•••

                 basic
G/HBF-1   TQGMDPADAKRVRRMLSNRESARRSRRRKQAHLTELETQVSQLRVENSSLLKRLTDISQKYNEAAVDNRVLK
 CPRF-2   •RNG••S••••••••••••••••••••••Q••M••L••••••L••••••L••••••I••R••DA••••••L•
  OHP-1   •GNGN•VQQRLQ••K•••••••••••S••A•••N•L•A••A•L••••••L••••A•VN••F••A••••••L•
  OHP-2   NGNGN•VQQRQQ••K•••••••••••S••A•••N•L•A••A•L••••••L••••A•VN••F••A••••••L•

                                                                   P4
                   D3                                          ••••••••••
G/HBF-1   ADVETLRTKVKMAEETVKRVTGLNPLFQAMS-EISSMVMPSYSGSPSDTSADAAVPVQDDPKHHYYQQPPNN
 CPRF-2   ••I••M•A•••••••••••••••••M••S••S•••TIG•Q•F•••••••••TT---••GS•Q•F•*PA*TS
  OHP-1   •••••••A••••••DS••••••M•T••P•V•-DM••LS••-FN•••••SAS•••••I•••LNSYFAN-----
  OHP-2   •••••••A••••••DS••••••M•A••P•V•-DM••LS••-FN•••••SAS•ST••••••LNSYFAN-----

                                                    D4
G/HBF-1   LMPTHDPRIQNGMV-DVPPIENVEQNPATAAVGGNKMGRTTSMQRVASLEHLQKRIRGEVSSCGTQGRGEQ
 CPRF-2   H••AQ•QK••••LL-Q•••VD•LQ•HS•SGP•E•••E••S••••••••••••••••G••••EA•VS•K•
  OHP-1   --•SEIGGS-••YMP•IASSAQEDDDFVNG•QVAG•••S•D•L••••••••••••MC•GPA•S•STS----
  OHP-2   --•SEIGGN-••YMP•IASSVQQDD•FVNGYQAAG•••••D•L••••••••••••MC•GPA•S•STS----
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Fig. 2. Comparison of the amino acid sequences of the bZIP transcription factors G/HBF-1, CPRF-2, OHP1 and OHP2. Conserved amino acid residues are indicated as dots, except those constituting the putative leucine zipper, which are shown in bold. The conserved domains D1–D4 and the basic region are enclosed in boxes and the peptides used for antibody production are marked by dotted lines.

A B

probe: -80/-42, H III, G box, box II-/, H con, as-1 | -80/-42

competitor: – – – – – – | –, -80/-42, -80/-42 MI, -80/-42 MII, -80/-42 MIII, HI, HII, HIII, HIII MI, HIII MII, G, G MI, box II-s, box II-/, as-1

free probe

Fig. 3. Gel retardation assay of G/HBF-1 binding to specific *cis* elements. (**A**) G/HBF-1(His_6) binding to the –80 to –42 region of *chs15* and indicated oligonucleotide sequences within this region. (**B**) DNA-binding specificity monitored by competition between test oligonucleotides and labeled wild-type –80 to –42 *chs15* sequence for binding to recombinant G/HBF-1(His_6).

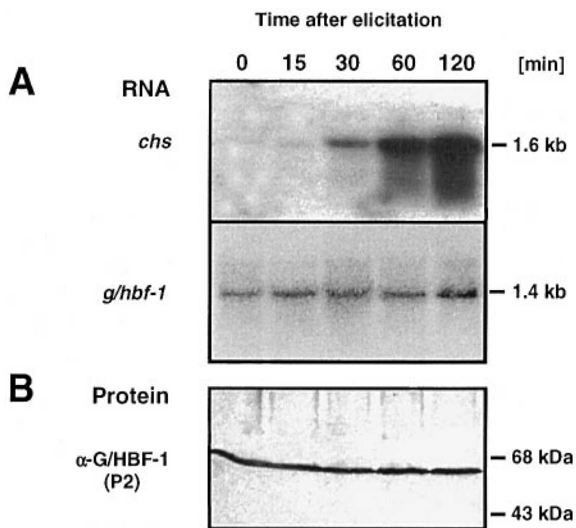

Fig. 4. G/HBF-1 transcripts and protein levels do not change during *chs* induction. Soybean cells were elicited with 0.5 mM glutathione. (**A**) *chs* and G/HBF-1 transcript levels. Northern blots of total RNA were hybridized with *chs1* or G/HBF-1 cDNA probes. (**B**) G/HBF-1 protein levels. Western blots of whole cell extracts were probed with α-G/HBF-1(P2).

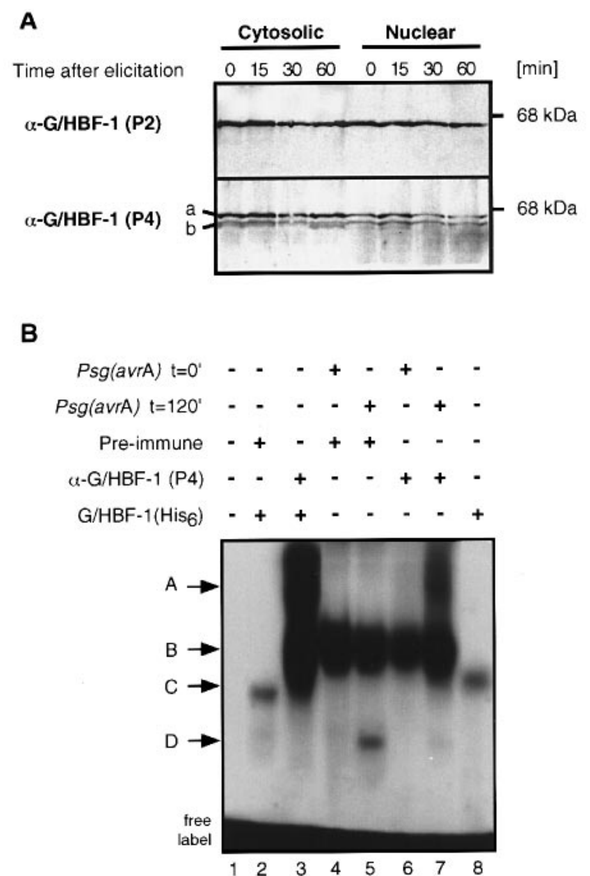

Fig. 5. Elicitor induces formation of a *chs15* promoter–nuclear protein complex containing G/HBF-1. (**A**) Cellular localization of G/HBF-1. Cytosolic and nuclear extracts, respectively, of uninduced soybean cells and cells at various times after elicitation with 0.5 mM glutathione were analyzed by probing Western blots with α-G/HBF-1(P2) and α-G/HBF-1(P4). The latter antibody recognizes two forms of G/HBF-1, labeled a and b respectively. (**B**) Antibody supershift analysis of the involvement of G/HBF-1 in DNA-binding complexes with the –80 to –42 region of the *chs15* promoter. The effects of pre-treatment of binding complexes with pre-immune serum (lanes 2, 4 and 5) or α-G/HBF-1(P4) (lanes 3, 6 and 7) on electrophoretic mobility were analyzed in gel retardation assays with nuclear extract from cells 2 h after elicitation with *Psg(avrA)* (lanes 5 and 7) and nuclear extract from equivalent control cells (lanes 4 and 6). Electrophoretic mobilities were compared with those of binding complexes formed with no added proteins (lane 1), recombinant G/HBF-1 alone (lane 8), recombinant G/HBF-1 plus pre-immune serum (lane 2) or recombinant G/HBF-1 plus α-G/HBF-1(P4) (lane 3). A, α-G/HBF-1(P4)-supershifted elicitor-regulated complexes; B, major complex not α-G/HBF-1(P4)-supershifted or elicitor regulated; C, complex with recombinant G/HBF-1; D, elicitor-regulated complex.

residues, with little detectable phosphothreonine (1–5%) and no phosphotyrosine (Figure 6C).

α-G/HBF-1(P4) gave strong immunoreactivity with recombinant G/HBF-1, whereas α-G/HBF-1(P2) did not react with the bacterially expressed protein (Figure 6E). However, incubation of recombinant G/HBF-1 with soybean whole cell extracts in the presence of ATP resulted in the appearance of strong α-G/HBF-1(P2) immunoreactivity *in vitro* (Figure 6E). Inhibition of the appearance of α-G/HBF-1(P2) immunoreactivity by inclusion of either EDTA or K252A and staurosporine in the protein kinase reactions closely followed the effects of these protein kinase inhibitors on the labeling of recombinant G/HBF-1 with ^{32}P from [γ-^{32}P]ATP in the same reactions (Figure 6E). Moreover, alkaline phosphatase treatment of soybean cytosolic extracts resulted in the loss of α-G/HBF-1(P2) immunoreactivity with native G/HBF-1, indicating that α-G/HBF-1(P2) recognizes a phosphorylation-dependent conformation of G/HBF-1 (Figure 6A).

Elicitor and pathogen activate G/HBF-1 kinase

To investigate further the phosphorylation control of G/HBF-1, Ni affinity-purified, recombinant factor was incubated with [γ-^{32}P]ATP and cytosolic or nuclear extracts prepared from soybean cells at various times after elicitation with glutathione (Figure 7A). No *in vitro* phosphorylation of recombinant G/HBF-1 was observed following incubation with nuclear extracts from control or elicited cells, although the nuclear extract from cells 30 min after elicitation gave substantial phosphorylation of an endogenous substrate of apparent M_r ~33 kDa. In contrast, *in vitro* phosphorylation of recombinant G/HBF-1 was observed following incubation with the cytosolic fraction, together with phosphorylation of an endogenous substrate of the same electrophoretic mobility as the endogenous nuclear substrate.

Glutathione induction of soybean cells caused a rapid, transient increase in extractable G/HBF-1 kinase activity as measured by the incorporation of ^{32}P from [γ-^{32}P]ATP into recombinant G/HBF-1 substrate (Figure 7A). As an internal control, no elicitor induction of protein kinase activity was observed using exogenous histone H1 as a substrate. There was little or no apparent lag for stimulation of G/HBF-1 kinase activity, with maximum activity, 5-fold above uninduced controls, in extracts prepared from cells ~30 min after elicitation, followed by rapid decay to basal levels after 60 min (Figure 7B). This transient increase in extractable G/HBF-1 kinase activity was concomitant with the onset of rapid accumulation of *chs* transcripts (Ryder *et al.*, 1984 and Figure 4). Inoculation of soybean cells with avirulent *Pseudomonas syringae* pv. *glycinea* also

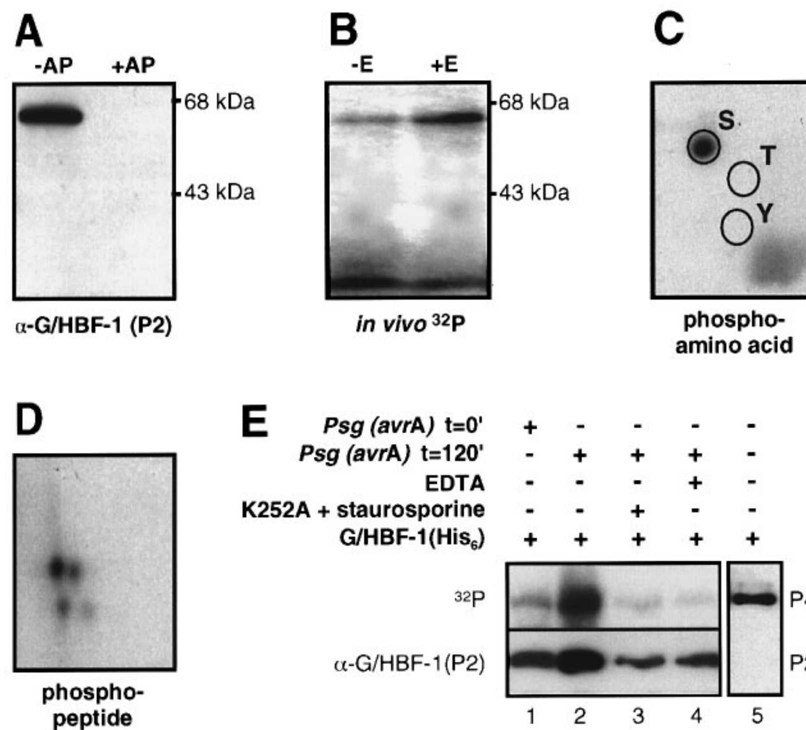

Fig. 6. Phosphorylation of G/HBF-1. (**A**) Effect of alkaline phosphatase treatment on G/HBF-1 reactivity in Western blots probed with α-G/HBF-1(P2). (**B**) Elicitor stimulation of G/HBF-1 phosphorylation *in vivo*. Cells were pre-labeled with [^{32}P]phosphate for 15 min. Incorporation of ^{32}P into immunoprecipitable G/HBF-1 was compared in cells then elicited with 0.5 mM glutathione for 30 min and in equivalent pulse-labeled control cells. (**C**) Phosphoamino acid analysis of *in vivo* ^{32}P-labeled G/HBF-1. The hydrolysate of immunoprecipitated G/HBF-1 was analyzed by two-dimensional thin layer chromatography and radiolabeled residues detected by autoradiography. (**D**) Two-dimensional mapping of G/HBF-1 phosphopeptides. Recombinant G/HBF-1(His$_6$) was incubated with [γ-^{32}P]ATP and a cytosolic extract from elicited cells. After immunoprecipitation, the trypsin digest of *in vitro* phosphorylated G/HBF-1(His$_6$) was analyzed by two-dimensional electrophoresis and autoradiography. (**E**) Relationship between G/HBF-1 phosphorylation and α-G/HBF-1(P4) immunoreactivity. Lanes 1–4: recombinant G/HBF-1 was pre-incubated with [γ-^{32}P]ATP and extracts from either control cells (lane 1) or cells 2 h after inoculation with *Psg(avrA)* (lanes 2–4). The protein kinase reaction in lane 3 contained 5 μM K252A and 5 μM staurosporine and in lane 4 contained 25 mM EDTA. The upper panel is an autoradiogram of ^{32}P labeling of recombinant G/HBF-1 and the lower panel is a Western blot analysis of α-G/HBF-1(P4) immunoreactivity of the same reaction products. Lane 5: Western blot analysis of the immunoreactivity of recombinant G/HBF-1 with α-G/HBF-1(P4) (upper panel) and α-G/HBF-1(P2) (lower panel).

caused a marked stimulation in extractable G/HBF-1 kinase activity in the cytosolic fraction, as measured either by the incorporation of ^{32}P from [γ-^{32}P]ATP into recombinant G/HBF-1 substrate or by probing Western blots of the products of equivalent non-radioactive assay reactions with α-G/HBF-1(P2), which is specific for phosphorylated G/HBF-1 (Figure 7C).

Phosphorylation promotes G/HBF-1 binding to DNA

As was demonstrated *in vivo*, phosphorylation of G/HBF-1 *in vitro* was also specific for serine (data not shown), and analysis of the pattern of ^{32}P-labeled peptides generated by trypsin digestion of recombinant G/HBF-1 immunoprecipitated from the *in vitro* protein kinase reaction with extracts from elicited cells revealed four labeled peptides, indicating phosphorylation at multiple sites (Figure 6D). To determine whether phosphorylation modulates the functional properties of G/HBF-1, we examined the effect of *in vitro* phosphorylation on factor binding to the *chs15* H-box III, monitored by Southwestern blot analysis of the protein kinase reaction products. *In vitro* phosphorylation of G/HBF-1 by cytosolic extracts from cells elicited with either glutathione or inoculation with avirulent *P.syringae* pv. *glycinea* resulted in markedly enhanced *cis* element-binding activity compared with that resulting from protein kinase reactions with extracts from equivalent unstimulated cells (Figure 8A).

The effect of *in vitro* phosphorylation on G/HBF-1 binding to H-box III was confirmed in gel retardation experiments. Incubation of recombinant G/HBF-1(His$_6$) with ATP and cytosolic extracts from cells isolated 20–30 min after elicitation with glutathione resulted in the generation of a high molecular weight DNA-binding complex substantially larger than that formed by non-phosphorylated recombinant factor and *cis* element in the absence of active cytosolic extracts (Figure 8B). Formation of this large complex was dependent on the addition of both recombinant factor and ATP (Figure 8B). Extracts from control cells only weakly supported formation of this complex, but elicitation caused a rapid, transient increase in the extractable activity driving complex formation, with similar kinetics to those for stimulation of extractable G/HBF-1 kinase activity.

Discussion

λG/HBF-1 cDNA encodes a 41 kDa protein with the characteristic features of bZIP transcription factors, including a basic domain involved in DNA binding adjacent to a leucine zipper involved in factor dimerization. G/HBF-1 is most similar to three other plant bZIP proteins, CPRF-2

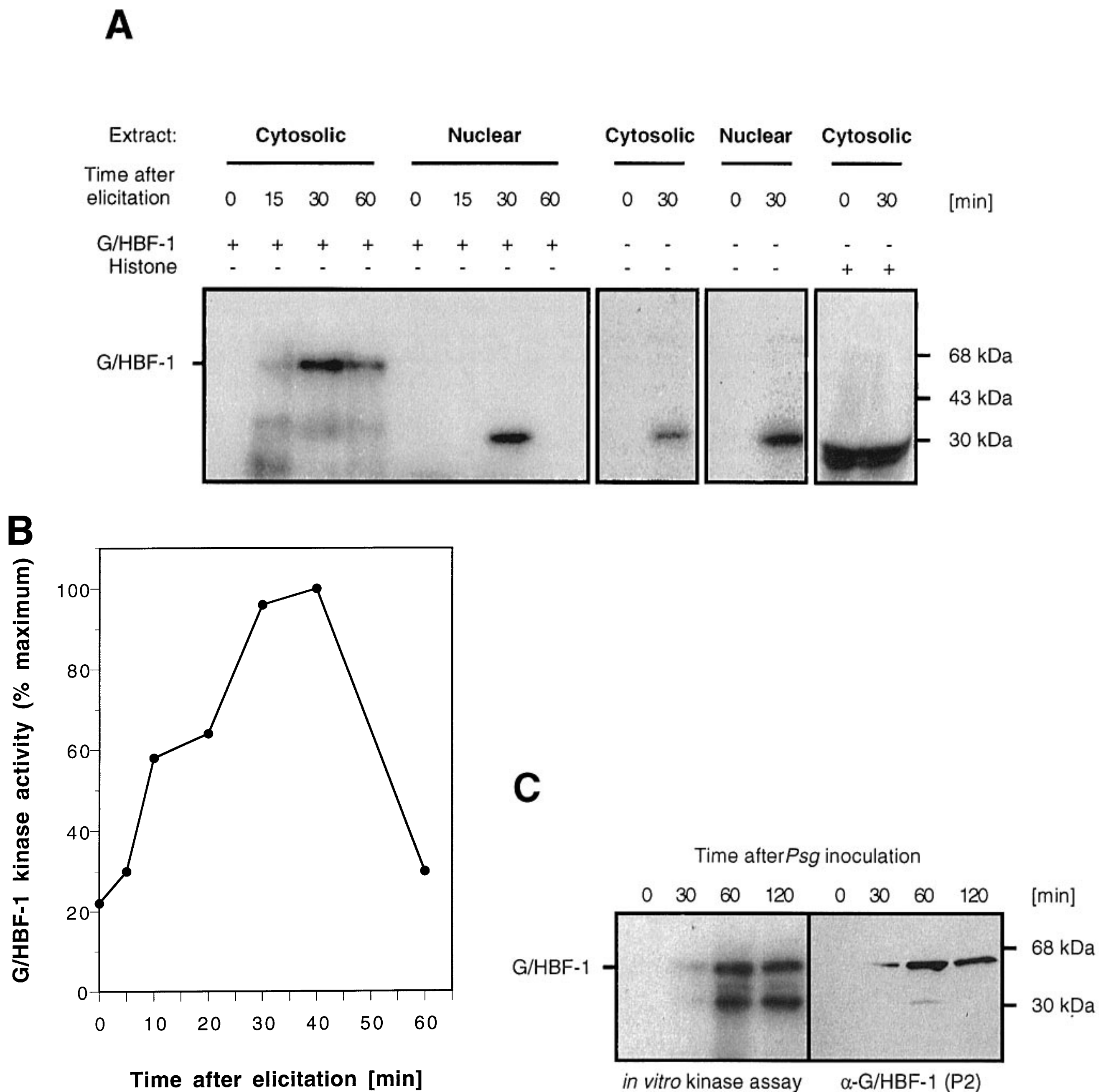

Fig. 7. Stimulation of extractable G/HBF-1 kinase activity in elicited soybean cells. (**A**) Glutathione stimulation of extractable cytosolic G/HBF-1 kinase activity. Cytosolic and nuclear extracts from control cells and cells at various times after elicitation with 0.5 mM glutathione were incubated with [γ-^{32}P]ATP and the indicated protein substrates. Protein kinase reaction products were analyzed by SDS–PAGE and autoradiography. (**B**) Rapid, transient stimulation of glutathione-extractable G/HBF-1 kinase activity by glutathione. G/HBF-1 kinase activity was measured by phosphorimager quantification of incorporation of ^{32}P from [γ-^{32}P]ATP into recombinant G/HBF-1(His$_6$) catalyzed by cytosolic extracts of cells isolated at various times after treatment with 0.5 mM glutathione. G/HBF-1 kinase activity is expressed relative to the maximum activity observed. (**C**) Stimulation of extractable cytosolic G/HBF-1 kinase activity by avirulent *P.syringae* pv. *glycinea* (*avrA*). G/HBF-1 kinase activity was assayed by incorporation of ^{32}P from [γ-^{32}P]ATP (left panel) or by probing Western blots of kinase reaction products with α-G/HBF-1(P2) (right panel).

from parsley (Weisshaar *et al.*, 1991) and OHP1 and OHP2 from maize (Pysh *et al.*, 1993). Four conserved domains, D1–D4, can be discerned in these four *trans* factors that are not found in other plant bZIP proteins. While D4 does not exhibit any distinctive structural features, the predicted helical structure of D1 may promote specific intermolecular interactions and, by analogy to the role of acidic domains in other transcription factors (Hope and Struhl, 1986), D2 may contribute to transcriptional activation. D3 is adjacent to the leucine zipper and deletion of the equivalent domain in CPRF-2 reduces DNA-binding affinity (Armstrong *et al.*, 1992). This domain is also found in certain helix–loop–helix transcription factors such as MyoD (Scales *et al.*, 1990) and may function in dimer stabilization. The conservation of these four domains in CPRF-2, OHP1, OHP2 and G/HBF-1 suggests that this group of bZIP proteins share distinctive functional attributes. While *opaque2* has been implicated in the expression of zein storage protein genes (Schmidt *et al.*, 1992), the biological functions of OHP1 and OHP2, which

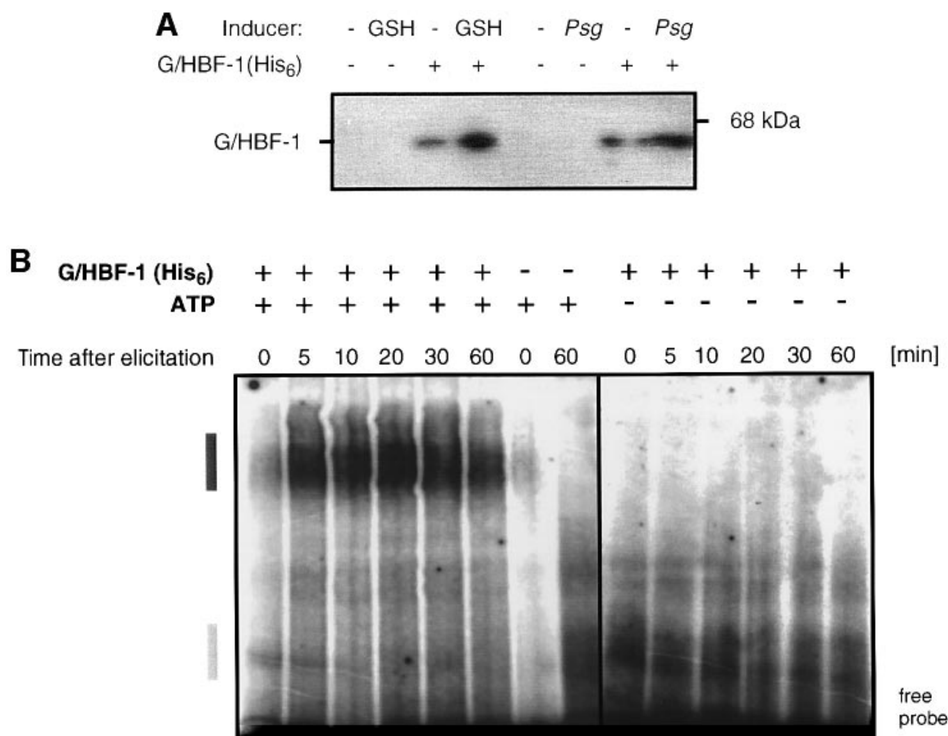

Fig. 8. G/HBF-1 phosphorylation *in vitro* promotes DNA-binding activity. Cytosolic extracts were isolated after elicitation with 0.5 mM glutathione or inoculation with avirulent *P.syringae* pv. *glycinea (avrA)* and from equivalent control cells. Cytosolic extracts were incubated with ATP and recombinant G/HBF-1(His_6) as indicated and the kinase reaction products analyzed for binding to the –80 to –42 region of the *chs15* promoter by (**A**) Southwestern blot analysis following SDS–polyacrylamide gel electrophoresis and (**B**) gel retardation assay. In (A), extracts were prepared 30 min after treatment with 0.5 mM glutathione or 60 min after bacterial inoculation and from equivalent control cells. The vertical lines mark the slow migrating, phosphorylation-dependent complex and the fast migrating, phosphorylation-independent complex formed by G/HBF-1, respectively.

were isolated through their interactions with *opaque2*, have not been established (Pysh, 1994). Likewise, while CPRF-1 appears to contribute to the light regulation of parsley *chs*, no function has yet been ascribed to CPRF-2 (Weisshaar *et al.*, 1991; Feldbrügge *et al.*, 1994). It is unlikely that G/HBF-1 is the soybean functional ortholog of parsley CPRF-2 since we have isolated sequences from tobacco and *Arabidopsis* much more closely related to G/HBF-1 (95% identity) than is CPRF-2 (our unpublished data).

One group of plant bZIP proteins including GBF, CPRF-1 and Taf-1 preferentially bind to the G-box CACGTG, while another group, the TGA1-like family, preferentially bind to the C-box, TGACGTC (Williams *et al.*, 1992; Izawa *et al.*, 1993). G/HBF-1 appears to fall in a third class, which also includes CPRF-2, *opaque2* and RITA-1, exhibiting relaxed binding specificity. For example, *opaque2* binds an imperfect C-box, ATCAGTCAT, lacking a complete ACGT core (de Pater *et al.*, 1994). Likewise, G/HBF-1 binds to the extended H-box III sequence, CACCTACC, which does not contain a perfect palindromic ACGT core. While disruption of the ACGT core in the *chs15* G-box GCACGTGA can prevent G/HBF-1 binding, this sequence is not sufficient for binding since G/HBF-1 does not recognize either the parsley *chs* G-box CCACGTGG or the *as-1* element TGACGTGG, and G/HBF-1 binding is clearly influenced by the nucleotides flanking the ACGT core. Moreover, G/HBF-1 does not bind to H-boxes I or II, which share with H-box III the core CCTACC motif, and G/HBF-1 probably binds to the H-box III region by virtue of its resemblance to an ACGT-like *cis* element when nucleotides immediately 5′ of this H-box are taken into account, rather than binding to the H-box core CCTACC, which is a canonical Myb-binding site (Sablowski *et al.*, 1994, 1995).

Binding to two adjacent ACGT-related *cis* elements implicated in the induction of phenylpropanoid biosynthetic genes in elicited cells is consistent with a role for G/HBF-1 in the activation of early transcription-dependent defenses, and several lines of evidence indicate that rapid phosphorylation of G/HBF-1 represents a terminal step in an elicitor-activated signal pathway. Thus, while G/HBF-1 transcript and protein levels do not change during the induction of *chs* transcription, antibody supershift analysis implicates G/HBF-1 in the formation of a binding complex with the –80 to –42 region of the *chs15* promoter specifically in elicited cells and there is a rapid, marked stimulation of G/HBF-1 phosphorylation *in vivo*. Moreover, recombinant G/HBF-1 is a substrate *in vitro* for a cytosolic protein-serine kinase that is stimulated rapidly in cells treated with glutathione, which closely mimics the effects of microbial elicitors (Wingate *et al.*, 1988), or in cells inoculated with avirulent *P.syringae* pv. *glycinea*, which induces a hypersensitive response (Levine *et al.*, 1994). Glutathione stimulation of extractable G/HBF-1 kinase activity occurs without detectable lag and hence precedes the stimulation of *chs* transcription first observed 5–10 min after elicitation, and maximal levels of extractable G/HBF-1 kinase activity are attained ~30 min after elicitation, concomitant with the onset of rapid accumulation of *chs* transcripts. Likewise, stimulation of extractable

G/HBF-1 kinase activity is an early event in the hypersensitive response to avirulent *P.syringae* pv. *glycinea*.

α-G/HBF-1(P2) was generated by immunization with a synthetic peptide hapten corresponding to a region spanning the C-terminus of the D1 domain. The loss of reactivity of native plant G/HBF-1 with this antibody following treatment of plant extracts with alkaline phosphatase implies that dephosphorylation causes a major conformational change in which the P2 peptide becomes inaccessible to the antibody. Moreover, α-G/HBF-1(P2) does not react with G/HBF-1(His_6) expressed in *E.coli*, and the observation that incubation of the recombinant factor with ATP and cytosolic extracts from elicited cells generates α-G/HBF-1(P2)-reactive G/HBF-1(His_6) in parallel with factor phosphorylation indicates that the *in vitro* kinase reaction faithfully reproduces a key effect of phosphorylation of native G/HBF-1 *in vivo* and that the phosphorylation-dependent conformational change is reversible.

Reversible phosphorylation controls the functional activity of many animal transcription factors by modulation of one or more of the following attributes: DNA-binding affinity, transactivation, interactions with other regulatory proteins and cellular localization (Hunter and Karin, 1992). Incubation with ATP and active cytosolic extracts promotes the binding of recombinant G/HBF-1(His_6) to the cognate *cis* elements in the proximal region of the *chs15* promoter as monitored by both gel retardation and Southwestern blot analysis of the kinase reaction products. The latter experiment, in which enhanced DNA binding to the recombinant substrate was monitored following electrophoretic fractionation of the reaction products, implies that phosphorylation of G/HBF-1 contributes to the enhanced DNA binding, although we do not rule out secondary effects, mediated by endogenous accessory factors in the cytosolic extracts, in the generation of the high molecular weight DNA-binding complex observed in gel retardation assays. In animals, phosphorylation control of the DNA-binding affinities of transcription factors is usually negative, e.g. c-Myb and c-Jun, and examples of phosphorylation directly promoting DNA binding are rare. However, G/HBF-1 regulation is reminiscent of the stimulation of serum response factor binding to the c-*fos* promoter following factor phosphorylation in response to epidermal growth factor (Janknecht *et al.*, 1992). The mechanism whereby phosphorylation affects DNA binding is unknown, but phosphorylation induces a conformational change in serum response factor (Manak and Prywes, 1991). In G/HBF-1, the phosphorylation-induced exposure of the D1 domain may reflect a conformational change, possibly activated by changes in charge distribution following phosphorylation, that concomitantly exposes the basic domain thereby promoting DNA binding. Such a phosphorylation-induced conformational change could also enhance dimerization, mediated by the adjacent leucine zipper region, or other protein–protein interactions, and indeed preliminary evidence indicates that G/HBF-1 undergoes conformational-sensitive interactions with the H-box-binding factor KAP-2, which also binds to the proximal region of the *chs15* promoter (W.P.Lindsay and W.Dröge-Laser, unpublished data).

Elicitor-stimulated G/HBF-1 kinase activity is exclusively cytosolic, with no corresponding activity detectable in nuclear extracts. Hence, unlike for example growth factor-stimulated ERK MAP kinase (Chen *et al.*, 1992), elicitation does not result in migration of the G/HBF-1 kinase to the nucleus. However, the phosphorylation-dependent conformational change that exposes the D1 domain of G/HBF-1 might also uncover nuclear localization sequences and hence promote migration of the *trans* factor substrate to the nucleus following elicitor stimulation of the cytosolic kinase. Strikingly, three serine residues are embedded within the cluster of basic amino acids constituting the putative nuclear localization signal within the basic domain (Varagona *et al.*, 1992), raising the alternative possibility that phosphorylation governs its interaction with the nuclear pore complex (Moll *et al.*, 1991). Western blot analysis failed to reveal major changes in the distribution of either total G/HBF-1 or the phosphorylated fraction between cytosol and nucleus during *chs* induction, and hence a second factor may be limiting for G/HBF-1 movement to the nucleus. KAP-2, which interacts with the activated conformation of G/HBF-1, is a likely candidate since this *trans* factor is present at very low abundance and, based on measurements of DNA-binding activity, appears to migrate from cytosol to nucleus in elicited cells (Yu *et al.*, 1992).

The involvement of protein phosphorylation in the activation of inducible defense mechanisms, inferred from physiological and pharmacological studies (Dietrich *et al.*, 1989; Felix *et al.*, 1991; Levine *et al.*, 1994; Suzuki and Shinshi, 1995), was confirmed recently by reports that the tomato *Pto* and rice *Xa21* disease resistance genes encode protein-serine/threonine kinases (Martin *et al.*, 1993; Loh and Martin, 1995; Song *et al.*, 1995). Moreover, the *Pto* kinase phosphorylates a second protein-serine kinase, *Pti1*, that is also involved in the hypersensitive response, demonstrating the operation of a phosphorylation signal cascade in the expression of disease resistance (Zhou *et al.*, 1995). Elicitation of potato tuber discs with arachidonic acid is correlated with a staurosporine-sensitive increase in the extractable activity of a *trans* factor designated PBF-1, which binds to the promoter of the PR-protein *PR-10a* gene (Després *et al.*, 1995). While these data suggest that induction of this defense gene involves protein phosphorylation, PRF-1 has not been isolated and it remains to be established whether phosphorylation control of *PR-10a* induction is exerted at the transcription factor level.

The protein kinase inhibitor K252A blocks *pal* and *chs* induction in elicited soybean cells (Levine *et al.*, 1994), and the present study demonstrated rapid phosphorylation of a specific transcription factor implicated in the activation of these immediate/early defense genes. This phosphorylation, which modifies the functional activity of G/HBF-1, thus represents the terminal step in a signal pathway activated by elicitors or pathogen avirulence signals, and the rapid stimulation of the cytosolic G/HBF-1 kinase without detectable lag suggests that the signal pathway between receptor and transcription factor is short. Transduction through a kinetically compressed pre-existing signal pathway and the potential signal amplification inherent in phosphorylation-mediated cascades may be crucial for rapid, massive deployment of transcription-dependent defenses and effective expression of disease resistance following perception of pathogen attack. Experi-

ments are in progress to clone the G/HBF-1 kinase and determine whether this gene is the soybean ortholog of the rice *Xa21* or tomato *Pto* resistance genes, the tomato *Pti1* gene which functions immediately downstream of *Pto*, or possibly an ortholog of a substrate of one of these protein-serine kinases.

Reversible phosphorylation of transcription factors is a common mechanism for selective regulation of gene expression in animals (Hunter and Karin, 1992). However, in plants, although various transcription factors have been implicated in the selective regulation of gene expression during development and in response to environmental cues, there is little information on the possible contribution of phosphorylation control exerted at the transcription factor level (Hunter and Karin, 1992). In a number of developmental processes, such as trichome formation, flower induction and determination of floral organ identity, selective gene expression appears to reflect changes in the abundance of specific transcription factors rather than post-translational modulation of the functional activities of pre-existing transcription factors (Meshi and Iwabuchi, 1995). The binding activity of the *Arabidopsis* bZIP protein GBF-1 to a G-box *cis* element implicated in light regulation of gene expression can be modulated *in vitro* by the nuclear protein kinase casein kinase II (Klimczak *et al.*, 1992, 1995). However, phosphorylation of this specific factor *in vivo*, regulation of its phosphorylation by light, or light activation of casein kinase II have not been reported. Experiments with antisera to *Arabidopsis* GBF-1 reveal a pool of cytosolic cross-reacting proteins in parsley cells and suggest that light may stimulate phosphorylation and relocalization from cytosol to nucleus (Harter *et al.*, 1994). While the specific G-box-binding factors and cytosolic protein kinases involved have not been characterized, this putative mechanism resembles that delineated here for elicitor and pathogen stimulation of the cytosolic G/HBF-1 kinase in soybean. Phosphorylation control of the functional activities of specific transcription factors by cytosolic protein kinases may thus prove to be a common mechanism in plants for rapid, flexible regulation of selective gene expression by environmental stimuli.

Materials and methods

Plant cells

Cell suspension cultures of soybean (*Glycine max* L.) cv. Williams 82 or cv. Harasoy 63 were elicited 3 days after transfer to fresh medium, either by treatment with 0.5 mM reduced glutathione (Dron *et al.*, 1988) or inoculation of 5×10^7 c.f.u./ml of *P.syringae* pv. *glycinea* (*Psg*) race 4 bacteria (Levine *et al.*, 1994) with a plasmid carrying the *avrA* gene (Keen and Buzzell, 1991).

Nucleic acid analysis

Total cellular RNA was isolated using the Tri-Reagent method (Molecular Research Center, Inc.). Northern blot hybridization was performed according to Levine *et al.* (1994), using *chs1* cDNA (Ryder *et al.*, 1984) and a PCR amplicon of nucleotides 248–637 of the G/HBF-1 cDNA (Figure 1) as probes. Soybean genomic DNA was isolated as described (Ausubel *et al.*, 1987). Southern blot hybridization and other standard molecular biology techniques were performed as described by Sambrook *et al.* (1989). Oligonucleotides were synthesized on a Cyclone Plus DNA synthesizer (Millipore).

cDNA libraries

Total cellular RNA was isolated from uninduced soybean cells (cv. Harasoy 63) and cDNA prepared using the Pharmacia cDNA synthesis kit. The cDNAs were cloned into the dephosphorylated arms of λgt11 using *Eco*RI linkers and packaged with commercially available extracts (Gigapack, Stratagene). The titer of the non-amplified expression library was 10^5 p.f.u./ml. For a second library, cDNAs from an RNA population 50% from control soybean (cv. Williams 82) cells and 50% from equivalent cells elicited with 0.5 mM reduced glutathione and 30 mg/ml oligogalacturonide fragments for 4 h were cloned into λZAP-II (Stratagene). This library contained 1.3×10^6 independent clones. For Southwestern screening of the λ phage libraries, plaque lifts were probed with an oligonucleotide corresponding to the –80 to –42 region of *chs15*, containing both the G-box and H-box III sequences (GTGTTGCACGTG-ATACTCACCTACCCTACTTCCTATCCA), end-labeled by T4 polynucleotide kinase (Yu *et al.*, 1992). G/HBF-1 cDNA sequences were subcloned in pKSII Bluescript (Stratagene) using the *Not*I site and sequenced by dideoxy chain termination (Sanger *et al.*, 1977).

Recombinant G/HBF-1

A *Kpn*I–*Nsi*I fragment containing the G/HBF-1-coding sequence was inserted into pQE30 (Qiagen) restricted with *Kpn*I and *Sal*I to give pWD11.24, carrying a translational fusion of the His_6 epitope tag at the N-terminus of the G/HBF-1-coding region. pWD16.1 was constructed by inserting the G/HBF-1 sequence, obtained as a *Sac*I fragment from pWD11.24, into pET-28a (Novagen). *E.coli* BL21 (pWD16.1) cells were grown in 0.8 mM IPTG for 4 h at 20°C to induce the expression of G/HBF-1(His_6) and the extracted fusion protein was purified under non-denaturing conditions by Ni-NTA affinity chromatography (Qiagen).

Antisera

Peptide synthesis was performed with an Applied Biosciences peptide synthesizer. Rabbit antisera to synthetic peptides corresponding to the G/HBF-1 amino acid sequences (C)SLNPQDSGSTAHD (P2) and (C)QDDPKHHYYQQ (P4) coupled to activated keyhole limpet hemocyanin (KLH) carrier protein, designated α-G/HBF-1(P2) and α-G/HBF-1(P4) respectively, were generated using standard immunization protocols (Harlow and Lane, 1988). An antibody to the C-terminal domain of the OHP1 transcription factor was kindly provided by R.Schmidt (University of California, San Diego).

Protein extraction

All manipulations were performed at 4°C. Snap-frozen soybean cells were extracted into RIPA buffer [50 mM Tris, pH 7.4, 100 mM NaCl, 5 mM EDTA, 20 mM Na pyrophosphate, 0.1% bovine serum albumin (BSA), 10 mM NaF, 1% Triton X-100, 0.1% dithiothreitol (DTT), 0.25% deoxycholate (DOC), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1.5 mg/ml aprotinin, 0.5 mg/ml leupeptin, 1 mM Na_3VO_4], and the extract cleared by centrifugation. Nuclear and cytosolic protein extracts were obtained by extraction into 300 ml of NH1 buffer (50 mM HEPES, pH 7.9, 1.1 M sucrose, 25 mM NaCl, 25 mM EDTA, 1.1 mM spermine, 1.1 mM spermidine, 5 mM DTT, 2 mM PMSF, 0.5 mg/ml leupeptin, 1.5 mg/ml aprotinin, 0.2% Triton X-100, 3.2% dextran T500, 0.6% polyvinylpolypyrolidine). After centrifugation for 3 min at 3000 *g*, the supernatant containing the cytosolic proteins was clarified by centrifugation. The pellet was resuspended in NH2 buffer (NH1 lacking Triton X-100, dextran T500 and PVPP) and the nuclei washed twice by centrifugation at 3000 *g* and resuspension. The washed nuclei were resuspended in an equal volume of 2× NE buffer (40 mM HEPES, pH 7.9, 1 M NaCl, 3 mM $MgCl_2$, 0.4 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 40% glycerol) and stirred on ice for 45 min, clarified by centrifugation at 16 000 *g* for 5 min twice, and the nuclear protein solution dialyzed against 20 mM HEPES, pH 7.9, 25 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 20% glycerol.

Protein analysis

An 8% SDS–PAGE system (Laemmli, 1970) was used to separate 50–100 mg of protein which was then blotted on PVDF membranes (Immobilon-P, Millipore), probed with the polyclonal rabbit antibodies described above (diluted 1:1000 with phosphate-buffered saline), and antibody binding detected with anti-rabbit horseradish peroxidase-linked antibody using the ECL detection kit (Amersham). Dephosphorylation of proteins in soybean cell extracts by alkaline phosphatase treatment was performed as described by Yu *et al.* (1992).

DNA binding assays

Gel retardation assays were performed with nuclear protein extracts or recombinant G/HBF-1 and ^{32}P-labeled cognate *chs15* sequences according to Yu *et al.* (1992). In antibody supershift experiments, DNA-binding reactions were performed in the presence of a 1:1000 dilution of α-G/HBF-1(P4) or the corresponding pre-immune serum prior to analysis

of electrophoretic mobility. Southwestern blotting was performed as described by Miskimins *et al.* (1985) except that incubation for renaturation and DNA binding was at 4°C for 8 h.

G/HBF-1 phosphorylation in vivo

Soybean cell suspensions (3 ml) were labeled with 1 mCi (3.7×10^7 Bq) of [^{32}P]phosphate for 15 min and then elicited with 0.5 mM reduced glutathione for 30 min. Harvested cells were frozen in liquid N_2, extracted into RIPA buffer and the extract clarified by centrifugation. The supernatant was diluted 5-fold and incubated with protein A/G (Pharmacia) for 1 h at 4°C. After centrifugation, antiserum was added and the immunoreaction incubated for 1.5 h at 4°C, followed by addition of protein A/G and further incubation for 1 h. The immunocomplex was collected by centrifugation, washed sequentially by centrifugation and resuspension in RIPA buffer, twice in TSA buffer (10 mM Tris, pH 8.0, 140 mM NaCl, 0.025% NaN_3, 0.1% BSA, 0.1% Triton X-100) and finally in 0.05 mM Tris, pH 6.8. The immunocomplex was boiled for 5 min in Laemmli buffer and then analyzed by SDS–PAGE, blotting on Immobilon-P membranes and autoradiography.

In vitro phosphorylation of recombinant G/HBF-1

Plant nuclear or cytosolic extracts were dialyzed against protein kinase buffer (20 mM MOPS, pH 7.0, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.1 mM Na_3VO_4). Plant extracts (40–60 μg protein) were incubated with ~10 μg of G/HBF-1 recombinant protein in the protein kinase buffer supplemented with protease inhibitors (1 mM PMSF; 0.5 mg/ml leupeptin; 1.5 mg/ml aprotinin), 0.1 mM ATP, 1 mCi (3.7×10^4 Bq) of [γ-^{32}P]ATP in a total reaction volume of 50 μl for 30 min at 25°C. Unreacted [γ-^{32}P]ATP was removed by passage through Sephadex G25 (Pharmacia) spin columns and ^{32}P-labeled G/HBF-1 analyzed by SDS–PAGE, transfer to PVDF membranes and autoradiography. Alternatively, the protein kinase reaction was performed with 5 mM ATP in the absence of [γ-^{32}P]ATP and G/HBF-1 phosphorylation analyzed by probing Western blots of the reaction products with α-G/HBF-1(P2), which reacts only with phosphorylated G/HBF-1. The effect of *in vitro* phosphorylation on the DNA-binding activity of recombinant G/HBF-1 was monitored by gel retardation assays and by Southwestern blotting following SDS–PAGE fractionation of the kinase reaction products as described above.

Phosphoamino acid analysis

Phosphoamino acid analysis and phosphopeptide mapping were performed as described by van der Geer *et al.* (1994).

Accession numbers

The DDBJ/EMBL/GenBank accession number for the G-max mRNA for G/HBF-1 described in this paper is Y10685.

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