Biol. Chem., Vol. 381, pp. 705 – 713, August 2000 ·

The Hepta-β-Glucoside Elicitor-Binding Proteins from Legumes Represent a Putative Receptor Family*

Axel Mithöfer[1,a], Judith Fliegmann[1,a], Gabriele Neuhaus-Url[2], Heinz Schwarz[3] and Jürgen Ebel[1,]**

[1] Botanisches Institut der Universität, Menzinger Str. 67, D-80638 München, Germany
[2] Friedrich Miescher-Institut, CH-4002 Basel, Switzerland
[3] Max-Planck-Institut für Entwicklungsbiologie, D-72076 Tübingen, Germany

* Dedicated to Professor Wolfgang Barz on the occasion of his 65th birthday
** Corresponding author

The ability of legumes to recognize and respond to β-glucan elicitors by synthesizing phytoalexins is consistent with the existence of a membrane-bound β-glucan-binding site. Related proteins of approximately 75 kDa and the corresponding mRNAs were detected in various species of legumes which respond to β-glucans. The cDNAs for the β-glucan-binding proteins of bean and soybean were cloned. The deduced 75-kDa proteins are predominantly hydrophilic and constitute a unique class of glucan-binding proteins with no currently recognizable functional domains. Heterologous expression of the soybean β-glucan-binding protein in tomato cells resulted in the generation of a high-affinity binding site for the elicitor-active hepta-β-glucoside conjugate (K_d = 4.5 nM). Ligand competition experiments with the recombinant binding sites demonstrated similar ligand specificities when compared with soybean. In both soybean and transgenic tomato, membrane-bound, active forms of the glucan-binding proteins coexist with immunologically detectable, soluble but inactive forms of the proteins. Reconstitution of a soluble protein fraction into lipid vesicles regained β-glucoside-binding activity but with lower affinity (K_d = 130 nM). We conclude that the β-glucan elicitor receptors of legumes are composed of the 75 kDa glucan-binding proteins as the critical components for ligand-recognition, and of an as yet unknown membrane anchor constituting the plasma membrane-associated receptor complex.

Key words: *Glycine max* / Heterologous expression / High-affinity glucan-binding site / Pathogen recognition / *Phaseolus vulgaris* / Reconstitution.

[a] These authors contributed equally to this work.

Introduction

The elicitor-receptor hypothesis may explain a key recognition event in plants which activates defense responses against pathogens (Ebel and Scheel, 1997). Elicitors are defined as signal compounds that are produced directly or indirectly by pathogens, and consist of different classes of substances (Ebel and Scheel, 1997; Ebel and Mithöfer, 1998). Although various plant defense mechanisms have been characterized in detail, recognition events occurring in plants after pathogen attack are not well understood. A number of high-affinity binding sites for purified elicitors have been characterized recently (Hahn, 1996; Ebel and Mithöfer, 1998). The properties of these sites reflect the high biological selectivity and specificity of the ligands. These binding sites are candidates for receptors. In none of the studies, however, an elicitor-binding protein has been isolated and its role as a receptor has been proven.

1,3-1,6-β-glucans of the oomycete *Phytophthora sojae*, a pathogen of soybean (*Glycine max* L.), were characterized as potent elicitors of a phytoalexin response in soybean tissues (Sharp *et al*., 1984). Membrane-associated β-glucan-binding sites were shown to exist in the same soybean tissues that were able to produce phytoalexins following β-glucan treatment (Schmidt and Ebel, 1987). Minimum structural requirements for both elicitor activity and ligand specificity were found in a defined 1,3-1,6-hepta-β-glucoside (HG) (Cosio *et al*., 1990b; Cheong and Hahn, 1991; Cheong *et al*., 1991). Furthermore, the HG represented the ligand for which the β-glucan-binding sites displayed highest affinity with an apparent dissociation constant (K_d) in the nanomolar range (Cosio *et al*., 1990b; Cheong and Hahn, 1991). The HG was also shown to possess the highest elicitor activity of all oligoglucosides tested so far. Radiolabeled derivatives of this compound were used to identify and characterize low abundance β-glucan-binding protein(s) (GBP) in soybean. The main component of the GBP was identified as a 75 kDa protein (Cosio *et al*., 1992; Mithöfer *et al*., 1996) and was recently purified to homogeneity using affinity chromatography (Mithöfer *et al*., 1996; Umemoto *et al*., 1997).

The corresponding cDNA encoding a GBP from soybean has been cloned and heterologously expressed (Umemoto *et al*., 1997). Binding activity for a β-glucan ligand with an average molecular mass of 10 kDa was detected for the recombinant proteins derived from transgenic tobacco cells and from *E. coli*. Saturation analyses were conducted only with a truncated β-glucan-binding protein using this high molecular weight ligand instead of the well-defined HG. Because the latter only partially inhibited binding of the high molecular weight ligand, the

binding affinity for the hepta-β-glucoside remained unresolved (Umemoto *et al.*, 1997). Moreover, the authors suggested the presence of a group of functionally distinguishable β-glucan-binding proteins in soybean, a hypothesis that was thought to be supported also by their observation of different cDNA sequences (Umemoto *et al.*, 1997).

The GBP used for amino acid microsequencing in the present study was isolated from microsomal membranes by using only derivatives of the HG for monitoring ligand binding during the process of its identification and purification (Mithöfer *et al.*, 1996). Therefore, the prerequisite for the isolation of the corresponding cDNA encoding the 1,3-1,6-hepta-β-glucoside-binding protein was fulfilled. In this study we describe the presence of related GBPs in plants which are responsive to β-glucan elicitors, the isolation of soybean and bean cDNAs encoding homologous β-glucan-binding proteins, the heterologous expression of the full-length soybean GBP, some unexpected features of the GBP, *i. e.* the existence of a 'soluble' fraction of the protein, and the functional reconstitution of this 'soluble' protein into lipid vesicles**.**

Results

Heterologous Expression and Functional Analysis of the 75 kDa Hepta-β-Glucoside-Binding Protein from Soybean

Microsequencing of the affinity-purified 75 kDa β-glucan-binding protein from soybean roots (Mithöfer *et al.*, 1996) established peptide sequences which facilitated the cloning of the corresponding cDNA. The deduced protein contained 668 amino acid residues including one putative transmembrane helix. The calculated molecular mass of 74.6 kDa agreed well with that of the purified GBP (Mithöfer *et al.*, 1996). The size of the corresponding transcript was estimated as 2400 bases by Northern blotting (see below) and supported the assumption that this clone represented a full-length cDNA for the GBP. The nucleotide sequence was found to be nearly identical to a cDNA for a β-glucan-elicitor binding protein from *G. max* cv. Green Homer (Umemoto *et al.*, 1997). Out of 2232 matching nucleotides five were different, resulting in two different amino acid residues in the deduced amino acid sequence.

To assure that the cloned cDNA represented the GBP from soybean, the full-length cDNA was expressed heterologously in eukaryotic cells. Expression in tomato cell lines resulted in the recovery of high-affinity binding sites for the hepta-β-glucoside. From ligand saturation studies with the radiolabeled HG conjugate (HG-APEA) in microsomal preparations of the well growing tomato cell line 11/1, a K_d value of 4.5 ± 1 nM (n = 3) was obtained (Figure 1). This value was similar to the K_d for this ligand in soybean (1–3 nM) as described previously (Cosio *et al.*, 1990b; Cheong and Hahn, 1991). The number of binding sites per mg of total membrane protein for independent transformants 3/2, 4, 11/1, and 11/5 was determined to be 225, 114, 115, and 70 fmol, respectively, similar to the value found for soybean cell suspension cultures (154 fmol/mg membrane protein). Saturable and specific binding activity for the HG ligand was detected neither in the soluble protein fraction of transgenic plant cells nor in non-transformed (Figure 1) or antisense-transformed tomato cells (lines 7/1, 14/4, 15/5, 15/6).

Although cDNA expression in baculovirus-infected insect cells (*S. frugiperda, Sf9*) produced high amounts of the 75 kDa protein, the major fraction of this protein was neither membrane-localized nor able to bind the HG. Membrane targeting of the 75 kDa protein in *Sf9* by introduction of the glycoprotein 67 membrane anchor (O'Reilly

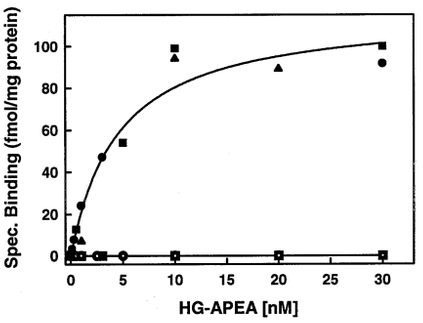

Fig. 1 Concentration Dependence of Specific HG-APEA Binding in Microsomes of Transgenic Tomato Cell Cultures Expressing the 75 kDa GBP from Soybean.
Microsomal membranes were incubated with the indicated concentrations of [^{125}I]-labeled HG-APEA. Data points show specific binding (total minus nonspecific binding). Different filled symbols denote different independent experiments with the tomato line 11/1, open symbols represent binding data from the non-transformed tomato cell line Msk8.

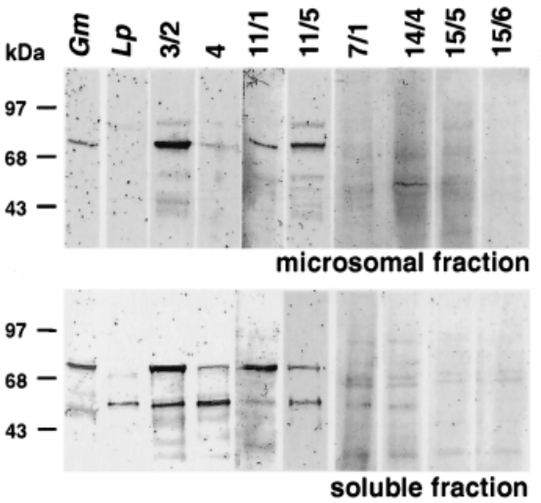

Fig. 2 Detection of Transgenic 75 kDa Protein by Western Blotting.
The 75 kDa β-glucan-binding protein was analyzed in microsomal and soluble fractions derived from soybean roots (*Gm*), tomato cell culture (*Lp*), and transgenic tomato cell cultures (sense transformants: 3/2, 4, 11/1, 11/5; antisense transformants: 7/1, 14/4, 15/5, 15/6) by Western blotting. Proteins (30 μg each) were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and probed with the anti-GBP antiserum.

et al., 1994) as a translational N-terminal fusion was not sufficient to restore binding activity, although membrane targeting was successful. Furthermore, extraction of infected insect cells using a detergent (Zwittergent 3 – 12) which was known to solubilize the soybean GBP in its active form (Cosio *et al*., 1990a; Mithöfer *et al*., 1996) was likewise insufficient to restore binding activity.

The high yield of expression of the 75 kDa protein in *Sf9* cells was exploited for the generation of a polyclonal anti-GBP antiserum. This antiserum exhibited GBP antigen specificity similar to the anti-peptide antiserum raised previously against a synthetic 15 mer internal oligopeptide sequence derived from the 75 kDa protein (residues V_{326}-Y_{340}) (Mithöfer *et al*., 1996), but was much more sensitive. Using this antiserum, the 75 kDa protein was detectable in non-purified protein fractions derived from soybean roots and transgenic tomato cell cultures (Figure 2). In addition to the microsomal fractions, soluble protein fractions contained a remarkable amount of the 75 kDa protein, which crossreacted with the anti-GBP-antiserum. This 'soluble' 75 kDa protein form did not display β-glucan-binding activity, neither in soybean nor in transgenic tomato cells.

In immunoprecipitation experiments using the antiserum combined with protein A-Sepharose, about 80% of the HG-binding activity was eliminated from crude solubilized soybean root membrane preparations. This result strongly supports the conclusion that the GBP represented the major elicitor-binding protein of the solubilized fraction initially used for protein purification (Mithöfer *et al.,* 1996).

Ligand Specificity of the Heterologously Expressed Soybean GBP

Ligand competition studies were performed to elucidate the ligand specificity of the recombinant GBP expressed in the tomato line 11/1. A variety of β-glucan fractions of increasing average degree of polymerization (DP), derived from *Phytophthora sojae* mycelial wall (1,3-1,6)-β-glucans (Cosio *et al.,* 1988, 1990b), were used as competing agents against radiolabeled HG-APEA in binding assays with microsomal fractions from soybean roots and transformed tomato cells (Table 1). The concentration of the unlabeled β-glucan that was required for half-maximal displacement of the radioligand (IC_{50}) decreased by two orders of magnitude or more in both protein fractions when apparent DP values increased from 5 to 15 – 25. The strong dependence of competition capacity on the apparent size of the competing glucan ligand fully agreed with our earlier experiments performed with the binding sites of soybean root membranes (Cosio *et al.,* 1990b: 12 μM and 100 nM, respectively). As also shown in Table 1, the recombinant GBP displayed a ten-fold lower affinity for a β-glucan fraction with a DP of 25 – 45 than the soybean GBP. For both the soybean and recombinant GBP from tomato the β-glucan fraction with a DP 25 – 45 was less well bound than that with a DP of 15 – 25. The ligand-binding characteristics of the soybean and of the recombinant GBP expressed in tomato were, thus, largely similar under the conditions tested.

Functional Reconstitution of a Non-Membrane Bound Form of the 75 kDa GBP

The detection of 'soluble' 75 kDa proteins by the antiserum in protein extracts from both soybean and transformed tomato cells that were devoid of binding activity prompted further studies on their relationship to the GBP. Using experimental conditions optimized for the reconstitution of the solubilized 75 kDa protein from soybean roots with crude soybean lipid or synthetic phospholipids (Mithöfer and Ebel, 1999), β-glucan-binding activity of

Table 1 Comparison of the Binding Affinity of Soybean Microsomes with Microsomes Prepared from the Transgenic Tomato Line 11/1 for a Variety of (1,3-1,6)-β-Glucan Fractions from *Phytophthora sojae*.

β-Glucan fraction	Soybean	Tomato 11/1
	IC_{50} (nM)	
DP = 5	6000 (n = 3)	11 000 (n = 2)
DP = 15 – 25	60 (n = 2)	70 (n = 2)
DP = 25 – 45	105 (n = 3)	1 200 (n = 2)

Binding affinity of the glucans is shown as the concentration necessary for half-maximal displacement (IC_{50}) of [^{125}I]-labeled HG-APEA from microsomal preparations in ligand competition assays.

Table 2 Reconstitution of Hepta-β-Glucoside Binding Activity of Soluble Proteins from Soybean Roots and Transgenic Tomato Cell Suspension Cultures in Lipid Vesicles after Treatment with Crude Soybean Phosphatidylcholine (PC).

Treatment	Soybean	Tomato 11/1
	HG-APEA binding activity (fmol × mg protein^{-1})	
Soluble fraction	n.d. (n = 3)	n.d. (n = 1)
Without lipid	n.d. (n = 3)	n.d. (n = 1)
Crude soybean-PC	18.2 ± 9.9 (n = 7)	10.5 ± 5.7 (n = 3)

Binding activity was analysed by determination of specific HG-APEA binding (total minus nonspecific binding). The results are the mean ± SE; n.d.: not detectable.

'soluble' 75 kDa protein derived from soybean and from transformed tomato cells was recovered in the lipid vesicles obtained with crude soybean lipid after separation from the protein extract by centrifugation at 200 000 *g* (Table 2). In contrast, soluble extracts from non-transformed tomato cells did not show lipid-dependent reactivation. In further control experiments, analyzing only crude soybean lipid in reconstitution buffer or soluble protein fractions from soybean and transgenic tomato without added lipid, no binding activity was measurable (Table 2). As the β-glucan-binding proteins did not show any specific binding in the absence of membranes or lipids, it was not possible to assess the true efficiency of reconstitution. Although the amount of reconstituted β-glucan-binding activity from soybean root extracts was rather low (18.2 fmol/mg protein) a ligand saturation experiment was performed to compare the binding affinity of reconstituted HG-binding proteins with that of microsomal and solubilized proteins. The apparent K_d value for the interaction of the reconstituted binding site with HG was estimated to be 130 nM (Figure 3), indicating a binding affinity that was

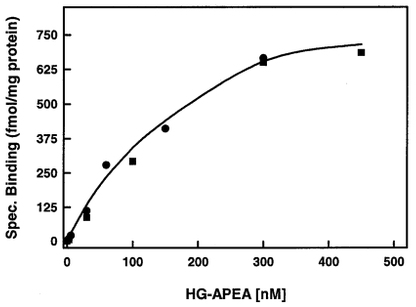

Fig. 3 Concentration Dependence of Specific [^{125}I]-Labeled HG-APEA Binding to the 'Soluble' 75 kDa Protein Reconstituted by Crude Phosphatidylcholine.
Data points show specific binding (total minus nonspecific binding). Nonspecific binding for each concentration was determined in the presence of a 1000-fold excess of unlabeled ligand. The data points represent two independent experiments indicated by different symbols, each point representing the average value of duplicate determinations.

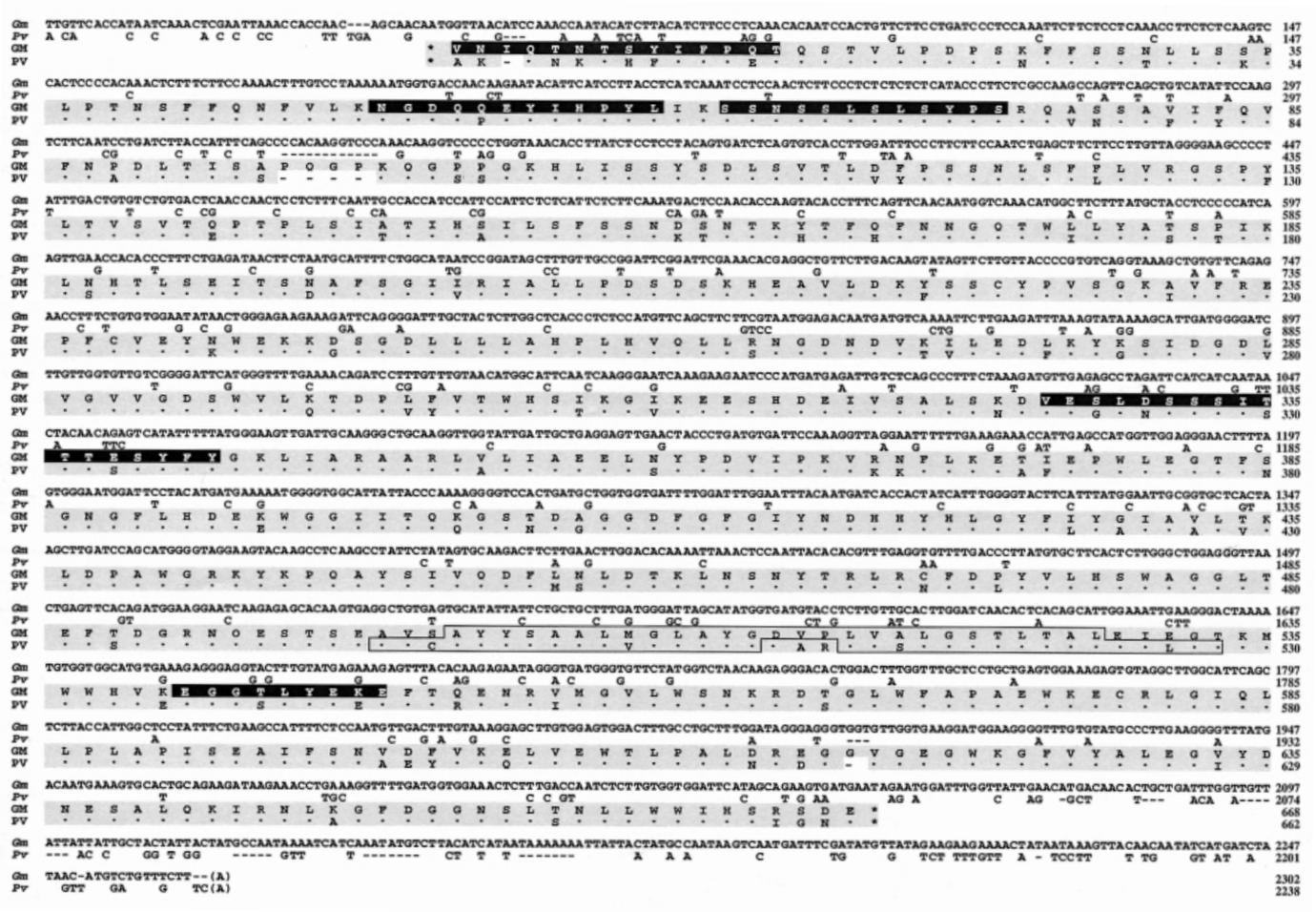

Fig. 4 Comparison of Soybean and French Bean GBP cDNA and Amino Acid Sequences.
For bean (*Phaseolus vulgaris*, *Pv*/PV), only nonidentical residues are written below the soybean (*Glycine max*, *Gm*/GM) nucleic (upper two lines) and amino acid (lower two lines, shaded in grey) sequences, respectively; identical amino acid residues are denoted by dots. Numbering of each sequence is given on the right. Microsequenced peptides from the soybean GBP are shown in black boxes and predicted integral transmembrane helices are denoted in frames. Asterisks denote start and stop codons, respectively. Gaps (shown as dashes) are introduced to maximize similarities.

around 40-times lower than that described for solubilized or microsomal soybean binding proteins (Cosio *et al.*, 1990b; Cheong and Hahn, 1991; Frey *et al.*, 1993).

Homologs of the 75 kDa GBPs in Legumes and Isolation of the GBP cDNA from French Bean

Previously we reported that several species of the legumes in addition to *G. max* showed a close correlation between high-affinity binding of β-glucan elicitors and the ability to respond to β-glucan elicitation (Cosio *et al.*, 1996). In addition, we have been able recently to isolate a putative β-glucan-binding protein from french bean (*Phaseolus vulgaris* L.) by exploiting the purification procedure developed for soybean (Mithöfer *et al.*, 1999b). A PCR-based approach was used to analyze the nucleotide sequence of the gene encoding a β-glucan-binding protein from french bean (Figure 4). Sequence comparisons revealed high similarities between the cDNAs (86% identity) and encoded proteins (85% identity plus 5% similarity) of french bean and soybean, respectively.

Northern blotting experiments with poly(A)-rich RNA from Fabaceae species used in the former study (Cosio *et al.*, 1996) and the soybean GBP cDNA as a probe detected the homologous transcript in French bean (*Phaseolus vulgaris)* (Figure 5A). A similar result was obtained with mRNA of alfalfa (*Medicago sativa)* and lupine (*Lupinus multilupa)* when higher amounts of mRNA were used (data not shown). In pea (*Pisum sativum)* and broad bean (*Vicia faba)*, no cross-hybridizing mRNA was detected. By using the internal bean GBP PCR probe homologous transcripts could be visualized for soybean, lupine, and, very faintly, for alfalfa (Figure 5A,B). The presence of homologous GBPs in other legumes was further confirmed by Western blotting analysis, showing cross-reactivity of the anti-GBP antiserum with microsomal proteins of French bean, very likely with the microsomal proteins of pea and alfalfa, and possibly with that of lupine (Figure 5C).

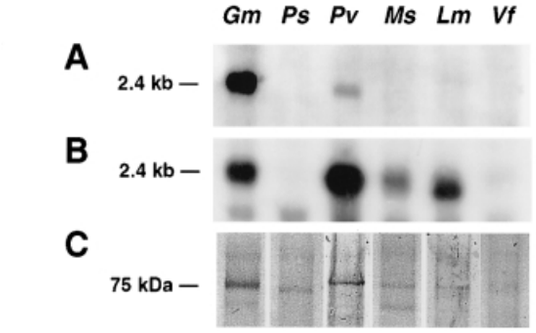

Fig. 5 Detection of GBPs in Leguminous Plants.
(A) Northern blot analysis of poly(A)$^+$ root RNA (2.7 μg) of different leguminous plants probed with the soybean GBP cDNA insert. The size of the hybridizing transcripts is shown on the left side.
(B) Northern blot analysis of the same blot as in (A), rehybridized with an internal PCR fragment of the bean GBP cDNA.
(C) Western blot analysis of microsomal fractions derived from root tissues of these plants and separated by SDS-PAGE (30 μg protein each), blotted onto nitrocellulose membranes, and probed with anti-GBP antiserum. *Gm, Glycine max; Ps, Pisum sativum; Pv, Phaseolus vulgaris; Ms, Medicago sativa; Lm, Lupinus multilupa; Vf, Vicia faba.*

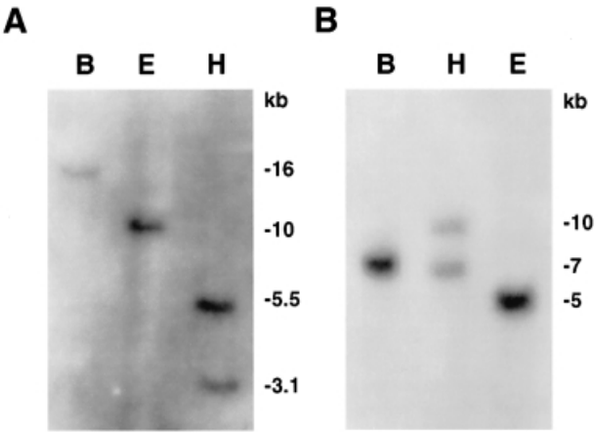

Fig. 6 Southern Blot Analysis of Soybean and Bean Genomic DNA.
Genomic DNA was restricted each with *Bam*HI (B), *Eco*RI (E), or *Hin*dIII (H). The size of the hybridizing DNA fragments is depicted on the right.
(A) DNA from soybean (20 μg per lane) was probed with the soybean GBP cDNA insert. Washing conditions used were as follows: 0.1 × SSC, 0.1% SDS, 55 °C, 1 h.
(B) DNA from French bean (50 μg per lane) was probed with the assembled coding sequence of the bean GBP cDNA. Washing conditions used were as follows: 0.5 × SSC, 0.1% SDS, 65 °C, 1 h.

Hybridization of genomic soybean and bean DNA at high stringency with full-length probes of the respective cDNAs suggested the existence of single genes for the GBP in soybean (Figure 6A) as well as in French bean (Figure 6B). For soybean and bean, respectively, one signal each was detected with *Bam*HI- and *Eco*RI-restricted DNA, whereas *Hin*dIII restriction identified two DNA species, corresponding to the presence of a *Hin*dIII recognition site within both cDNAs. The genomic 5′-fragment of the GBP gene from bean produced by restriction with *Bam*HI (position 561 of the cDNA) was not detectable with the probe used, probably because of the short overlaps.

Discussion

We have demonstrated that the recombinant protein expressed in tomato cells from the cDNA encoding the 75 kDa GBP of soybean retained the binding activity and, at least in part, the ligand specificity towards the hepta-β-glucoside, the pure synthetic ligand that was bound with highest affinity by the soybean GBP (Figure 1, Table 1). The similarity of the affinity for the HG of the recombinant protein and the soybean GBP (Cosio *et al.*, 1990b; Cheong and Hahn, 1991) supports the conclusion that the 75 kDa protein is a hepta-β-glucoside-binding protein constituting the major fraction of the HG-binding site of soybean.

The differences in the results obtained for heterologous expression in insect cells and tomato cells were quite striking. The recombinant protein in insect cells appeared

to be primarily cytosolic and did not possess elicitor-binding activity in a cell-free extract. Attemps to restore binding activity by reconstitution of the insect cell-expressed protein into lipid vesicles, exploiting the experience with the 'soluble' forms of the 75 kDa protein derived from soybean or transgenic tomato cells, or by guidance of the polypeptide to the plasma membrane of *Sf9* cells as a fusion protein with an N-terminal membrane anchor derived from the glycoprotein 67 (O'Reilly *et al*., 1994) were unsuccessful. These results indicate that insect cells are not suitable for the functional expression of the GBP.

The finding that the 75 kDa protein can be detected in 'soluble' protein fractions of soybean roots and transgenic tomato cells (Figure 2) where it is devoid of binding activity indicates that the compartmentalization may be more complex than thought earlier (Cosio *et al*., 1988). It would be of interest to learn whether the distribution observed between the membrane-associated and soluble forms of the 75 kDa protein has any biological significance. For example, we do not yet know whether the 'soluble' form represents an intermediate before assembly of the active form associated with the plasma membrane or whether it resembles an inactivated form upon release from the membrane. Conversely, the 'soluble' protein could represent an artifact generated during tissue extraction. However, the reconstitution of the 'soluble' GBP (Table 2, Figure 3) clearly demonstrates the capacity of restoring β-glucan-binding activity by incorporation of the inactive binding protein in lipid vesicles, thereby indicating the need of a lipid environment for the maintenance of activity of the β-glucan elicitor-binding proteins. We have previously shown that restoration of the binding activity of the soybean GBP was critically dependent on the lipid environment provided by the phospholipid used in the reconstitution experiments. Highest levels of reconstituted binding activity were obtained when the acyl chain length of the phospholipid was C10 and C12 (Mithöfer and Ebel, 1999).

We have now observed that reconstitution of binding activity was restricted to proteins expressed in plant cells. It remains thus far unclear whether the (re)activation of binding activity by lipids is due to a direct specific requirement for these compounds or to their effects on the binding protein conformation. Differing membrane environments may explain the differences in binding affinity as disclosed in ligand competition analyses with the soybean GBP and the GBP expressed in tomato. However, in the ligand competition analyses, the differences were rather subtle and only apparent when a β-glucan fraction with a high DP range was used.

In contrast to other membrane-associated proteins, there was neither evidence for a membrane-targeting leader peptide nor for glycosylation of the GBP in the plant systems analyzed. The latter observation was remarkable in view of the presence of seven potential glycosylation sites in the GBP sequence, which were, at least partially, functional in insect cells after targeting the protein to the ER by using the glycoprotein 67 membrane anchor (results not shown). Although little is known about protein transport into and through the plasma membrane of plant cells, these results suggest that an as yet unknown component or mechanism appears to be necessary for both membrane association and expression of binding activity, but does not work in insect cells. Moreover, oligomerization, either through self-association or through association with an additional factor, might represent an intrinsic property of the GBP (Mithöfer *et al*., 1996). Concerning self-association or complex formation with other components, it might be important to analyze whether the putative transmembrane helix (Figure 4) is critical for these suggested interactions.

Umemoto *et al*. (1997) draw a number of conclusions from their studies that differ from those in our present work. These authors were not certain whether their recombinant protein expressed from the cDNA was indeed a HG-binding protein. Reasons that might have caused ambiguity in their experiments include the use of a radioligand other than the hepta-β-glucoside, the fusion of a truncated domain of the GBP to MalE for assessing ligand binding, and the use of *E. coli* as the source for the recombinant protein used in ligand affinity and competition experiments. Further, they did not analyze subcellular localization in the two organisms, tobacco and *E. coli*, that were used for heterologous expression and its possible influence on binding activity. Umemoto *et al*. (1997) also described different possible homologs to the cDNA in soybean. We were able to detect multiple signals performing Southern blotting experiments with soybean genomic DNA at low stringency (data not shown), but we suppose a very low similarity of these crosshybridizing fragments with the gene for the GBP. The relationship of these genomic fragments with the GBP cDNA remains unknown at present.

In an earlier study, we demonstrated that in certain members of the plant family Fabaceae there was a close correlation between the existence of β-glucan-binding sites and the ability to respond to β-glucan treatment with phytoalexin production (Cosio *et al*., 1996). We now propose that the basis of the responsiveness to this glucan elicitor lies in the presence of single-copy genes encoding closely related glucan-binding proteins, as demonstrated for soybean and bean. These findings are supported by immunoblotting experiments using anti-soybean-GBP antiserum (Figure 5C).

An interesting result was the high degree of similarity between the deduced amino acid sequences for the soybean and the putative bean GBP (Figure 4), although the ligand-binding specificity and affinity of the French bean GBP was rather different in comparison to the soybean GBP (Cosio *et al*., 1996; Mithöfer *et al*., 1999b). Together with results from the hybridization experiments using both the soybean and bean cDNA probes, the data indicate that the β-glucan-based signaling components of these two species are structurally more closely related to each other than to the other legumes analyzed (Figure 5). The results are also consistent with the phylogenetic grouping of

these species as members of the tribe *Phaseolae*. Although an overall similarity in the binding activity for the HG ligand was documented earlier for several legume species (Cosio *et al*., 1996), subtle differences in the cross-reactivity with the GBP antiserum (Figure 5C) and the distinct cross-hybridization patterns as obvious in Northern blotting experiments (Figure 5A,B) suggest different levels of similarity of the GBPs. The isolation of the gene encoding the β-glucan-binding protein from bean enables us to perform structure-function analyses. This offers, for the first time, the possibility of exploring a family of membrane-bound elicitor-binding proteins in plants.

Of the candidate elicitor-binding proteins that have been identified at present (Hahn, 1996; Ebel and Mithöfer, 1998), only a few have been isolated apart from the hepta-β-glucoside-binding protein of soybean (Tang *et al*., 1996; Shibuya *et al*., 1996; Ji *et al*., 1998). An important aspect of future studies will be to verify the function of these proteins as receptors in the signal transduction cascade to activate defense responses. With the exception of Pto kinase that appears to physically interact with the bacterial AvrPto protein elicitor (Tang *et al*., 1996) and whose physiological function may be protein phosphorylation, no obvious clue as to the nature of the immediate signaling function is apparent with the other elicitor-binding proteins isolated so far (Shibuya *et al*., 1996; Ji *et al*., 1998).

The earliest β-glucan elicitor-stimulated responses analyzed to date in soybean are intracellular reactions, including the transient increase of the cytosolic Ca^{2+} level (Mithöfer *et al.,* 1999a) as well as the activation of a putative MAP-kinase cascade (Daxberger *et al.,* unpublished results). They thus require the conversion of an extracellular binding event into an intracellular response. The plasma membrane-located β-glucan receptor of soybean was shown to be represented by a 240 kDa complex (Mithöfer *et al.,* 1996) which most likely contains an as yet unidentified membrane protein in addition to the 75 kDa GBP. As the 75 kDa GBP itself does not contain any known functional domains which may be involved in signal transduction, this proposed function could be accomplished by the unknown component of this receptor complex.

Materials and Methods

Plant Material, RNA and DNA Methods, and Protein Sequencing

Plant material and growth conditions for soybean (*Glycine max* L. cv. 9007), French bean (*Phaseolus vulgaris* L. cv. Reina mora), pea (*Pisum sativum* L. cv. Lincoln), alfalfa (*Medicago sativa* L. var. Aragón), lupine (*Lupinus multilupa* L.), and broad bean (*Vicia faba* L. cv. Helda) were as described previously Cosio *et al*. (1996). Total RNA from roots was prepared according to Chang *et al*. (1993) or Schröder *et al*. (1979) and DNA according to Draper *et al*. (1988). Standard protocols were used for restriction enzyme digestions, RNA and DNA blots (Ausubel *et al*., 1987; Sambrook *et al*., 1989). Hybridization conditions were according to Fliegmann and Sandermann (1997) unless otherwise noted. Approximately 5 μg of the 75 kDa GBP from soybean roots were purified as described (Mithöfer *et al*., 1996) and derived peptides were sequenced using a gas-phase sequencer (Applied Biosystems, model 470).

cDNA Synthesis and Selection

Soybean root cDNA was synthesized using the Superscript System and cloned into λZipLox *Not*I/*Sal*I-arms (Gibco-BRL, Karlsruhe, Germany). For the first selection step, the initial cDNA was used as template for PCR amplification (annealing at 40 °C) with degenerated primers derived from two peptide sequences, forward primer corresponding to residues D_{51}-I_{56}: 5′-GAC/TCAICAIGAA/GTAC/TAT-3′; reverse primer corresponding to residues K_{548}-E_{541}: 5′-TTC/TTCA/GTANAA/GNGTNCCNCCC/TTC-3′. The PCR product (approx. 1500 base pairs) contained an open reading frame which included two more peptide sequences and was used for the second cDNA selection step. Three positive clones were recovered from a root cDNA library screen of which the largest cDNA (2302 bp) was sequenced and used further (accession number Y10257).

Bean cDNA was recovered from reverse transcription (RT) of root RNA followed by PCR amplification of GBP-related sequences. First, RT-PCR was performed with random primers and internal oligonucleotides derived from the soybean GBP cDNA (forward: 5′-CAAGTTGAACCACACCCT$_{613}$-3′, reverse: 5′-GCAGCAGAATAATATGC$_{1548}$-3′). A modified protocol for the rapid amplification of cDNA ends (RACE) was then used for the generation of overlapping cDNA fragments (Frohman *et al*., 1988; Fliegmann and Sandermann, 1997). The same set of dT-adaptor primer (*bsh-t17* and *bsh*) (Fliegmann and Sandermann, 1997) was exploited for 5′- and 3′-RACE. For 5′-RACE, a poly(A)-tail was synthesized after reverse transcription of bean RNA with Pv-GSP1 (5′-CCACTTGTACTCCACACA$_{744}$-3′). After chromatographic purification of the synthesized complementary DNA (S-400 spin column, Pharmacia, Freiburg, Germany) unidirectional amplification was performed by single use of the adaptor primer *bsh-t17* at low temperature (10 cycles with an annealing temperature of 40 °C), followed by the addition of adaptor primer *bsh* and gene-specific nested primer Pv-GSP2 (5′-TTGTCAAGAACCGCCTCGTG$_{672}$-3′) for 35 cycles with annealing at 52.5 °C. 3′-RACE was performed as described (Frohman *et al*., 1988; Fliegmann and Sandermann, 1997) with Pv-GSP3 (5′-ATGCAATTGCAGCGCTCG$_{1343}$-3′. The complete cDNA sequence composed of the three partly overlapping fragments comprised 2238 bp (accession number AF088188) and included an open reading frame which encoded 662 amino acids.

Heterologous Expression of the Soybean β-Glucan-Binding Protein

The Bac-To-Bac Baculovirus system (Gibco-BRL, Karlsruhe, Germany) was used for the expression of the soybean GBP in *Spodoptera frugiperda (Sf9)* cells (Invitrogen, Heidelberg, Germany) according to the manufacturer's instructions. Insect cells were harvested 3 days postinfection in PBS, pH 8.0.

For binding studies and Western blot analyses, we used transgenic tomato (*Lycopersicon peruvianum* L.) cell lines carrying stably integrated pDH51 (Pietrzak *et al*., 1986) derivatives. For the generation of the constructs, the GBP cDNA was inserted 3' to the CaMV 35S promoter into pDH51 either in sense or in antisense orientation, as well as the kanamycin resistance cassette from pSHI 913 (Schnorf *et al*., 1991). The plasmids were introduced to the tomato cell line Msk8 by particle bombardment. The transformants were selected by addition of 20 mg/l kanamycin to Murashige-Skoog medium (Felix *et al*., 1991) and grown at 28 °C in the dark.

Binding Studies

Two-week old transgenic tomato cell cultures or soybean roots were extracted and binding assays were performed as described previously (Cosio *et al*., 1988, 1992) with appropriate amounts of protein. All data were corrected for nonspecific binding determined in the presence of a 1000-fold excess of unlabeled β-glucan. The preparation of β-glucan fractions with various degrees of polymerization (DP) used for the ligand competition assays and the synthesis of [^{125}I]-labeled 2-(4-aminophenyl)ethylamine conjugate of HG (HG-APEA) were reported earlier (Cosio *et al.,* 1990b). Binding constants from ligand saturation experiments and values for half-maximal displacement (IC_{50}) were calculated from the data by nonlinear regression using Sigmaplot 5.0 (Jandel Scientific).

Reconstitution of β-Glucan-Binding Proteins

The supernatants obtained after preparation of microsomes (Cosio *et al*., 1988) were subjected to a further centrifugation step at 200 000 *g* for 40 min at 4 °C. The resulting supernatants containing 'soluble' 75 kDa proteins were treated for reconstitution with crude soybean phosphatidylcholine according to Mithöfer and Ebel (1999). The lipid was dissolved in 25 mM Tris/HCl, pH 8.0, containing 1 mM EDTA, 2 mM DTT, and 20% (v/v) glycerol and sonicated for 10 min before it was added to the reconstitution mixture. The solution was incubated with gentle stirring for 12 h at 4 °C. The lipid:protein ratio was 3:1. The lipid vesicles thus generated were precipitated by centrifugation at 200 000 *g*. The pellet was dissolved in 25 mM Tris/HCl, 10 mM $MgCl_2$, 100 mM NaCl, pH 8.0 and used immediately for binding analyses.

Preparation of Anti-GBP Antiserum and Western Blot Analyses

The 75 kDa soybean GBP expressed in *Sf9* cells was purified by SDS-PAGE (Laemmli, 1970) and the minced gel piece containing the protein band was injected in a rabbit to raise the 75 kDa protein-specific antiserum. Preimmune serum was collected before the first immunization was done. Immunoblotting experiments were performed as described (Mithöfer *et al*., 1996) using a 1:10 000 dilution of the anti-GBP antiserum.

Acknowledgements

We are indebted to Dr. F. Lottspeich for performing protein sequencing. We thank Dr. A. Werenskiold and M. Löwel for the introduction into insect cell culture methodology, Dr. T. Boller for continuous support, I. Arlt and E. Kock for expert technical assistance, D. Becker for experimental assistance, and Dr. J. Shiozawa and E. Schroeder-Reiter for critical comments. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 369) and Fonds der Chemischen Industrie.

References

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1987). Current Protocols in Molecular Biology (New York, USA: John Wiley and Sons).

Chang, S., Puryear, J., and Cairney, J. (1993). A simple and efficient method for isolating RNA from pine trees. Plant Mol. Biol. Rep. *11*, 113 – 116.

Cheong, J.-J., and Hahn, M.G. (1991). A specific, high affinity binding site for the hepta-β-glucoside elicitor exists in soybean membranes. Plant Cell *3*, 137 – 147.

Cheong, J.-J., Birberg, W., Fügedi, P., Pilotti, A., Garegg, P.J., Hong, N., Ogawa, T., and Hahn, M.G. (1991). Structure-activity relationships of oligo-β-glucoside elicitors of phytoalexin accumulation in soybean. Plant Cell *3*, 127 – 136.

Cosio, E.G., Pöpperl, H., Schmidt, W.E., and Ebel, J. (1988). High-affinity binding of fungal β-glucan fragments to soybean (*Glycine max* L.) microsomal fractions and protoplasts. Eur. J. Biochem. *175*, 309 – 315.

Cosio, E.G., Frey, T., and Ebel, J. (1990a). Solubilization of soybean membrane binding sites for fungal ß-glucans that elicit phytoalexin accumulation. FEBS Lett. *264*, 235 – 238.

Cosio, E.G., Frey, T., Verduyn, R., van Boom, J., and Ebel, J. (1990b). High-affinity binding of a synthetic heptaglucoside and fungal glucan phytoalexin elicitors to soybean membranes. FEBS Lett. *271*, 223 – 226.

Cosio, E.G., Frey, T., and Ebel, J. (1992). Identification of a high-affinity binding protein for a hepta-β-glucoside phytoalexin elicitor in soybean. Eur. J. Biochem. *204*, 1115 – 1123.

Cosio, E.G., Feger, M., Miller, C.J., Antelo, L., and Ebel, J. (1996). High affinity binding of fungal β-glucan elicitors to cell membranes of species of the plant family Fabaceae. Planta *200*, 92 – 99.

Draper, J., Scott, R., Armitage, P., and Walden, R. (1988). Plant Genetic Transformation and Gene Expression (Oxford, UK: Blackwell Scientific Publications).

Ebel, J., and Mithöfer, A. (1998). Early events in the elicitation of plant defense. Planta *206*, 335 – 348.

Ebel, J., and Scheel, D. (1997). Signals in host-parasite interactions. In: The Mycota V. Plant Relationships, Part A, G.C. Caroll and P. Tudzynski, eds. (Berlin, Germany: Springer), pp. 85 – 105.

Felix, G., Grosskopf, D.G., Regenass, M., Basse, C.W., and Boller, T. (1991). Elicitor-induced ethylene biosynthesis in tomato cells. Characterization and use as a bioassay for elicitor action. Plant Physiol. *97*, 19 – 25.

Fliegmann, J., and Sandermann, H., Jr. (1997). Maize glutathione-dependent formaldehyde dehydrogenase cDNA: a novel plant gene of detoxification. Plant Mol. Biol. *34*, 843 – 854.

Frey, T., Cosio, E.G., and Ebel, J. (1993). Affinity purification and characterization of a binding protein for a hepta-β-glucoside phytoalexin elicitor in soybean. Phytochem. *32*, 543 – 550.

Frohman, M.A., Dushi, M.K., and Martin, G.R. (1988). Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA *85*, 8998 – 9002.

Hahn, M.G. (1996). Microbial elicitors and their receptors in plants. Annu. Rev. Phytopathol. *34*, 387 – 412.

Ji, C., Boyd, C., Slaymaker, D., Okinaka, Y., Takeuchi, Y., Midland, S.L., Sims, J.J., Herman, E., and Keen, N. (1998). Characterization of a 34-kDa soybean binding protein for the syringolide elicitors. Proc. Natl. Acad. Sci. USA *95*, 3306 – 3311.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680 – 685.

Mithöfer, A., and Ebel, J. (1999). Functional reconstitution of β-glucan elicitor-binding activity upon incorporation into lipid vesicles. FEBS Lett. *458*, 129 – 132.

Mithöfer, A., Lottspeich, F., and Ebel, J. (1996). One-step purification of the β-glucan elicitor-binding protein from soybean (*Glycine max* L.) roots and characterization of an anti-peptide antiserum. FEBS Lett. *381*, 203 – 207.

Mithöfer, A., Ebel, J., Bhagwat, A.A., Boller, T., and Neuhaus-Url, G. (1999a). Transgenic aequorin monitors cytosolic calcium

transients in soybean cells challenged with β-glucan or chitin elicitors. Planta *207*, 566 – 574.

Mithöfer, A., Fliegmann, J., and Ebel, J. (1999b). Isolation of a french bean (*Phaseolus vulgaris* L.) homolog to the β-glucan elicitor-binding protein of soybean (*Glycine max* L.). Biochim. Biophys. Acta *1418*, 127 – 132.

O'Reilly, D.R., Miller, L.K., and Luckow, V.A. (1994). Baculovirus Expression Vectors (New York, Oxford: Oxford University Press).

Pietrzak, M., Shillito, R.D., Hohn, T., and Potrykus, I. (1986). Expression in plants of two bacterial antibiotic resistance genes after protoplast transformation with a new plant expression vector. Nucleic Acids Res. *14*, 5857 – 5868.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd Ed. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Schmidt, W.E., and Ebel, J. (1987). Specific binding of a fungal glucan phytoalexin elicitor to membrane fractions from soybean *Glycine max*. Proc. Natl. Acad. Sci. USA *84*, 4117 – 4121.

Schnorf, M., Neuhaus-Url, G., Galli, A., Iida, S., Potrykus I., and Neuhaus, G. (1991). An improved approach for transformation of plant cells by microinjection: molecular and genetic analysis. Transgenic Res. *1*, 23 – 30.

Schröder, J., Kreuzaler, F., Schäfer, E., and Hahlbrock, K. (1979). Concomitant induction of phenylalanine ammonium-lyase and flavanone synthase mRNAs in irradiated plant cells. J. Biol. Chem. *254*, 57 – 65.

Sharp, J.K., Valent, B., and Albersheim, P. (1984). Purification and partial characterization of a β-glucan fragment that elicits phytoalexin accumulation in soybean. J. Biol. Chem. *259*, 11312 – 11320.

Shibuya, N., Ito, Y., and Kaku, H. (1996). Perception of oligochitin (*N*-acetylchitooligosaccharide) elicitor signal in rice. In: Biology of Plant-Microbe Interactions, G. Stacy, B. Mullin, and P.M. Gresshoff, eds. (St. Paul, USA: International Society Molecular Plant-Microbe Interactions), pp. 83 – 88.

Tang, X., Frederick, R.D., Zhou, J., Haltermann, D.A., Jia, Y., and Martin, G.B. (1996). Initiation of plant disease resistance by physical interaction of AvrPto and Pto kinase. Science *274*, 2060 – 2063.

Umemoto, N., Kakitani, M., Iwamatsu, A., Yoshikawa, M., Yamaoka, N., and Ishida, I. (1997). The structure and function of a soybean beta-glucan-elicitor-binding protein. Proc. Natl. Acad. Sci. USA *94*, 1029 – 1034.

Received April 28, 2000; accepted June 29, 2000