An Ancient Enzyme Domain Hidden in the Putative β-Glucan Elicitor Receptor of Soybean May Play an Active Part in the Perception of Pathogen-associated Molecular Patterns during Broad Host Resistance*

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A successful defense against potential pathogens requires that a host organism is able to discriminate between self and nonself structures. Soybean (Glycine max L.) exploits a specific molecular pattern, a 1,6-β-linked and 1,3-β-branched heptaglucoside (HG), present in cell walls of the oomycetal pathogen Phytophthora sojae, as a signal compound eliciting the onset of defense reactions. The specific and high affinity HG-binding site is contained in the β-glucan-binding protein (GBP), which in turn is part of a proposed receptor complex. The ability to perceive and respond to Phytophthora cell wall-derived β-glucan elicitors is exclusive to plants that belong to the Fabaceae. However, we propose that the presence of the GBP is essential, but not sufficient for β-glucan elicitor-dependent disease resistance, because genes encoding GBP-related proteins can be retrieved from many plant species. Furthermore, we show that the GBP is composed of two different carbohydrate-active protein domains, one containing the β-glucan-binding site, and the other related to glucan endoglucosidases of fungal origin. The glucan hydrolase displays most likely an endo-specific mode of action, cleaving only 1,3-β-D-glucosidic linkages of oligoglucosides consisting of at least four moieties. Thus, the intrinsic endo-1,3-β-glucanase activity of the GBP is perfectly suited during initial contact with Phytophthora to release oligoglucoside fragments enriched in motifs that constitute ligands for the high affinity binding site present in the same protein. The concept of innate immunity in plants receives substantial support by this highly sophisticated system using ancient enzyme modules as an active part of the recognition mechanism.

Plants are exposed to a wide range of potential pathogens, such as viruses, bacteria, fungi, nematodes, and damaging insects. Only a few of these organisms actually succeed in efficiently attacking plant species, which implies that most plants are not susceptible to most putative pathogens. Effective plant-pathogen interactions display either broad host species, or even race-specific resistance phenomena based on respective perception systems. The evolution of host-pathogen interactions thus is primarily related to the evolution of recognition systems of both partners. The products of plant disease resistance (R) and corresponding avirulence (Avr) genes of the pathogens are the critical components of species or race specificity (1, 2). They appear to evolve more rapidly than other regions of the plant genomes, a phenomenon that may be based on gene duplications, intragenic and intergenic recombinations, gene conversions, and/or adaptive selection (3). In contrast, broad host resistance cannot be described with a single matching R gene/Avr gene pair, and thus is genetically much less defined.

The concept of broad host resistance is better described from a biochemical point of view, for example, during colonization of plants by pathogens that requires the mutual identification of extracellular structures and/or compounds. Substantial progress was made in characterizing structures, called elicitors, which are perceived by plants during the infection process (4–7). Elicitors are believed to interact with host plant receptors with both high specificity and sensitivity. The elicitor receptors, in turn, transduce the elicitor signal into cellular reactions that result in the activation of plant defense reactions (8–10).

According to a more general concept, the long-known general elicitors may be collectively termed pathogen-associated molecular patterns (PAMPs).1 PAMPs were defined as invariant structures shared by large groups of microorganisms that are essential for survival of the microbes, are absent from the host, and thus are determinants of nonself recognition as a prerequisite for innate immunity in humans and animals (11, 12). Recently, the concept of innate immunity has been broadened to insects and plants because of related putative receptors and similar signaling functions (2, 13–17).

However, the receptors responsible for the detection of PAMPs in broad host resistance of plants are mostly unknown. The receptor for flagellin, a subunit of bacterial flagella, was identified as a leucine-rich repeat-containing receptor kinase in Arabidopsis thaliana (14, 18). In contrast, the β-glucan-binding proteins (GBP) from Fabaceae, which are believed to be part of the β-glucan elicitor receptors, do not feature any of the functional domains found in other innate immunity receptors (19–22). A distinct oligo-β-glucosidic component of the cell walls of the Oomycete Phytophthora was described as the main deter-

1 The abbreviations used are: PAMP, pathogen-associated molecular pattern; ANTS, 8-aminoantiphalene-1,3,6-trisulfonic acid; Avr, avirulence; DP, degree of polymerization; GBP, glucan-binding protein; βGRP, β-glucan recognition protein; HG, hepta-β-glucoside; MES, 2-(N-morpholino)ethanesulfonic acid; OS, oligosaccharide; PACE, polysaccharide analysis using carbohydrate gel electrophoresis; PBS, phosphate-buffered saline; R, resistance; TBS, Tris-buffered saline.

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Glucan Hydrolase Activity of the β-Glucan-binding Protein

minor of broad host resistance in soybean (reviewed in Refs. 5 and 23). *Phytophthora sojae*, the causative agent of root and stem rot in soybean, interacts with its host both in a cultivar/race-specific manner, depending on corresponding *R* and *Acr* genes, as well as in a broad host resistance type. How these different mechanisms contribute to plant defense is not yet understood (24). The existence of GBP-related proteins and transcripts was observed in other species of the Fabaceae, such as *Medicago sativa*, *Lupinus mutatus*, and very likely *Pisum sativum* by Northern and Western blotting experiments (21).

These plants have been previously shown to display high affinity binding sites for the β-glucan elicitor and to respond to these compounds by inducing the production of phytoalexins (25). The β-glucan elicitors used for these studies consisted either of purified structural isomers of 1,3-β-linked fragments of the cell walls of *P. sojae* with a degree of polymerization (DP) up to 25, or of the synthetic 1,3-β-linked and 1,3,β-branched heptaglucoside (HG) (25), the oligosaccharide with the highest biological activity in eliciting phytoalexin accumulation in soybean (26). Moreover, several experimental attempts failed to detect high affinity binding sites or any biological activity of the purified β-glucan fractions or of the synthetic HG in plants outside the Fabaceae (27–29). Consequently, the ability to specifically perceive and respond to β-glucans derived from the cell walls from *Phytophthora* seems to be restricted to species belonging to the Fabaceae, although not every single species inside this group must necessarily possess this ability.

In this study, we show that despite the absence of biological activity or binding sites for β-glucans, related genes for putative GBPs exist in presumably all plant species. Moreover, we show that these proteins are very likely descendants of carbohydrate-active enzymes and that they still retain glycoside hydrolytic activity. We postulate a model of pathogen perception using ancient enzyme modules by the host for the amplification of lytic activity. We postulate a model of pathogen perception using ancient enzyme modules by the host for the amplification of lytic activity. We postulate a model of pathogen perception using ancient enzyme modules by the host for the amplification of lytic activity.

EXPERIMENTAL PROCEDURES

Data Retrieval and Multiple Sequence Alignment—Similarity searches were performed by using the coding regions of the cDNAs and the encoded protein sequences of the GBPs from *Glycine max* and *Phaseolus vulgaris* using BLAST and TBLASTN, respectively (30), to different sequence data sets. Gene and expressed sequence tag sequences were translated and aligned with the GBPs using MultAlin (31). Pairwise identities were calculated using the BioEdit Sequence Alignment Editor (32). Sequence accessions from plants outside of the Fabaceae that were judged to encode GBP-related proteins (similarities of the overlapping regions were found to be ~40%) were retrieved from *A. thaliana*, *Brassica napus*, *Capsicum annuum*, *Glycyrrhiza uralensis*, *Hordeum vulgare*, *Lactuca sativa*, *Loysopersicum esculentum*, *Oryza sativa*, *Phycomyrt ella patens*, *Pinus pinaster*, *Pinus taeda*, *Sorghum bicolor*, *Triticum aestivum*, *Zea mays*, and *Zinnia elegans*. The data set containing a list of the retrieved accessions and the pairwise identities were calculated using the BioEdit Sequence Alignment Editor (32). Sequence accessions from plants outside of the Fabaceae were used, including 1 using the endogenous translational initiator codon, and the second, which was engineered to additionally express a *3′*-terminal signal peptide encoding a synthetic glycoprotein 67 (GP67) as present in the vector series pAGP67 (BD Pharmingen, Dianova). GP67 constitutes a major membrane-bound structural component of the wild type baculovirus (34). Site-directed mutagenesis was performed according to the protocol of the QuikChange site-directed mutagenesis kit (Stratagene) using the recombinant pFastBac plasmids as templates and matching oligonucleotides (forward: 5′-AAGC-GAA CACTGGACTTTGCTTGTTGATCC-3′, reverse: 5′-ACTCAGCCTGACTTTGCTTGTTGATCC-3′) spanning the region 1514 to 1548 of the GsGBP cDNA (21). The PCR introduced two sequence alterations resulting in the conversion of the two glutamic acid codons (positions 494 and 498 of the GBP) into glutamine codons.

### Enzymatic Studies—β-Glucosidase assays were conducted using p-nitrophenyl β-n-glucoside at 2.5 mg/ml as substrate in 100 mM imidazole-citrate, pH 6.0, and monitoring the liberation of p-nitrophenol at 405 nm. Endo-1,3-β-glucanase activity was assayed as described (35) with some modifications. Laminarin (Paesel) and pestulan (Calbiochem) were reduced with 25 mmol NaCNBH3 in 1 N NH4OH overnight at room temperature. Standard tests were performed in 0.1 M acetic acid/NaOH or MES/KOH, pH 5.0–6.5, at 40 °C, using 1–20 μg of protein and 100 μg/ml reduced laminarin (1,3-β-glucan) in a total volume of 250 μl. Alternative substrates were tested at 250 μg/ml. Inhibitors tested were glucano-1,5-lacton (Sigma), β-norjirimycin, β-n-glucosidase, and gluco-configured imidazole (provided by A. Vasella, ETH Zurich, Switzerland). Hydrolysis of the substrates was quantified by determining the amount of liberated reducing sugars according to Ref. 36. Additional glucan polymers used were 1,3-β-glucoooligosaccharides (Mobitec), cyclic 1,3-1,6-β-glucan from *Bradyrhizobium japonicum* (37), and β-glucan preparations derived from *P. sojae* cell walls as described (38). Briefly, mycelial cell wall-derived glucans were size-fractionated and used for gel-permeography on a calibrated Bio-Gel P-4 column. The flow-through contained polymers of at least 50 glucose residues with a mean DP of 139 (DP139). The fraction DP15–25 refers to glucan oligomers in the range of 15 to 25 glucose residues according to the calibration. Enzyme tests for analytical gel electrophoresis were incubated in 25 mM pyridine/acetate, pH 5.5, at 40 °C, for the times indicated, using 1 mg/ml laminarin, 1 mg/ml DP139 β-glucan, or 0.1 mg/ml DP15–25 β-glucan. To test the ability of cyclic 1,3-1,6-β-glucan as an inhibitor of the glucoside hydrolase, the protein extract was incubated with 0.5 mg/ml cyclic β-glucan for 30 min at 4 °C prior to the addition of laminarin (0.5 mg/ml). Protein concentrations were adjusted to 5 μg/ml (Fig. 5), 10 μg/ml (Figs. 6 and 7, A and B, lanes 15 and 16, and C), or 0.1 mg/ml (Fig. 7, lanes 15 and 16, and B). Quantamxyme (Qiogene), a recombinant 1,3-β-glucanase originating from *Cellulomonas cellulosa* (39), was used as a control at 2400 units per ml. Typically, one-tenth of the reaction products (total volume of the enzyme assays 50 μl) were analyzed after derivatization (see below).

**Carbohydrate Gel Electrophoresis—**Polysaccharide analysis using carbohydrate gel electrophoresis (PAGE) was performed as described (40). Briefly, dry glucans were treated with equal volumes of both, 0.2 mol of 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS, Molecular Probes, Mobitec), prepared in acetic acid/water (3/17, v/v), and 1 mol NaCNBH3 (in Me2SO) at 37 °C overnight. After vacuum evaporation the derivatized glucans were dissolved in 6 μl urea and applied to 8% gels separated in 17.5 or 20% (w/v) polyacrylamide gels containing 0.5% (w/v) N,N'-methylenebisacrylamide using 0.1 M Tris borate, pH 8.2.

**Polycarylamide Gel Electrophoresis and Immunoblotting—**Proteins were separated on 10% SDS-polyacrylamide gels (41) and transferred to nitrocellulose membranes (Pall). Western blot analysis was carried out using rabbit-anti-GGBP antibodies (21) diluted 1:1000 overnight after blocking in 1% (w/v) gelatin and 5% (w/v) milk powder at room temperature. Anti-rabbit anti-IgG secondary antibody, coupled with alkaline phosphatase (Sigma), was used at a dilution of 1:20,000 in TBS including 1% (w/v) milk powder.

**Immunocytochemical Analyses—**Tissue pieces of 2-day-old soybean roots were fixed with 2.5% (v/v) glutaric dialdehyde in 75 mM sodium cacodylate, 2 mM MgCl2, pH 7.0, for 2 h at 0–4 °C. After two washing steps in fixative buffer and distilled water, the tissue pieces were dehydrated with a graded acetone series. Tissue samples were then infiltrated and embedded in Spurr's low-viscosity resin (42). Sections
Comparison of specific binding and biological activity of \( \beta \)-glucan fractions in different Fabaceae species

The values for specific HG-APEA binding in microsomes (K\textsubscript{d} HG, B\textsubscript{max} HG) and microsomal binding of a radiolabeled APEA conjugate of a mixture of 1,3-1,6-\( \beta \)-glucans derived from mycelial walls of P. sojae (average degree of polymerization (DP) = 18, expressed as ligand competition with \( \beta \)-glucan, IC\textsubscript{50} OS) are summarized under “HG binding”. The column “Biological activity” refers to either the values for the concentrations of the above mentioned \( \beta \)-glucan fraction necessary to induce a half-maximal phytoalexin synthesis in these plants (EC\textsubscript{50} OS) or the ability of both a \( \beta \)-glucan fraction with a DP20–25 and HG to induce medicarpin in Medicago truncatula (27). Values are from published data as denoted or from this work.

| Fabaceae species | HG binding | K\textsubscript{d} HG | B\textsubscript{max} HG | IC\textsubscript{50} OS | Biological activity | EC\textsubscript{50} OS | Ref. |
|------------------|------------|----------------------|------------------------|-----------------------|---------------------|-----------------------|-----|
| G. max           | +          | 3.0                   | 1.2                    | 17.0                  | +                   | 17                    | 25, 38 |
| Medicago sativa  | +          | 5.3                   | 1.2                    | 28.2                  | +                   | 43                    | 25  |
| H. vulgare       | +          | 0.06                  | 0.08                   | +                     | -                   | ND                    | 27  |
| P. sativum       | +          | 4.7                   | 0.08                   | 15.9                  | +                   | 30                    | 25  |
| V. faba          | +          | ND*                   | 0.25                   | ND                    | -                   | ND                    | 25  |
| V. hisuana       | +          | 8.5                   | 0.56                   | 44                    | +                   | 1900                  | 25  |
| Cicer arietinum   | +          | ND                    | 0                      | 144                   | +                   | 1900                  | 27  |
| Lotus japonicus  | +          | 8.9                   | 0.14                   | 21.5                  | +                   | 51                    | 25  |
| P. vulgaris      | +          | 33                    | 2.8                    | 5.0                   | (--)                | 25                    |     |
| Lupinus albus    | +          | 3.7                   | 0.15                   | 25                    |                     |                       |     |
| **Note:**        |            | **ND,** not detectable. |                        |                       |                     |                       |     |

TABLE I

**Results**

**Responsiveness to \( \beta \)-Glucan Elicitors**—Plant species belonging to the family Fabaceae were able to detect surface structures of *Phytophthora* using high affinity binding sites for branched 1,3-1,6-\( \beta \)-glucans, which are the main polysaccharide components of the oomycetal cell walls (25, 27, 38). Table I summarizes the available data for both the glucan binding activity and the elicitor activity using a branched HG or a mixture of 1,3-1,6-\( \beta \)-glucans (oligosaccharide, OS) as ligand and elicitor, respectively, in different Fabaceae plants. Interestingly, plants outside of the Fabaceae showed no responsiveness to these compounds. The failure to trace binding sites for HG has been reported for *A. thaliana*, *Brassica nigra*, *Nicotiana tabacum*, *Lycopersicon peruvianum*, *Z. mays*, *T. aestivum*, *H. vulgare*, and *O. sativa* (21, 27–29). However, it is noteworthy that, like *Vicia faba*, not every member of the Fabaceae displays these capacities (25).

**\( \beta \)-Glucan-binding Proteins**—As part of the putative \( \beta \)-glucan receptor, GBPs have been isolated from soybean and bean (19–22). Receptors, by definition, must perceive the biologically active signal with high affinity and specificity, and must be able to convert it into a cellular response. Moreover, the localization of a putative receptor must facilitate the binding of its ligands, which, like the oomycete-derived cell wall fragments, may not be able to traverse the plasma membrane, and thus must be perceived at the outside of responsive cells. We therefore analyzed the localization of the soybean GBP by immuno-histochemistry (Fig. 1). Electron microscopical analyses of soybean roots with immunogold labeling showed that the 75-kDa GBP was mostly restricted to the cytoplasmic face of the cell wall (Fig. 1, b–e). Sporadically, the gold label was detectable in vesicle-like structures adjacent to the plasma membrane (Fig. 1, b and e; asterisk), as well as in the cytoplasm. The endoplasmic reticulum did not show significant labeling (Fig. 1, c and d). Comparing different tissues of longitudinal sections of roots shows that essentially all cells from rhizodermis to xylem cells are labeled as well as from the apex (Fig. 1, b and c) to the differentiation zone (Fig. 1, d and e). No labeling was observed in calyptra cells. The control assay with preimmune serum showed very little binding (Fig. 1a). The GBP therefore resides at a site of the cell to eventually come into contact with the \( \beta \)-glucan elicitor.

**GBP-related Proteins**—Data base searches were conducted for sequences that may be related to the GBP genes characterized for Fabaceae. Surprisingly, genes and expressed sequence tags encoding GBP-related proteins were identified from plants outside of the Fabaceae. Sequence accessions were derived from a moss (*Physcomitrella patens*), from gymnosperms (*Pinus* species), as well as from mono- and dicotyledonous angiosperms (for example rice, barley, maize, Arabidopsis, and tomato) (data not shown). For example, putative full-length sequences from Arabidopsis and rice have been aligned with the GBPs from soybean and French bean (Fig. 2), demonstrating the overall conservation of the proteins. However, these gene sequences contained coding capacities for amino- and carboxy-terminal extended proteins, missing in the GBPs. Interestingly, both genes did contain methionine codons at positions similar to the initiator codons of the genes from *G. max* and *P. vulgaris*. The values of pairwise identity, calculated for the regions defined by the open reading frames of the soybean and French bean GBP sequences, were shown to be rather high, which may suggest a functional constraint on the evolution of these proteins (Table II).

Recently, fungal protein accessions have been added to the protein data bases that were structurally related to the GBPs. They included predicted open reading frames from *S. cerevisiae* and *S. pombe* as well as putative 1,3-\( \beta \)-glucanases from *C. albicans* and *A. fumigatus*. A recent report established the enzymatic functions of the encoded proteins of the two open reading frames from *S. cerevisiae* as a new type of endo-1,3-\( \beta \)-glucanase (33). Significant sequence similarities were found to be restricted to the carboxyl-terminal parts of the respective proteins, including two highly conserved peptides (Fig. 2). The corresponding amino acid sequences of ScENG1p, encoded by one of the previously mentioned open reading frames, contained 12 of 24 and 13 of 21 identical amino acid residues (Fig. 2), whereas the overall similarities between the plant and the fungal proteins has been reported to be only between 20 and 25% (33). This indicated that the GBPs may be enzymatically active glucanases.

**Enzymatic Activity of the Glucan-binding Protein**—The GBP from *G. max* was produced in baculovirus-infected insect cells with and without a membrane targeting leader peptide (21). As
a control, insect cells infected with baculovirus containing the empty vector (wild type virus) were utilized. By using p-nitrophenyl β-D-glucose as substrate, an enzymatic activity corresponding to an exo-acting glucoside hydrolase was detected, albeit at a very low level. Similar glucosidase activity was present in protein fractions of insect cells after infection with both the control virus as well as with the GBP-expressing virus, and thus was not caused by the expression of the transgene (data not shown).

Following expression of the transgene, reduced laminarin (1,3-β-glucan containing 2–3% 1,6-β-linkages) but not reduced pustulan (1,6-β-glucan, 10–20% O-acetylated) was efficiently hydrolyzed. The hydrolysis of laminarin showed a broad activity peak at a pH of 5 to 6 (data not shown). The hydrolytic activity was present in soluble and microsomal fractions derived from insect cells that produced the unmodified or the membrane-targeted GBP from soybean (Fig. 3, Table III), but was absent in protein extracts derived from Sf9 cells infected with control virus. The targeting of the heterologously synthesized GBP was not confined solely to either the microsomal or the soluble protein fraction, as shown by Western blotting analysis (Fig. 4). The microsomal fractions derived from both the membrane-tagged and the unmodified GBP displayed higher substrate (laminarin) affinity compared with soluble protein fractions (Table III). We tested the effect of β-glycosidase inhibitors on the enzymatic degradation of laminarin by the GBP. Neither glucono-1,5-lacton itself (up to 2 mM) nor structures derived from it, like D-nojirilactam, D-gluconhydroximo-1,5-lactam, and gluco-configured imidazole (all at 50 μM), showed any effect on laminarin hydrolysis.

To establish substrate acceptance, laminarioligosaccharides of different sizes were tested in enzyme assays. The analysis of the reaction products using carbohydrate gel electrophoresis (PACE, Ref. 40) supported the proposed endo-specific mechanism of hydrolysis executed by the GBP from soybean (Fig. 5). Neither laminaribiose nor laminaritriose were hydrolyzed. Oligomers of this size were the predominant products detectable after degradation of laminaripentaose (equimolar amounts of DP 2 and 3) or laminarihexaose (DP 2 to 3 in the ratio of ~2 to 1). Trace amounts of monomeric glucose were detectable in some of the assays (Fig. 5, lanes 5 and 6), which could point to some laminaritriose hydrolysis. Overall, laminaritetraose very likely represented the minimal 1,3-β-oligoglucoside that was efficiently hydrolyzed by the GBP.

To confirm further the presence of an intrinsic enzymatic activity of the GBP, variants of the protein were produced in

**Fig. 1.** Electron micrographs of ultrathin sections (50–70 nm) of 2-day-old soybean root tissue after immunogold labeling with preimmune serum (a) or anti-GBP antiserum (b–e) and detection with anti-rabbit IgG conjugated with 10 nm gold. Specific immunostaining was mostly detected on the cytoplasmic face of the cell wall (CW) (b–e, arrows) and associated in some cases with vesicle-like structures adjacent to the plasma membrane (b and e; asterisk). The endoplasmic reticulum did not show any labeling (c and d), nor did the vacuole (d and e) and other subcellular compartments. ER, endoplasmic reticulum; P, plastid.
recombinant insect cells containing amino acid alterations in the second of two conserved regions shared by the GBPs and endo-1,3-β-glucanases (Fig. 2). Two glutamic acid residues have been proposed as putative nucleophiles of the catalytically active site of the endoglucanase of A. fumigatus (43). Substituting these conserved residues for glutamine in the soybean GBP resulted in the GBP-Gln494-Gln498 variant. Western blotting experiments demonstrated the production of the mutant...
Concerning the comparison of the soybean and bean frames of the Fabaceous mutants in respect to the hydrolytic activity of the GBP (Fig. 6).

Interestingly, these Gln-Gln variants proved to be loss-of-function lanes 2 Glycine max 9:GP67/GmGBP Sf 9:GmGBP G

Representative data are shown for the soluble (duced the soluble or the membrane-targeted GBP from soybean. luments on laminarin concentration in the presence of microsomal enzymes of the GBP defines a previously unknown type of glucanase in plants. To ascertain that the 75-kDa GBP expresses the hydrolytic activity in its native form, affinity purified solubilized microsomal proteins of soybean roots were assayed for β-glucan degrading activity. Laminarin, but not pustulan, was found to be hydrolyzed by eluates from the affinity matrix, supporting the GBP-intrinsic 1,3-β-glucanase activity. However, eluates from the β-glucan matrix did not only contain the 75-kDa protein. Other, less abundant protein species with molecular masses of approximately 100 and 33 kDa were observed in slightly different experimental conditions (19, 46). Because of the similarity of kinetic data between the native and the recombinant protein (Table III) we conclude that at least the major portion of the enzyme activity in the eluate from the affinity column is based on the native GBP.

Degradation of Phytophthora Cell Wall Glucans—Diverse 1,3-β-glucans were incubated with recombinant soybean GBP. For comparison, a commercially available 1,3-β-glucanase from Cellulomonas cellulosas (Quantzyme, Ref. 39) was used as well. The analysis of the degradation products by PAGE

![Fig. 4. Western blotting analysis of the recombinant soybean GBP in Sf9 cells. Protein (5 µg each) was separated on 10% SDS-PAGE and transferred to nitrocellulose membranes. Anti-GBP antiserum was diluted 1:10,000 in TBS and detected with alkaline phosphatase-coupled anti-rabbit secondary antibody (1:20,000, in TBS + 1% milk powder). The upper blot displays soluble proteins, microsomal protein fractions are shown on the lower blot. The samples were loaded in identical order: lane 1, Sf9 x GmGBP; 2, Sf9 x GmGBP-Gln494-Gln498, 3, Sf9 x GP67/GmGBP; 4, Sf9 x GP67/GmGBP-Gln494, Gln498, 5, Sf9 x wild type virus. The position of the 75-kDa GBP is denoted by an arrow for each blot, membrane-localized GBP was detectable as multiple protein bands possibly because of post-translational modifications on putative glycosylation sites (21). Molecular weight markers are denoted on the right.](image)

![Fig. 5. Analysis of the substrate specificity of the glucan hydrolase activity of the soybean GBP by PAGE (40). Soluble GBP recovered after heterologous expression in Sf9 cells, was incubated for 60 min with laminarioligosaccharides of different sizes (lanes 5–8), the reaction products were derivatized with ANTS and separated on a 20% (w/v) polyacrylamide gel. The products were visualized by UV light (366 nm) and video documentation. As a control, a soluble protein extract derived from wild type virus-infected Sf9 cells was used (lanes 1–4). Glucans used for the assays (lanes 1–8, 400 pmol each) and as standards (lanes 9–13, 250 pmol each) were: laminariheptaose (L7, lanes 1, 5, and 9), laminaripentaose (L5, lanes 2, 6, and 10), laminarinotetraose (L4, lanes 3, 7, and 11), laminaribiose (L2, lanes 4, 8, and 12), and glucose (Glc, lane 13). The positions of the derivatized oligoglucoside standards are denoted on the right.](image)
showed distinct profiles (Fig. 7). Laminarin, the model substrate, which, in comparison to the Phytophthora glucans, contains a much lower degree of branching (2–3% as opposed to 13% in the Phytophthora glucan fraction, Ref. 47), was cleaved by the GBP after limited hydrolysis into a complete ladder of laminarioligosaccharides (Fig. 7A). Depending on the resolution capacity of the gel system used, glucans from DP2 to approximately DP15 were visible in similar amounts (Fig. 7A, lanes 7–9). After enzyme treatment for up to 1 h, little glucose was detectable (Fig. 7A, lane 12). Higher glucose amounts were produced following extended hydrolysis overnight (Fig. 7A, lane 16). Quantzyme released predominantly laminarinpentaose (Fig. 7A, lane 13), as reported for the structurally related enzyme from Streptomyces matensis (48). Hydrolysis of Phytophthora cell wall-derived β-glucans was evident only after prolonged incubations using higher amounts of enzyme preparations, and only by use of the soybean GBP (Fig. 7B). Quantzyme was not able to liberate oligosaccharides from Phytophthora cell wall glucans (Fig. 7B, lanes 9 and 13). The main product after digestion with the recombinant GBP appeared to be laminaribiose (Fig. 7B, lanes 8 and 12), as judged by comparison with the oligosaccharide standards. Accordingly, the saturating hydrolysis with laminarin as substrate yielded laminaribiose, too, as the main end product (Fig. 7A, lane 16). In contrast to laminarin, the Phytophthora glucan was only partially degraded and a ladder of oligosaccharides with higher DPs remained visible (Fig. 7B, lanes 8 and 12). The production of glucose after prolonged enzyme incubations could be attributed to the glucosidase activity present in protein preparations of baculovirus-infected insect cells (Fig. 7, A and B). Moreover, different protein preparations exhibited different degrees of glucosidase activity (compare for example, Fig. 6, lanes 1–3 with Fig. 7C, lanes 7–9). In any case, the glucosidase activity was shown to be independent of the expressed transgene because also wild type virus-infected insect cells, as well as recombinant insect cells expressing the Gln-Gln variant of the GBP contained this activity (Fig. 6).

**DISCUSSION**

The perception of defined β-glucan cell wall fragments of the phytopathogenic oomycetes Phytophthora spp., which results in the initiation of a multitude of plant defense responses, was demonstrated in recent years exclusively for species belonging to the plant family Fabaceae (25, 27). The recognition molecule, a GBP, was isolated and the corresponding cDNA cloned from soybean and French bean (19–22). Thus, it was concluded that this specific broad host resistance phenomenon was causally linked to the occurrence of the GBPs exclusively in this plant family (21). We now realized that genes coding for GBP-related proteins exist in presumably all plant species. Similar expressed sequence tags were found in cDNA libraries derived from distinct tissues of a variety of plants (supplementary data not shown), which indicates that the encoded proteins are actually synthesized in these different tissues. There is an overall conservation of approximately 60% identical amino acid residues in the cross-taxa comparison of selected full-length sequences, as opposed to 85% identical and 5% similar positions between the GBPs from soybean and French bean (21). The authentic β-glucan-binding sites in soybean and French bean display different ligand-binding specificities: the soybean protein bound the hepta-β-glucoside ligand with higher affinity than any other β-glucan fraction, whereas the reverse situation was found for French bean (25). The binding characteristics have largely been retained by the recombinant soybean GBP (21) and thus are an intrinsic property of the encoded protein. Consequently, 10% or fewer differing residues in the GBPs can...
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be responsible for the diversity of the binding properties of the β-glucan-binding sites in soybean and French bean. The effect of 40% differing positions encoded by the genes from A. thaliana or rice on the protein function may be rather striking and thus may be consistent with the absence of high affinity binding sites for HG in these plants.

Genes encoding GBP-related proteins were not retrieved from cyanobacterial sources, or from organisms that do not belong to either plants or fungi. Accordingly, this β-glucan binding ability seems to be invented by evolutionary progenitors that developed glucan-based cell walls. Whereas Fabaceous species deployed β-glucan-binding proteins for the generation of their defense mechanisms, the function of the GBP-related proteins in non-Fabaceous species remains to be elucidated.

Negative results obtained in this and the preceding study (21) may have important implications. The soybean Gbp cDNAs have been heterologously expressed in different hosts for proof of their identity and function. The eukaryotic recipient cells were not equally suitable for this purpose. The production of the GBP in either soluble or membrane-anchored forms in both yeast (data not shown) and insect cells (Fig. 4) did not generate high affinity binding sites for HG. Only the use of plant cells allowed the more thorough characterization of the heterologously produced soybean GBP. The binding sites in tomato cells, which resulted from the expression of the full-length Gbp cDNA, displayed high affinity toward the radiolabeled hepta-β-glucoside (4.5 nm) and ligand specificity comparable with the endogenous binding sites from soybean microbes (21). However, the artificial high affinity binding site in tomato cells was not able to transduce the elicitor signal into cellular reactions, e.g. protein kinase activation or induction of ethylene production (data not shown). Preliminary results obtained after heterologous expression of the soybean GBP together with the luminescent calcium indicator aequorin in hairy roots of Vicia hirsuta, a member of the Fabaceae, suggested that the overexpressed soybean GBP could stimulate a transient and fast elevation of the intracellular calcium concentration above the control levels in this tissue after elicitation with HG. This situation is reminiscent of the restricted cross-taxa function of certain R genes as observed in earlier studies (49). Whereas R genes from one Solanaceae species have been shown to function in other species of the same family, no successful transfers outside of a single plant family have been reported (Ref. 49 and references therein). The failure of the cross-taxa function in all these systems may be because of missing components of the putative receptor complexes or other essential signaling components that may not be conserved between different taxa (49). Thus, we postulate that an unknown factor, which is not identical with the GBP itself, is responsible for the exclusive ability of Fabaceous species to respond to defined β-glucan structures. Moreover, the existence of GBPs seems to be essential but not sufficient for a functional β-glucan receptor because the data base searches revealed the existence of GBP-related proteins also in those plants that are unable to specifically perceive and respond to β-glucan oligomers derived from the cell walls from Phytophthora.

In contrast to the purely hypothetical additional factor postulated above, the essential recognition molecule of the Fabaceous broad host defense mechanism itself, the GBP, has been characterized thoroughly at the biochemical and the molecular level (9, 19–23). The discovery of an additional function of the GBP, namely glucoside hydrolase activity, presents intriguing aspects of evolution and function of broad host resistance. The presumed catalytically active site is located in the carboxy-terminal part of the protein that displays low but significant similarity to fungal endo-1,3-β-glucanases (Fig. 2) (33). This annotation was confirmed by displacement of the carboxyl-containing side chains from two glutamate residues in one of the two conserved regions between the endoglucanases and the plant GBPs that abolished the hydrolytic activity of the soybean GBP. However, the β-glucan binding site is very likely not identical with the hydrolytically active site as suggested recently (33). A suppressor of β-glucan-induced defense reactions in soybean, a bradyrhizobial cyclic 1,3–1,6-β-glucan, was previously shown to bind to the GBP by ligand competition assays (50). Using heterologously produced soybean GBP we showed on the one hand that the cyclic β-glucan was not hydrolyzed and on the other hand that it did not inhibit the degradation of the model substrate, laminarin (Fig. 7C), indicating two different β-glucan-binding domains in the GBP.

The specificity of catalysis executed by the glucoside hydrolase of the GBP appears to be comparable with the fungal enzymes described (33, 35). The hydrolytic activity was restricted to 1,3-β-glycosidic linkages and appeared to be endo-specific because glucose was not detected as a specific product (Figs. 5–7). The assignment of an endo-specific mode of action might also be supported by the inability of the inhibitors to interact with laminarin hydrolysis by the enzyme and the lack of enzyme activity with p-nitrophenyl β-D-glucose as substrate. The minimal substrate hydrolyzed by the GBP was laminariotetraose, as reported for the endo-1,3-β-glucanase from A. fumigatus (35). The results may indicate that the catalytic activities remained well preserved both in the fungal and in the plant endo-1,3-β-glucanases of this type despite a low level of overall conservation of the protein structures, confirming the common classification of these enzymes in the family GH-81 of glycoside hydrolases (Ref. 45).4 However, a final conclusion can only be drawn after more thorough characterization of the enzymatic mechanism executed by the GBP.

Particular note should be taken of the subcellular localization of the soybean GBP. The high affinity HG-binding activity in soybean was reported to be exclusively membrane-localized (38, 51, 52). Accordingly, the presence of soluble GBP in soybean was surprising (21) and can now be explained by considering an additional role of the GBP during pathogen defense. This function would comprise a prime attack on the pathogen by degrading the cell walls and a concomitant release of soluble cell wall components. The GBP showed best hydrolytic activity in acidic conditions, indicative of an apoplastic localization, where the first encounter with an approaching pathogen will take place. The released cell wall fragments could be ultimately enriched in units that are recognized by the elicitor-binding site because 1,6-β-linkages will remain untouched, whereas linear 1,3-β-glucan chains will disintegrate. This mechanism might result in an amplification of the number of elicitor molecules that have been calculated to be represented by only one of 150 to 300 different heptaglucoside structures present in the Phytophthora cell walls (53). Pioneering work on plant defense already proposed this mechanism of elicitor release by an endo-1,3-β-glucanase from soybean (54, 55). More recently, a number of pathogenesis-related proteins have been shown to be glucanases or chitinases, which may play an active role during plant defense by attacking fungal cell walls. Moreover, the liberation, maturation, and destruction of chitin-derived signaling molecules represents another activity of pathogenesis-related proteins (16). In contrast to “classical” 1,3-β-glucanases (family GH-17), which partly have been classified as pathogenesis-related proteins, the GBP displays a unique capability to

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3 J. Fliegmann, unpublished data.

4 afmb.cnrs-mrs.fr/~pedro/CAZY/db.html.
use the products of the intrinsic hydrolytical activity as ligands of a disparate binding site localized in the same protein part as in the pathogen receptor.

Glucanases have also been detected in a different set of defense proteins, the \( \beta \)-glucan recognition proteins (\( \beta \)GRPs), which participate in the innate immune response in insects (56–58). Linear 1,3-\( \beta \)-glucans represent molecular patterns specific for fungi, which, after binding to the \( \beta \)GRPs, lead to the activation of insect-specific defense pathways, i.e. the prophenoloxidase pathway or the induction of antimicrobial peptide genes (57, 58). \( \beta \)GRPs and the GBP from plants share other properties also. Signal transmitter domains have not been found in either of these proteins. With the exception of the \( \beta \)GRP from Drosophila melanogaster, which was shown to be carboxyl-terminal anchored via a glycosylphosphatidylinositol modification (58), what may be the case for the \( \beta \)GRP from Bombyx mori as well (56), no obvious membrane targeting signal could be detected. Moreover, the recognition proteins from soybean, B. mori, and D. melanogaster existed in soluble as well as membrane-bound forms. However, neither the full-length recognition proteins nor the glucanase-like domains of plants and insects show any similarities of the primary structures to each other. In contrast to the glucanase domain of the insect proteins, the respective domains of the insect proteins did not retain the active site residues of their bacterial counterparts.

In summarizing these recent advances in the understanding of the perception of invading pathogens by organisms as diverse as plants and insects, it is intriguing how similarly these mechanisms have evolved. Both systems used ancient, but different carbohydrate-active enzymes as modules for the assembly of versatile defense systems. The identification of elusive components of the respective receptors will yield valuable information concerning both the mechanism and the evolution of these complex structures.

The concept of innate immunity in plants gains an important extension by the identification of the dual function of the GBP from Fabaceae. The similarity of disease resistance proteins participating in the innate immunity in mammals, insects, and plants has been stated in recent reviews (2, 13, 59, 60). However, for most of these putative receptors either the ligand is not known or the binding of the corresponding PAMP could not be demonstrated (for a recent summary, see Ref. 61). Furthermore, for the GBP from plants and the \( \beta \)GRPs from insects the essential components that enable the signal transduction are still unknown. It is therefore tempting to speculate that at least some of the orphan disease resistance proteins that contain signal transmitter domains are responsible for this part of the broad host resistance mechanism, supporting the guard hypothesis of plant-pathogen interaction (2, 62). In addition, the concept is confirmed by the conservation of common perception principles as observed in the orthologous evolution of glucanase domains into glucan-recognition receptors in insects and in plants. To our knowledge, the \( \beta \)-glucan perception of Fabaceae represents the first example for a sophisticated receptor system in plants, where the ligand is processed by an intrinsic part of the receptor complex itself resulting in the amplification and tailoring of the best fitting ligand molecules.

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