

Molecular Cloning and Characterization of Glucanase Inhibitor Proteins: Coevolution of a Counterdefense Mechanism by Plant Pathogens

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A characteristic plant response to microbial attack is the production of endo- β -1,3-glucanases, which are thought to play an important role in plant defense, either directly, through the degradation of β -1,3/1,6-glucans in the pathogen cell wall, or indirectly, by releasing oligosaccharide elicitors that induce additional plant defenses. We report the sequencing and characterization of a class of proteins, termed glucanase inhibitor proteins (GIPs), that are secreted by the oomycete *Phytophthora sojae*, a pathogen of soybean, and that specifically inhibit the endoglucanase activity of their plant host. GIPs are homologous with the trypsin class of Ser proteases but are proteolytically nonfunctional because one or more residues of the essential catalytic triad is absent. However, specific structural features are conserved that are characteristic of protein-protein interactions, suggesting a mechanism of action that has not been described previously in plant pathogen studies. We also report the identification of two soybean endoglucanases: EGaseA, which acts as a high-affinity ligand for GIP1; and EGaseB, with which GIP1 does not show any association. In vitro, GIP1 inhibits the EGaseA-mediated release of elicitor-active glucan oligosaccharides from *P. sojae* cell walls. Furthermore, GIPs and soybean endoglucanases interact in vivo during pathogenesis in soybean roots. GIPs represent a novel counterdefensive weapon used by plant pathogens to suppress a plant defense response and potentially function as important pathogenicity determinants.

INTRODUCTION

In response to continual challenge by a broad spectrum of pathogenic microorganisms, plants have evolved a diverse battery of defense responses, some of which are actively induced upon detection of the potential invader, whereas others are passive preexisting defensive measures (Paxton and Groth, 1994; Hutcheson, 1998). One such innate defense response is provided by the cell wall, a resilient and structurally heterogeneous barrier that in many cases must be compromised before colonization of the plant is possible. To accomplish this, microbial pathogens secrete a cocktail of enzymes that depolymerize polysaccharides in the plant host wall (Walton, 1994). In response, plants secrete proteins that inhibit these degradative enzymes, including polygalacturonase inhibitor proteins (Leckie et al., 1999; Stotz et

al., 2000), xylanase inhibitor proteins (McLauchlan et al., 1999), and pectin lyase inhibitor proteins (Bugbee, 1993).

Conversely, a characteristic plant defense response is the production of enzymes that degrade polysaccharides in the cell wall of the invading pathogen. These include endo- β -1,3-glucanases and chitinases that, in many cases, can be categorized as pathogenesis-related (PR) proteins, because expression often is induced upon infection (Kauffmann et al., 1987; Bowles, 1990; Linthorst, 1991; Stinzi et al., 1993). Both endo- β -1,3-glucanases and chitinases have been studied extensively, and considerable evidence supports the hypothesis that these PR proteins play a protective role through two distinct mechanisms. First, the enzymes can impair microbial growth and proliferation directly by hydrolyzing the β -1,3/1,6-glucan and chitin components of the cell walls of the pathogen, rendering the cells susceptible to lysis and possibly to other plant defense responses. Second, an indirect defensive role is suggested by the observation that specific chitin and β -1,3/1,6-glucan oligosaccharides, termed oligosaccharide elicitors or oligosaccharins, which are released from the pathogen walls by the action of chitinases and glucanases, respectively, can induce a wide range of plant defense responses (Côté and Hahn, 1994; Ebel and Cosio, 1994).

Thus, the overexpression in crop plants of enzymes that

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degrade pathogen cell walls represents an attractive strategy for improving disease resistance. However, although there are examples of this approach affording some protection against specific pathogens (Honeé, 1999), a number of reports have described transgenic plants with substantially reduced or increased levels of endoglucanase or chitinase expression but with no consequent effect on pathogen resistance (Neuhaus et al., 1991; Jongedijk et al., 1995). Although endoglucanases and chitinases have been shown to inhibit the *in vitro* growth of fungal or oomycete plant pathogens, the effect is inconsistent (Mauch et al., 1988; Sela-Buurlage et al., 1993; Joosten et al., 1995; Kim and Hwang, 1997). Furthermore, certain fungal strains that are susceptible initially to growth inhibition by endoglucanases become resistant after a few hours of exposure to the enzyme (Ludwig and Boller, 1990), suggesting an adaptive response by the pathogen. Some of the observations regarding the inconsistency of endoglucanases as inhibitors of fungal growth *in vitro* and in transgenic plants might be explained if pathogens secrete inhibitors of plant endoglucanases.

The interaction between soybean and the oomycete pathogen *Phytophthora sojae* provides an attractive, well-characterized experimental system in which to identify putative endoglucanase inhibitors. For example, inducible and constitutively expressed soybean endoglucanases have been studied in some detail (Keen and Yoshikawa, 1983; Takeuchi et al., 1990), and purified soybean endoglucanases have been shown to release glucan oligosaccharide elicitors from *P. sojae* cell walls (Yoshikawa et al., 1981; Ham et al., 1991). Moreover, glucan elicitors were first detected in the culture medium of *P. sojae* cultures (Ayers et al., 1976), an observation that led to the identification of the minimum β -1,3/1,6-oligoglucoside structure required for elicitor activity (Sharp et al., 1984).

Glucan elicitor binding proteins have been purified from soybean plasma membrane extracts (Cosio et al., 1992; Frey et al., 1993; Mithöfer et al., 1996), and the corresponding cDNA has been cloned (Umemoto et al., 1997), although conclusive proof that this gene encodes a functional glucan elicitor receptor has yet to be reported (Ebel, 1998). Furthermore, numerous reports have described downstream defense responses in soybean tissues that are induced by glucan elicitors and, in particular, the accumulation of the antimicrobial phytoalexin glyceollin. Most of the enzymes in the phytoalexin biosynthetic pathway in soybean have been characterized biochemically, and in many cases, the corresponding genes have been cloned (Ebel, 1998).

Our group recently reported the purification of a soybean endoglucanase inhibitor protein (Glucanase Inhibitor Protein1; GIP1) from *P. sojae* culture filtrates that inhibited ~45% of the endoglucanase activity in extracts of soybean seedlings (Ham et al., 1997). However, >85% of the activity was inhibited using crude *P. sojae* culture filtrate, suggesting the presence of multiple GIPs. Interestingly, GIP1 inhibited the activity of one endoglucanase isoform, EGaseA (formerly EnGL_{soy}-A; Ham et al., 1997), but it had no effect on the ac-

tivities of another soybean endoglucanase, EGaseB (formerly EnGL_{soy}-B; Ham et al., 1997), an endoglucanase from tobacco, or an α -1,3-glucanase from *P. sojae* itself (Ham et al., 1997), establishing a high degree of specificity. Thus, the possibility was addressed that GIP1 acts as a protease and degrades specific host endoglucanases. However, evidence was obtained that the formation of a stable GIP1-EGaseA complex, rather than proteolysis, is the basis of enzyme inhibition (Ham et al., 1997). To date, the nature of GIP action has remained unresolved, and the identities of GIP1 and the corresponding ligand, EGaseA, have not been determined.

In this article, we report the cloning and localization of a GIP and the identification of a GIP gene family from *P. sojae*. We demonstrate that GIPs and endoglucanases form complexes both *in vitro* and *in vivo* during pathogenesis and that a consequence of this interaction is the inhibition of glucan elicitor release from *P. sojae* cell walls. We also describe the molecular identification of both EGaseA and EGaseB and present a model in which GIPs represent a novel counterdefense mechanism used by plant pathogens to suppress a plant defense response.

RESULTS

GIP cDNA Cloning and Sequence Analysis

The mature GIP1 polypeptide was purified from the media of *P. sojae* cultures, as described by Ham et al. (1997), and the N-terminal sequence was determined by Edman degradation (5'-VMGGGTVPVGAKTYYTGLXXXAEGDTF-3'). Sequencing of peptides derived from trypsinized GIP1 by mass spectrometry identified the following amino acid sequences: 5'-DGERLK-3', 5'-FSPVK-3', 5'-LPAADGSDI-APSMSSK-3', 5'-LMGWGD-3', 5'-NGSGDADDI-3', and 5'-DVASVYA-3'. Using degenerate oligonucleotide primers designed from the internal peptide sequences underlined above, a 244-bp cDNA fragment was amplified by PCR from *P. sojae* mycelia-derived cDNA. This was used to identify a GIP1-specific sequence to be used for a second round of PCR, together with a degenerate oligonucleotide primer designed from the GIP1 N-terminal sequence underlined above, resulting in the amplification of a 529-bp GIP1 cDNA fragment.

Subsequent screening of a *P. sojae* mycelia cDNA library identified a 977-bp full-length GIP1 cDNA encoding a 257-amino acid precursor polypeptide with a predicted molecular mass of 26.5 kD and a pI of 5.9. The sequenced peptides described above showed 100% identity with the equivalent regions of the predicted sequence derived from the GIP1 cDNA. Post-translational processing to remove the first 28 amino acids, corresponding to the N-terminal signal sequence for protein secretion, was predicted to generate a mature protein with a molecular mass of 23.6 kD and a pI of

5.6. Two closely related sequences, designated *GIP2* and *GIP3*, also were identified from the library screen; these encode proteins with 62 and 67% amino acid identity, respectively, with *GIP1*. The gene designation is based on the high degree of sequence identity with *GIP1* and the conservation of predicted structural motifs (Figure 1A). The *GIP3* sequence is truncated by an estimated 380 bp at the 5' end.

Database searches indicated that GIP orthologs had not been identified previously, other than a 442-bp cDNA (EST 3-10C-HA) from a *P. sojae*-infected soybean hypocotyl EST library (Qutob et al., 2000), which corresponded to a portion of the *GIP1* sequence. However, all three GIP sequences showed sequence homology with Ser proteases, which typically share a low overall degree of amino acid sequence identity (~20 to 40%), but possessed a number of conserved sequence motifs, a similar geometric arrangement of the catalytic residues, and a common reaction mechanism (Perona and Craik, 1995).

A characteristic feature of Ser proteases is the "catalytic triad" charge relay system, comprising a Ser nucleophile, an Asp that acts as an electrophile, and a His base (Kraut, 1977). The relative linear order of these residues is diagnostic of the Ser protease class to which the protease belongs. *GIP1*, -2, and -3 show the greatest overall sequence homology with trypsin proteases of the chymotrypsin (also designated SA) clan, in which the order of the residues of the catalytic triad is His-Asp-Ser (Rawlings and Barrett, 1994). A numbering system has been adopted for amino acids of proteases in the SA clan to facilitate structural comparisons. By convention, residues are numbered according to those of bovine chymotrypsin (Schechter and Berger, 1967), and the catalytic triad is referred to as His-57, Asp-102, and Ser-195. Critically, none of the predicted GIPs has an intact catalytic triad (Figure 1A). Thus, the triad of *GIP1* is His-57→Thr-57, Asp-102→Asn-102, and Ser-195→Thr-195. The triad of *GIP2* is His-57→Met-57, Asp-102 is intact, and Ser-195→Thr-195. Although only a partial sequence of *GIP3* has been identified, its third triad position also is Ser-195→Thr-195 (Figure 1A).

Therefore, although GIPs are proteolytically inactive, several stretches of amino acids and motifs that are highly conserved among Ser proteases are present over the length of the GIPs. This is illustrated by the sequence alignment in Figure 1A, which includes sequences of the two most closely related genes identified in the databases, both of which encode trypsin-like proteins; these are from the bacterium *Saccharopolyspora erythraea* (Yamane et al., 1991) and the oomycete *Aphanomyces astaci* (Bangyeekhun et al., 2001). In addition, trypsins from three fungal pathogens of plants, *Fusarium oxysporum* (Rypniewski et al., 1993), *Cochliobolus carbonum* (Murphy and Walton, 1996), and *Phaeosphaeria nodorum* (Carlile et al., 2000), are included for comparison. *GIP1* exhibits between 19 and 28% amino acid identity with these five trypsin homologs.

Several key structural features that are diagnostic of Ser proteases and that are present in GIPs are highlighted in Figure 1A. First, an N-terminal signal sequence is present

that targets the enzyme for secretion. N-terminal sequencing of native *GIP1* confirmed the location of the predicted cleavage site. Clan SA is unique among the known Ser protease clans in that members typically are extracellular (Krem and Di Cera, 2001). Second, the GIPs possess three characteristically spaced pairs of Cys residues (Figure 1A) that are involved in the formation of disulfide bonds. Third, key functional residues are present in the S₁ specificity substrate binding pocket (Volanakis and Narayana, 1996) that is constructed from three β-strands and spans residues 189 to 195, 214 to 220, and 225 to 228 (Figure 1A). Two regions that flank the catalytic residues in the binding pocket form so-called variable surface loops 1 and 2 and are composed of residues 185 to 188 and 221 to 224 (Figure 1A), respectively. These surface loops contribute to the geometry of the Ser protease specificity pocket and act synergistically to influence selective substrate binding and catalysis (Hedstrom et al., 1992; Kim et al., 1995).

A phylogenetic analysis of the GIP sequences aligned with other SA clan Ser proteases from a number of evolutionarily diverse organisms revealed that the *P. sojae* GIPs form a distinct group (A in Figure 1B), together with the two most closely related sequences that encode the trypsin-like proteins from *S. erythraea* and *A. astaci*. The trypsin homologs from plant pathogenic fungi that were used in the alignment shown in Figure 1A, and a trypsin from the insect pathogenic fungus *Metarhizium anisopliae*, form a separate group (B in Figure 1B). Plant Ser proteases from Arabidopsis, tomato, and melon are shown as divergent group C, whereas diverse trypsin homologs from mammalian and insect species group together and exhibit a similar degree of sequence identity with each other as with the GIP sequences (20 to 40%).

DNA gel blot analysis of genomic DNA from *P. sojae*, using the full-length *GIP1* cDNA as a probe, identified a small GIP gene family with two or three cross-reacting fragments detected on membranes washed at high stringency (Figure 2B). Additional more distantly related sequences were identified on the same membranes probed under less stringent conditions (Figure 2A). Similar analyses identified homologous sequences in genomic DNA from other *Phytophthora* species, including *P. megasperma*, *P. infestans*, *P. nicotiana* (a generous gift from T. Nürnberger, University of Halle, Germany), and *P. medicaginis*. However, no related sequences were detected in the genomes of the yeast *Saccharomyces cerevisiae* or of several species of plant pathogenic fungi, including *Cladosporium fulvum*, *Colletotrichum lindemuthianum*, *Fusarium moniliforme*, *Cochliobolus sativus*, *Magnaporthe grisea*, and *Aspergillus niger* (data not shown).

Detection of GIP Proteins

The coding sequence of the mature *GIP1* polypeptide was ligated into an expression vector, and *GIP1* was expressed

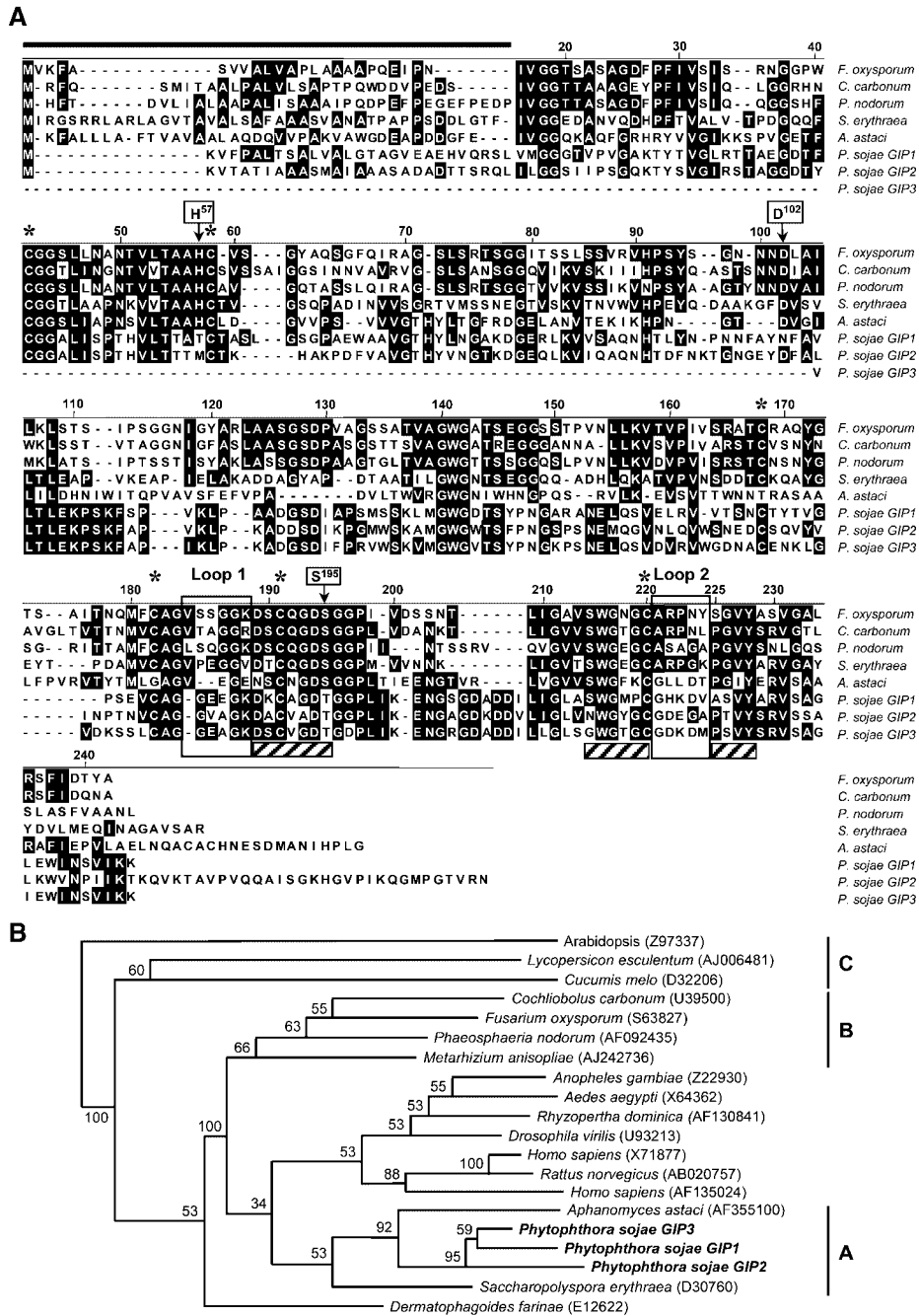


Figure 1. Sequence Alignment and Phylogenetic Analysis of GIP Genes and Ser Proteases.

(A) The *P. sojae* GIP1, GIP2, and partial-length GIP3 deduced amino acid sequences were aligned with those of Ser proteases and orthologs from fungi and bacteria using the ClustalW algorithm of DNASTAR Lasergene software. Amino acids conserved between any three sequences are indicated in reverse contrast, and residues are numbered according to the convention for Ser proteases using the consensus sequence for bovine chymotrypsin (Schechter and Berger, 1967). The N-terminal signal sequences and conserved Cys residues involved in disulfide bond formation are indicated by a horizontal bar and asterisks, respectively. The positions of the His (H⁵⁷), Asp (D¹⁰²), and Ser (S¹⁹⁵) residues of the catalytic triad are indicated with arrows. Amino acids predicted to form surface loops 1 and 2 are boxed, and residues forming the walls of the S₁ substrate binding pocket are underlined with cross-hatched boxes.

(B) The full-length GIP1, GIP2, and partial-length GIP3 deduced amino acid sequences were aligned using ClustalW within DNASTAR Lasergene software, and a phylogram was generated using PAUP. Bootstrap values are shown at the branch points. Groups A, B, and C are indicated by vertical lines, and accession numbers are shown in parentheses.

in *Escherichia coli* as a fusion protein with a poly-His tag. Substantial amounts of recombinant GIP1 protein were obtained, but it was localized in insoluble inclusion bodies, and GIP activity was not detected upon refolding and resolubilization in vitro (data not shown). Attempts to express GIP1 in *Pichia pastoris* similarly failed to yield active soluble protein. However, the recombinant protein from *E. coli* was purified and used to generate a polyclonal antiserum.

Protein extracts from the filtered cell-free extracellular media of *P. sojae* cultures were separated by two-dimensional gel electrophoresis and electroblotted to membranes, and the membranes were incubated with GIP antibodies (Figure 3A). Three major cross-reacting polypeptides were detected, with estimated molecular masses of 33 to 36 kD and pI values of 5.5 to 6.0, in accordance with the pI values predicted from the sequences of the three GIPs and the previous observation that native GIP1 migrates on a denaturing SDS-PAGE gel with an apparent molecular mass of 34 kD (Ham et al., 1997). Preimmune antiserum exhibited no cross-reactivity with a duplicate two-dimensional protein gel blot (data not shown).

Proteins extracted from soybean roots 28 h after inoculation with *P. sojae* zoospores were subjected to two-dimensional protein gel blot analysis with the GIP antibodies, and a pattern of three immunoreactive proteins, similar to that seen in Figure 3A, was detected (Figure 3B), whereas no

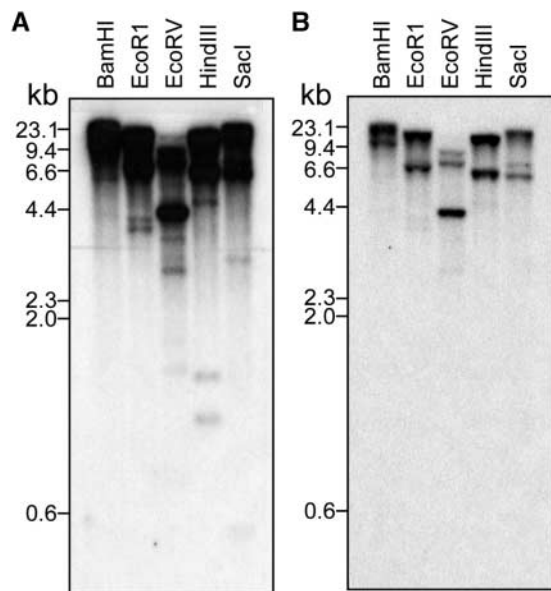


Figure 2. Genomic DNA Analysis of *GIP1*.

Genomic DNA from *P. sojae* race 1 (10 μ g per lane) was digested with the indicated restriction enzymes, and the resulting DNA gel blots were hybridized with a radiolabeled *GIP1* cDNA. The membrane was washed at low (A) and then high (B) stringency. Molecular mass markers are indicated in kilobases.

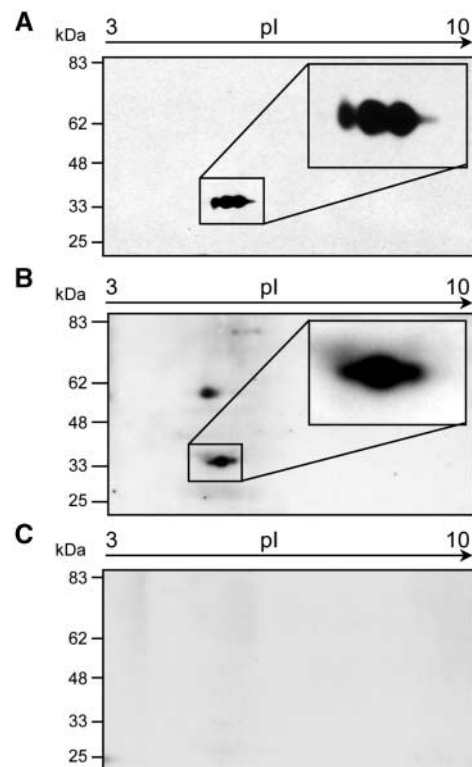


Figure 3. Two-Dimensional Protein Gel Blot Analysis of GIP Expression.

Immunoblot analysis of GIP protein expression in *P. sojae* culture filtrate (A), soybean roots inoculated with *P. sojae* zoospores (B), and uninfected soybean roots (C). Proteins were separated by two-dimensional gel electrophoresis spanning the indicated pI range and transferred to membranes, and the blots were incubated with antibodies to GIP1. Inset boxes show magnified portions of the gel highlighting the presumed GIP polypeptides. Molecular mass markers are indicated in kilodaltons.

cross-reacting proteins were detected in the extracts from uninfected roots (Figure 3C). This finding indicates that GIPs are expressed in vivo during pathogen infection. An unidentified higher molecular mass cross-reacting polypeptide was detected in the infected root sample (Figure 3B), with an apparent molecular mass of 60 kD.

The GIP antiserum was used for immunolocalization studies with culture-grown *P. sojae* mycelia (Figure 4). Pre-immune antiserum used in conjunction with fluorescent secondary antibodies showed no cross-reactivity with the mycelia (Figures 4A and 4C), whereas the GIP1 antiserum detected abundant epitopes in the mycelial cell walls (Figures 4B and 4D). No specific sites of accumulation were apparent, suggesting that GIP proteins were distributed widely over the mycelial surface. Washing the mycelia with 1 M NaCl before incubation with the GIP antibodies substantially reduced the fluorescent signal (data not shown), suggesting

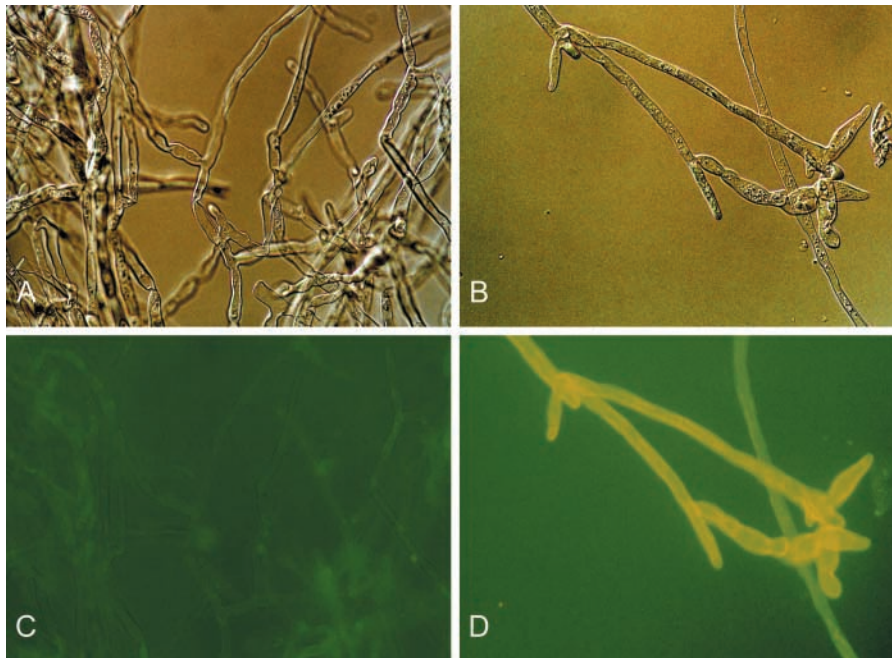


Figure 4. Immunolocalization of GIP.

Mycelia from *P. sojae* race 1 cultures were incubated with preimmune serum (**[A]** and **[C]**) or antibodies to GIP (**[B]** and **[D]**), followed by fluorescently labeled secondary antibodies, and viewed under bright-field (**[A]** and **[B]**) or dark-field (**[C]** and **[D]**) conditions.

that GIPs are, at least in part, ionically associated with the mycelial wall, in addition to being soluble in the extracellular medium.

Identification of Soybean Endo- β -1,3-Glucanases

Previous studies examined the differential inhibition by GIP1 of two soybean endo- β -1,3-glucanases, EGaseA and EGaseB (formerly EnGL_{soy}-A and EnGL_{soy}-B, respectively; Ham et al., 1997). However, the corresponding endoglucanase genes have not been identified. An important goal now is to characterize both partners in the inhibitor–ligand interaction and to assess the potential consequences of GIP action. Therefore, EGaseA and EGaseB were purified from mercuric chloride–treated soybean leaves as described previously (Ham et al., 1991, 1997). After purification, both proteins were proteolytically digested, internal peptides were sequenced by tandem mass spectrometry, and the resulting sequence tags were used to search for consensus sequences with known proteins in the databases using the Sequest algorithm (Eng et al., 1994).

EGaseA, the isozyme that is inhibited by GIP1 (Ham et al., 1997), was identified as corresponding to a previously cloned ethylene-regulated soybean endo- β -1,3-glucanase that catalyzes the release of glucan elicitors from *P. sojae* cell walls in vitro (Takeuchi et al., 1990). The tryptic peptide

sequences covered \sim 44% of the coding sequence (Figure 5A). EGaseB, the isozyme that is not inhibited by GIP1 (Ham et al., 1997), corresponded to *SGLu5*, a partial-length gene sequence identified from a soybean genomic DNA library (Jin et al., 1999). The tryptic peptide sequences covered \sim 34% of the known coding sequence (Figure 5A). For both EGaseA and EGaseB, the sequenced peptides showed 100% identity with the equivalent regions of the predicted sequences derived from the EGaseA and EGaseB cDNAs. EGaseA and EGaseB share \sim 50% deduced amino acid sequence identity, regardless of whether EGaseA is truncated to the same equivalent length as EGaseB. Homologous stretches of sequence are distributed over the whole length of the proteins, and distinct divergent regions are apparent (Figure 5A).

A phylogenetic alignment (Figure 5B) of the EGaseA and EGaseB amino acid sequences with those of three other previously described soybean endo- β -1,3-glucanases (SGLu1, SGLu7, and SGN1), an anonymous soybean glucanase, and the tobacco PR-2 endo- β -1,3-glucanase revealed that EGaseA groups together with SGN1, the corresponding gene of which has been shown to be upregulated by a number of defense-related signals (Cheong et al., 2000). In contrast, EGaseB aligns more closely with tobacco PR2. Neither EGaseB nor PR2 acts as a ligand for GIP1 (Ham et al., 1997). The predicted phylogenetic relationship of the sequences in Figure 5B is the same whether full-length se-

quences are used or all of the sequences are truncated to the size of EGaseB (data not shown).

Interaction between GIP1 and EGaseA in Vitro

The differential affinity of GIP1 for distinct soybean endo- β -1,3-glucanase isozymes was characterized using polyclonal antibodies raised against recombinant GIP1 and polyclonal antibodies to tobacco PR-2c (a generous gift from S. Kauffmann, Institut de Biologie Moléculaire des Plantes, Centre National de la Recherche Scientifique, Strasbourg, France) that cross-react with both EGaseA and EGaseB. Aliquots of native GIP1 (GIP1n) or refolded recombinant GIP1 (GIP1r) were incubated with either EGaseA or EGaseB and electrophoresed on polyacrylamide gels under nondenaturing conditions at pH 8.0. The gels then were electroblotted onto nylon membranes that were subjected to protein gel blot analysis using the GIP or endoglucanase antisera (Figure 6).

Because EGaseA and EGaseB have pI values of 8.0 and 8.3, respectively, they exhibited little or no net charge under the basic electrophoresis conditions used and did not migrate into the gels. Therefore, no corresponding bands were detected with either antiserum (Figures 6A and 6B, lanes 1 and 2). Conversely, the mature GIP1n polypeptide, which has a predicted pI of 5.6, migrated rapidly through the gel and was detected with the GIP1 antibody (Figure 6A, lane 3). GIP1r also was detected (Figure 6A, lanes 4, 6, and 8), but it migrated more slowly than GIP1n and was detected as two cross-reacting bands.

Preincubation of GIP1n with EGaseA under conditions that resulted in the elimination of detectable endoglucanase activity (data not shown) resulted in severely retarded migration of the GIP1n when electrophoresed subsequently on a nondenaturing gel (Figure 6A, lane 5). A duplicate membrane blot probed with the endoglucanase antibody showed a strong cross-reactive band at the same position (Figure 6B, lane 5). We concluded that the GIP1 migrated more slowly because it was in the form of a GIP1-EGaseA complex, in which the movement of GIP1 was slowed substantially by being bound to EGaseA, whereas the complex formation with GIP1 caused the bound EGaseA to enter the gel. Incubation of EGaseB with GIP1n or GIP1r did not result in complex formation with EGaseB (Figure 6) or inhibition of EGaseB activity (data not shown).

Surface plasmon resonance also was used to evaluate the interaction between GIP1 and EGaseA in vitro (Schuster et al., 1993). This technique has been used to measure the affinity between plant polygalacturonase inhibitor proteins and fungal polygalacturonases (Desiderio et al., 1997; Leckie et al., 1999). Purified GIP1 was used as the ligand and was immobilized on the biosensor surface, whereas purified EGaseA functioned as the analyte and was passed over the immobilized GIP1. The use of native proteins allowed any post-translational modifications that might be present in vivo, and that might play a potentially critical role in influencing

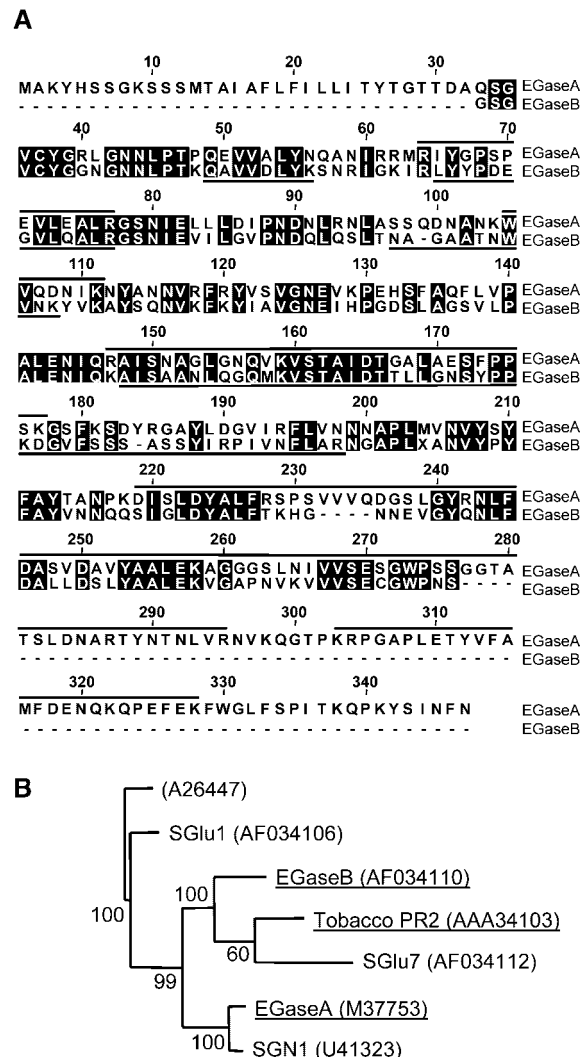


Figure 5. Sequence Alignment and Phylogenetic Analysis of Endo- β -1,3-Glucanases.

(A) The soybean EGaseA and EGaseB deduced amino acid sequences were aligned using the ClustalW algorithm of DNASTAR Lasergene software. Sequences confirmed by analysis of internal tryptic peptides are indicated by a horizontal bar above or below the alignment for EGaseA and EGaseB, respectively. Conserved residues are boxed and shaded, and residue numbers are shown.

(B) Deduced amino acid sequences derived from six partial-length soybean endo- β -1,3-glucanases and an endo- β -1,3-glucanase from tobacco were aligned using the ClustalW algorithm, and a phylogenetic tree was generated using PAUP. Bootstrap values are shown at the branch points. The genes corresponding to the proteins that have been tested as ligands for GIP1 are underlined, and accession numbers are shown in parentheses.

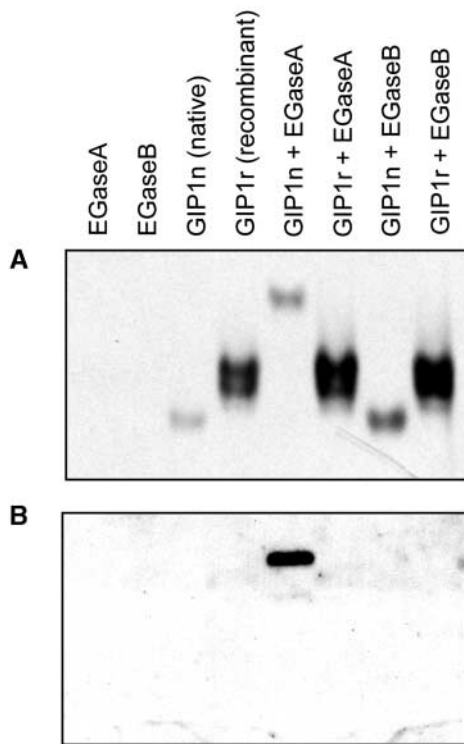


Figure 6. Protein Gel Blot Analysis of in Vitro Interactions between GIP1 and Soybean Endoglucanases.

Immunoblot analysis of purified native GIP (GIP1n), recombinant GIP1 (GIP1r), and purified soybean EGaseA and EGaseB, with or without a 1-h coincubation, separated by native gel electrophoresis, transferred to membranes, and incubated with antibodies to GIP1 (**A**) or endoglucanase (**B**).

the interaction, to be taken into account. Kinetic analyses showed that EGaseA interacted with GIP1 and bound with such high affinity that the interaction could not be reversed, even under stringent elution conditions. Although this prevented a determination of kinetic values such as the dissociation constant, the result provides further evidence for a high-affinity interaction between GIP1 and EGaseA.

Interaction between GIPs and Endo- β -1,3-Glucanases in Vivo

To examine the expression of GIPs and endoglucanases during pathogen infection, proteins were extracted from the roots of 3-day-old soybean seedlings 14 and 28 h after the roots had been dip inoculated with *P. sojae* zoospores. Proteins from infected or uninfected control roots were separated by SDS-PAGE, electroblotted to membranes, and subjected to protein gel blot analysis using the GIP and en-

doglucanase antisera described above for the in vitro binding studies. Strong immunoreactive bands of the predicted molecular masses of GIPs were detected by the GIP antiserum in extracts from infected roots by 14 h after inoculation but not in control roots (Figure 7A). The endoglucanase antibodies detected a polypeptide of \sim 32 kD in control and infected roots, whereas a second, higher molecular mass endoglucanase, with an estimated molecular mass of \sim 35 kD, was induced upon infection (Figure 7B). These values correspond to the previously observed molecular masses for EGaseA and EGaseB, respectively (Ham et al., 1997).

The protein extracts from infected and control roots also were subjected to native gel electrophoresis under basic

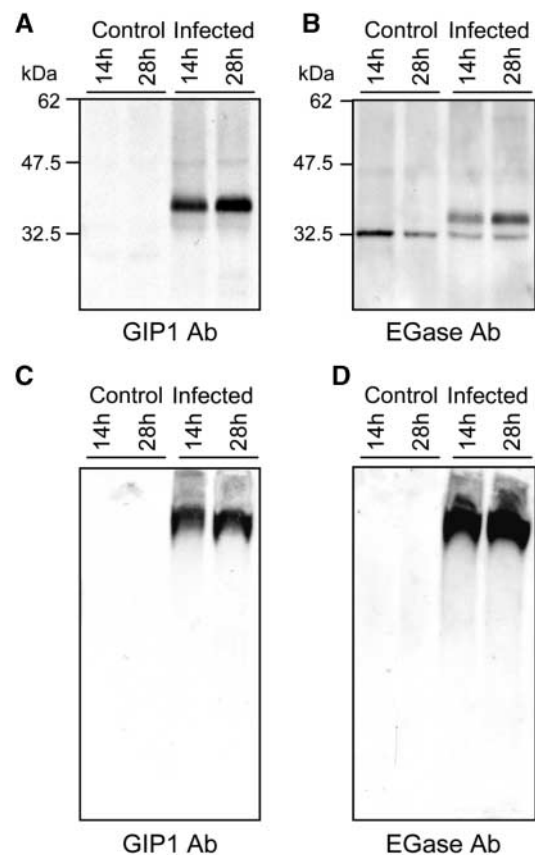


Figure 7. Denaturing and Nondenaturing Protein Gel Blot Analyses of GIP1 and Endoglucanase Expression in Soybean Roots Infected with *P. sojae*.

Immunoblot analysis of proteins extracted from uninfected soybean roots or roots at 14 or 28 h after inoculation with *P. sojae* zoospores. Proteins were separated by denaturing (**A**) and (**B**) and native (**C**) and (**D**) SDS-PAGE, transferred to membranes, and incubated with antibodies (Ab) raised to GIP1 (**A**) and (**C**) or endoglucanase (**B**) and (**D**). Molecular mass markers are indicated in kilodaltons.

conditions, as shown in Figure 6, and protein gel blot analyses were performed with the GIP1 and endoglucanase antisera (Figures 7C and 7D). No immunoreactive proteins were present in uninfected root extracts, whereas strong comigrating cross-reactive bands were detected in the infected root extracts by both the GIP and endoglucanase antisera. This finding indicates that GIPs and endoglucanases form interacting complexes *in vivo* during *P. sojae* infection.

Suppression of Glucan Elicitor-Mediated Defense Responses by GIP1

Two observations suggest that GIPs might suppress the endoglucanase-catalyzed release of glucan elicitors from *Phytophthora* cell walls during pathogen infection. First, EGaseA, the ligand of GIP1, was identified originally by its ability to release elicitors from *P. sojae* cell walls (Takeuchi et al., 1990). Second, application of EGaseA directly onto *P. sojae* (Yoshikawa et al., 1990; data not shown) had no apparent effect on hyphal growth or cell viability, but inclusion of EGaseA with *P. sojae* zoospores in soybean inoculation experiments improved disease resistance in the plant.

To determine whether GIP1 suppresses the release of defense-inducing elicitors, cell walls were extracted from *P. sojae* mycelia and incubated with buffer, native GIP1, purified EGaseA, or GIP1 that had been preincubated with EGaseA. The samples then were boiled and centrifuged, and the supernatant was filtered to remove insoluble wall material. Aliquots of the solubilized extract then were shaken for 24 h with suspension-cultured soybean cells. After treatment, the soybean cells were filtered and the intracellular proteins were extracted. Proteins also were isolated from the cell-free extracellular media.

These two protein extracts were assayed for Phe ammonia-lyase (PAL) and endo- β -1,3-glucanase activities, respectively (Figure 8). Basal levels of intracellular PAL activity and extracellular endoglucanase activity were detected in the extracts from suspension-cultured cells treated with the control enzyme-free wall incubations (Figures 8A and 8B, lane 1) and the GIP1-treated wall extracts (Figures 8A and 8B, lane 2). However, both activities were induced substantially by the EGaseA-treated wall extracts (Figures 8A and 8B, lane 3). Preincubation of EGaseA with GIP1 eliminated this induction (Figures 8A and 8B, lane 4), because activity levels from these cell extracts were not significantly different from those of the controls.

DISCUSSION

GIPs Are Ser Protease Orthologs

Database searches revealed GIPs to be Ser protease orthologs belonging to the chymotrypsin clan, and the two

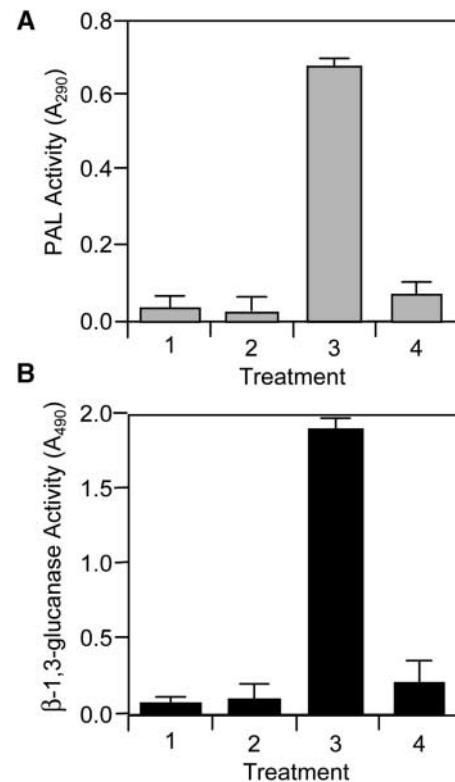


Figure 8. Suppression of EGaseA-Mediated Release of Oligoglucoside Elicitors from *P. sojae* Cell Walls by GIP1.

PAL (A) and endo- β -1,3-glucanase (B) activity detected in protein extracts from suspension-cultured soybean cells and the extracellular culture filtrate, respectively, after the following treatments. Suspension cells were incubated for 24 h with solutions released from the cell walls of *P. sojae* race 1 after incubation with buffer (lane 1), GIP1 (lane 2), EGaseA (lane 3), and EGaseA and GIP1 that had been coincubated for 1 h before application to the wall extracts (lane 4).

most closely related sequences were identified as bacterial and oomycete trypsins (Figure 1). Ser proteases are among the most studied enzymes at the structural and biochemical levels, and the resolution of multiple crystal structures has established that, despite considerable variability at the primary sequence level, they have similar basic structures (Greer, 1990). Extensive analyses have identified critical conserved features of these enzymes and have determined their contribution to Ser protease structure and function. This information can be used to deduce the functions of the corresponding features in structural orthologs (Krem et al., 1999), such as GIPs.

In particular, four features have been identified in Ser proteases that are essential for proteolytic activity: a catalytic triad, an oxyanion binding hole, a substrate specificity pocket, and a nonspecific binding site that also associates with the substrate (Perona and Craik, 1995).

The catalytic triad is partly or entirely absent in GIP1, -2, and -3, so although other characteristics of proteases are conserved, such as high-affinity binding to specific substrates, proteolysis cannot occur. This confirms the previous observation (Ham et al., 1997) and data in this study (Figure 6) that GIP1 binds to but does not fragment EGaseA.

Regions of the protein that influence substrate binding also may be predicted by homology with proteins in previous studies. The C-terminal portion of Ser proteases is believed to determine most of the functional diversity (Figure 1A), and residues 189 to 220 (chymotrypsin numbering) account for >95% of the area around the primary specificity pocket (Krem et al., 1999), whereas the two adjacent variable surface loops and Gly-216 are additional important determinants of substrate specificity and catalysis (Hedstrom et al., 1992, 1994; Perona et al., 1995). Loop 2 is believed to define the geometry of the water channel of the primary specificity pocket and is regulated fundamentally by the nature of residue 225 (Krem et al., 1999), which has been implicated with altering the substrate binding affinity by up to 5 orders of magnitude. The Asp residue at position 189 is diagnostic for the members of the trypsin family (Volanakis and Narayana, 1996), as is the GDSGG motif around position 195, although the catalytic Ser-195 is replaced by Thr in the GIPs.

In addition, GIP2 and -3 have alterations in the first and last Gly residues, respectively, of the GDSGG motif. A number of residues within the S₁ binding pocket and adjacent surface loops are divergent among the GIPs, or are conserved among the GIPs but distinct from other Ser proteases, suggesting differences in the geometry of the substrate binding surfaces. For example, at position 226, a normally conserved Gly has been replaced by a Ser or a Thr in all three GIPs. Loss of Gly-216 or Gly-226 has been shown to decrease the catalytic efficiency of Ser proteases by 40- to 10,000-fold (Perona and Craik, 1995).

Additionally, residue 225 is a conserved Pro or Tyr in >95% of known Ser proteases (Guinto et al., 1999), and although the Pro is present in GIP2 and -3, it is replaced by Ala-225 in GIP1, a unique substitution among known Ser proteases. Although Pro-225 is thought not to make contact with the substrate directly, it is believed to have a stabilizing function (Guinto et al., 1999). Regions outside of the C-terminal binding pocket also have been implicated in modulating substrate binding. For example, residue 172, which is normally Tyr in trypsin and Trp in chymotrypsin, also is an important determinant of substrate specificity (Hedstrom et al., 1994) because it occupies the base of the specificity pocket. Interestingly, Tyr-172 is present in GIP2, but it is replaced by Val-172 and Leu-172 in GIP1 and GIP3, respectively.

Members of the SA clan of Ser proteases, with which GIPs share the greatest sequence identity, perform a broad range of developmental processes. It has been demonstrated that changes in the environment around the

active site have influenced functional divergence significantly (Krem and Di Cera, 2001) and that the impetus for this process is substrate recognition (Krem et al., 1999). Ser proteases exhibit a fast rate of evolution (de Haën et al., 1975) and can be envisaged as versatile protein modules that are recruited for diverse physiological functions. Their protein binding and hydrolytic properties are thought to undergo independent evolutionary selection, and the catalytic triad has been described as an independently evolving motif that is associated with different binding sites to perform diverse functions (Iengar and Ramakrishnan, 1999).

This apparently is the case with GIPs and other catalytically inactive Ser protease homologs, which exhibit a wide range of biological functions but which share a unifying theme of high-affinity protein-protein recognition (Kurosky et al., 1980; Isackson and Bradshaw, 1984; Højrup et al., 1985; Nakamura et al., 1989; Lindsay et al., 1999; Murugasu-Oei et al., 1995; Lindsay et al., 1999; Huang et al., 2000). However, to our knowledge, GIPs represent the first example of proteolytically inactive Ser protease homologs that function as enzyme inhibitors.

***Phytophthora* Species Express Multiple GIPs**

GIPs are present as a gene family in *P. sojae* and several other *Phytophthora* species, as determined by genomic DNA gel blot analysis, and protein gel blot analysis suggests that GIP proteins are expressed in *P. infestans* cultures (data not shown). The fact that *Phytophthora* cell walls are composed of glucans but not chitin, in contrast with most fungi, may have resulted in a particularly strong evolutionary pressure to develop an endoglucanase inhibitor. GIP activity was reported originally in culture filtrates of *Colletotrichum lindemuthianum*, a fungal pathogen of plants (Albersheim and Valent, 1974), although we have not detected orthologous sequences in genomic DNA from a range of plant pathogenic fungi, including *C. lindemuthianum* (data not shown). However, Ser proteases from phylogenetically divergent organisms exhibit low sequence identity, typically 20 to 30%, so it is possible that GIP functional homologs also are expressed by fungi but are too divergent at the DNA sequence level to be detected by DNA gel blot analysis.

At least three GIP polypeptides were detected by two-dimensional protein gel blot analysis (Figure 3A) in protein extracts from *P. sojae* cultures, corresponding to the number of related genes as assessed by genomic DNA gel blot analysis (Figure 2). The same isozymes appear to be expressed during *P. sojae* infection of soybean roots (Figure 3B). Immunolocalization studies suggest that GIPs are distributed widely in the mycelial walls (Figure 4), in addition to being released into the surrounding milieu, and so are well placed to provide protection from plant host endoglucanases.

GIPs Associate with Plant Endoglucanases *In Vitro* and *In Vivo* during Pathogenesis and Suppress the Release of Oligoglucoside Elicitors

We used GIP and endoglucanase antisera to confirm that GIP1 binds specifically to EGaseA, but not EGaseB, when coincubated *in vitro* and determined that the absence of GIP activity exhibited by recombinant GIP1 was attributable to the lack of binding to EGaseA (Figure 6). This, in turn, probably was a consequence of misfolding of the protein, which had to be solubilized and refolded from inclusion bodies. GIPs are predicted to have the three pairs of disulfide bonds that are characteristic of Ser proteases, and inappropriate bond formation may have contributed to the lack of success in generating active recombinant GIP1 in *E. coli* or *P. pastoris*. Indeed, the inactive doublet that was apparent in protein gel blot analysis of recombinant GIP1 (Figure 6A) suggests that at least two aberrantly folded forms were generated.

The native GIP1 protein migrates on a denaturing gel as a protein of ~33 kD (Figure 6A) (Ham et al., 1997), whereas the predicted mass from the amino acid sequence is 23.6 kD. It has been reported that the trypsin-like protease from *S. erythraea*, the database sequence that has the greatest homology with GIP1 (Figure 1), exhibits similar anomalous migration behavior when analyzed by SDS-PAGE, because the native protein (23.3 kD as determined by mass spectrometry) has an apparent molecular mass of 35 kD (Nagamine-Natsuka et al., 1995). The basis of this inconsistency is unknown.

In addition to the *in vitro* studies, we observed that GIPs not only are expressed *in vivo* during *P. sojae* infection of soybean seedlings but also form complexes with endoglucanases in infected roots (Figure 7). The identities of the GIPs and endoglucanases that associated *in vivo* were not determined; however, the 32- and 35-kD molecular masses of the two soybean endoglucanases that were identified by denaturing SDS-PAGE (Figure 7B) correspond to the known masses of EGaseB and EGaseA, respectively (Ham et al., 1997). The higher molecular mass endoglucanase was induced in the infected roots by 14 h after inoculation, coincident with the detection of GIPs, so although it is likely that the complex shown in Figure 7D represents GIP1-EGaseA, it is possible that other GIP-endoglucanase combinations comigrate and are not resolved under the native conditions used.

To further elucidate the potential roles of GIPs, we obtained supporting evidence that GIP1 suppresses the release of elicitors from *P. sojae* cell walls by coincubation bioassays (Figure 8). The activities of PAL and endo- β -1,3-glucanase were selected because PAL is a key enzyme in the biosynthesis of phenylpropanoids, including the soybean phytoalexin glyceollin, whereas endoglucanase activity has long been associated with defense responses.

EGaseA Has Been Shown to Release Elicitor-Active Oligoglucosides from *Phytophthora* Mycelial Walls

To provide further insight into the interaction between specific pairs of GIPs and endoglucanases, and to connect the GIP-related research with previous studies of soybean endoglucanases, an important objective was to identify the genes corresponding to EGaseA and EGaseB. Sequencing of the purified proteins revealed that EGaseA was purified originally as a major endoglucanase enzyme responsible for releasing phytoalexin elicitors from *P. sojae* cell walls (Keen and Yoshikawa, 1983). Subsequent studies have shown that EGaseA can release a highly complex population of elicitors from *P. sojae*, with molecular masses ranging from several hundred to >50,000 D (Okinaka et al., 1995). The gene was cloned subsequently, and mRNA levels were shown to be upregulated by ethylene (Takeuchi et al., 1990).

Application of a solution of purified EGaseA to *P. sojae* has been reported to have no toxic effect on zoospore motility, cytospor germination, or mycelial growth (Yoshikawa et al., 1990). However, when soybean hypocotyls were inoculated with *P. sojae* zoospores, the presence of supplemental purified EGaseA in the inoculum reduced the infection rate compared with the application of zoospores alone (Yoshikawa et al., 1990). This finding suggests that EGaseA-mediated elicitor release is an important component of disease resistance.

EGaseB corresponds to a partially sequenced gene that was amplified from soybean genomic DNA as part of a study of soybean endoglucanases (Jin et al., 1999). That report identified 12 classes of soybean endoglucanases, and EGaseA was referred to as a member of class *SGlu2*, whereas EGaseB belonged to *SGlu5*. The soybean endoglucanase classes were aligned with several proposed functional classes of tobacco endoglucanases. EGaseB grouped with tobacco class II, which includes the tobacco PR endoglucanase PR2, whereas EGaseA aligned closely with tobacco class III proteins, which also are induced by pathogens and are predicted to be localized to the cell wall (Jin et al., 1999), as was suggested to be the case for EGaseA (Takeuchi et al., 1990). The results of that study agree well with the denrogram shown in Figure 5B, in which EGaseB and tobacco PR2, neither of which is inhibited by GIP1, align together and separately from EGaseA. This suggests that primary sequence homology between subclasses of endoglucanases correlates with a facility to function as a ligand for GIP1. Stretches of variable sequence apparent in the alignment shown in Figure 5A presumably correspond to regions of the endoglucanase proteins that contribute to the differential binding to divergent GIPs.

A Model of GIP Function

The intimate relationship between plants and phytopathogens has led to the coevolution of a number of complex

strategies for attack and defense. For a pathogen to colonize a host successfully, it must develop mechanisms either to evade detection or, failing that, to subvert the defense responses. Models have been proposed in which pathogen-derived effector molecules interfere with elicitor binding, signal transduction, gene activation, or the activities of the defense responses (Knogge, 1996; Staskawicz et al., 2001).

A number of structurally diverse “suppressors” have been identified that constrain active resistance in plants (Shiraishi et al., 1997). They are considered to be determinants of pathogenicity and are defined as factors that are produced at the infection site, participate in suppressing general resistance, and induce local susceptibility and that are nontoxic to plants and host specific. Certain glycoproteins and glucans have been classified as suppressors (for review, see Shiraishi et al., 1997), and although additional studies may reveal that GIPs also should be classified as suppressors, unlike GIPs, none of the currently defined suppressor molecules has been shown to inhibit the generation of elicitors as well as the activity of a downstream defense response.

A model summarizing GIP function is presented in Figure 9. During infection of soybean, *P. sojae* secretes multiple GIPs. One isoform, GIP1, binds with high affinity to the host endo- β -1,3-glucanase, EGaseA, inhibiting its hydrolytic activity. Because a major role for EGaseA, which is predicted to be localized in the plant cell wall, appears to be the release of glucan elicitors from *P. sojae* mycelia, GIP1 is depicted as inhibiting oligoglucoside elicitor release. Consequently, GIPs suppress the perception of the glucan elicitors by the putative plasma membrane receptor and the downstream signaling cascade that would result in the induction of a range of plant defenses, including endoglucanases and PAL activity. However, it is likely that other GIPs also provide a more direct protective role and suppress mycelial cell lysis by other soybean endoglucanases.

In this system, the glucan elicitors probably are one of the earliest pathogen-derived molecules that are perceived by the plant, so GIPs may play a critical role in determining the early outcome of pathogen challenge. Our future studies will address the importance of GIPs in pathogenicity through the analysis of GIP-suppressed transgenic pathogen strains. In addition, the identities and specific interactions of other GIP-endoglucanase combinations will be determined.

GIPs appear to be an effective mechanism for the inhibition of plant-derived endoglucanases, and this inhibition is based on high-affinity protein–protein interactions using a catalytically nonfunctional Ser protease domain. This system is the converse of the interaction between wall-degrading enzymes from pathogens and the corresponding inhibitor proteins that are synthesized by plants as part of the defense response, such as the well-characterized polygalacturonase–polygalacturonase inhibitor protein interaction (Leckie et al., 1999; Stotz et al., 2000). It seems likely that additional classes of proteinaceous inhibitors of glycanases and glycosidases will be identified, from both phytopathogens and their plant hosts, that interact with other categories of wall-

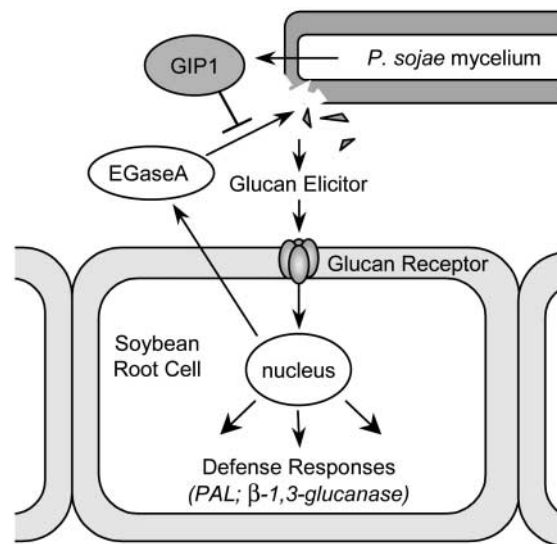


Figure 9. Model of GIP Action during the Interaction between *P. sojae* and Soybean Roots.

Key features of this model include the release of glucan elicitors from the *P. sojae* cell wall by extracellular EGaseA, binding of the elicitors to a plasma membrane–localized receptor, and the subsequent induction of a range of defense responses, including increased levels of PAL and endo- β -1,3-glucanase activities. The secretion of GIP1 by *P. sojae* during the infection of soybean roots and the subsequent high-affinity, highly selective binding of GIP1 to EGaseA reduces the release of elicitors and consequently suppresses the induction of defense responses. GIPs probably also directly protect the mycelia against endo- β -1,3-glucanase–mediated cellular lysis.

modifying proteins, thus suppressing wall disassembly and the release of wall-derived elicitors.

METHODS

Plant and Oomycete Material and Growth Conditions

Soybean (*Glycine max* cv Williams 82) seedlings were grown as described previously (Ham et al., 1997), and suspension-cultured soybean cells of the same cultivar, a generous gift from R. Dixon and F. McAlister (The Noble Foundation, Ardmore, OK), were grown as described by Guo et al. (1998). *Phytophthora sojae* (race 1) cultures were maintained as described by Ham et al. (1997).

Peptide Sequencing of GIP1, EGaseA, and EGaseB

Native GIP1 polypeptides were purified from *P. sojae* race 1 culture medium, as described by Ham et al. (1997), fractionated by SDS-

PAGE, excised from the gel, and sequenced at the Macromolecular Structure Facility at Michigan State University (East Lansing). Amino acid sequences of the N-terminal region and constituent tryptic peptides were obtained by digestion with Lys-C followed by Edman degradation sequencing.

The native soybean EGaseA and EGaseB proteins (referred to previously as EnGL_{soy}-A and EnGL_{soy}-B, respectively [Ham et al., 1997]) were purified from mercuric chloride-treated soybean leaves as described by Ham et al. (1991, 1997). After confirmation of purity by matrix-assisted laser-desorption ionization time-of-flight mass spectrometry, the proteins were sent for tryptic peptide microsequencing to the Harvard Microchemistry Facility at Harvard University (Cambridge, MA).

RNA Extraction, PCR Amplification, and cDNA Library Screening

RNA was extracted from *P. sojae* race 1 culture-grown mycelia using the hot borate protocol described by Rose et al. (1996). Total RNA was used with reverse transcriptase (Moloney murine leukemia virus; Gibco BRL) for cDNA synthesis and to generate a cDNA library in the HybriZAP 2.1 vector (Stratagene, La Jolla, CA), according to the manufacturer's instructions.

Degenerate oligonucleotide primers were designed based on GIP1 internal amino acid sequences (sense primer, 5'-GCNGAYGGNWSNGAYATHGCNCC-3'; antisense primer, 5'-CRTICGRT-CICCSIWICCRTT-3', corresponding to amino acid sequences ADGSDIA and NGS GDAD, respectively) and used to amplify a 244-bp cDNA fragment from cDNA derived from *P. sojae* mycelia by touchdown PCR using *Taq* polymerase (Qiagen, Valencia, CA). PCR conditions were 10 initial touchdown cycles (94°C for 1 min, 10 cycles decreasing from 69 to 59°C in 1°C increments per cycle for 1.5 min, and 72°C for 1.5 min) followed by 35 cycles of annealing at 59°C. Subsequently, the amplified 244-bp cDNA fragment was gel purified, subcloned into the PCR2.1-TOPO vector (Invitrogen, Carlsbad, CA), and sequenced at the BioResource Center at Cornell University.

A gene-specific primer was designed corresponding to the 3' end of the 244-bp fragment (antisense primer, 5'-CAGTGTGCCAGCGCACTTGTC-3') and used, together with a degenerate PCR primer (sense primer, 5'-ATGGGNGGGNACNGTNCNG-3', corresponding to MGGGTVP of the N-terminal amino acid sequence of the mature GIP1 polypeptide), to amplify a 529-bp cDNA fragment that was subcloned and sequenced as described above. This fragment was radiolabeled subsequently by random hexamer priming using α -³²P-dATP (3000 Ci/mmol; DuPont, Wilmington, DE) and Klenow DNA polymerase (New England Biolabs, Beverly, MA) and used to screen the *P. sojae* cDNA library as described by Sambrook et al. (1989).

Hybridization of the library filters was performed at 42°C in 50% (w/v) formamide, 6 × SSPE (1 × SSPE is 0.115 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7.4), 0.5% (v/v) SDS, 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA), and 100 mg/mL sonicated salmon sperm DNA. Membranes were washed three times in 5 × SSC and 1% (w/v) SDS at 42°C for 15 min, followed by three washes in 0.2 × SSC and 0.5% (w/v) SDS at 55°C. Eight positive colonies were identified, and the inserts were sequenced as described for the PCR product described above. In addition to *GIP1*, two homologous sequences were identified and designated *GIP2* and *GIP3*.

DNA Sequence Alignment and Phylogenetic Analysis

The deduced amino acid sequences of *GIP1*, -2, and -3 were aligned with those of Ser protease orthologs using ClustalW within LaserGene version 5.0 software (DNASTAR, Madison, WI). The deduced amino acid sequences of EGaseA and EGaseB were aligned similarly with four soybean endoglucanase sequences and a related tobacco endoglucanase sequence. Phylogenetic analyses were performed using PAUP version 4.0b8 (Sinaur Associates, Sunderland, MA). Parsimony analyses used branch-and-bound (endoglucanases) or heuristic searches with 100 random taxon entries and tree-bisection-reconnection branch swapping (Ser proteases). Bootstrap values were calculated from 100 replicates of either branch-and-bound (endoglucanases) or heuristic searches with simple taxon addition and tree-bisection-reconnection branch swapping.

Equally weighted parsimony analysis of the aligned Ser protease amino acid sequences using thorough heuristic searching identified two equally parsimonious trees with tree length values of 2640, a consistency index of 0.81 (0.75 excluding uninformative characters), and a retention index of 0.56. Equally weighted branch-and-bound parsimony analysis of the aligned EGase amino acid sequences identified a single most parsimonious tree of tree length 471, a consistency index of 0.88 (0.82 excluding uninformative characters), and a retention index of 0.62. Distance analyses using neighbor joining on uncorrected distances gave similar results (data not shown).

DNA Gel Blot Analysis

Genomic DNA was extracted from the mycelia of *P. sojae*, *P. infestans*, *P. medicaginis*, *Cladosporium fulvum*, *Colletotrichum lindemuthianum*, *Fusarium moniliforme*, *Cochliobolus sativus*, *Magnaporthe grisea*, and *Aspergillus niger* as described previously (Laugé et al., 1997), and 10- μ g aliquots were digested with the appropriate restriction enzymes, fractionated on agarose gels, and transferred to nylon membranes as described previously (Rose et al., 1997). The membranes were hybridized with the radiolabeled 529-bp *GIP1* cDNA fragment described above, washed three times for 15 min in 5 × SSC and 1% (w/v) SDS at 42°C, and then exposed to film for 24 h. This was followed by three 15-min washes in 0.2 × SSC and 0.5% (w/v) SDS at 62°C and reexposure to film.

Recombinant Protein Expression and Antibody Production

The *Nco*I and *Bam*HI restriction sites (underlined below) were introduced by PCR into the 5' and 3' ends, respectively, of the *GIP1* coding sequence minus the first 19 amino acids, which corresponded to the cleaved N-terminal signal sequence, using the 5' primer ACGTGCGGCTCCATGGTCATG and the 3' primer GAAGTTGATGAGGATCCGAGCC. PCR was performed for 35 cycles (94°C for 1 min, 60°C for 1.5 min, and 72°C for 1.5 min) in 50- μ L final volumes using 1 unit of AmpliTaq (Perkin-Elmer, Norwalk, CT), 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 200 μ M deoxynucleotide triphosphates, 3 mM MgCl₂, and 1 μ M of the primers described above, with *GIP1* cDNA as a template. The resulting 750-bp DNA fragment was gel purified and cloned into the *Nco*I and *Bam*HI restriction sites of the pET32a vector (Novagen, Madison, WI), which added a 6 × His tag to the N terminus of the recombinant protein, to promote binding to nickel-primed gel purification columns.

Expression of recombinant *GIP1* followed the procedures outlined in the pET handbook (Novagen). Briefly, the pET32a vector harboring

the *GIP1* coding sequence was transformed into *Escherichia coli* AD494 cells, and cultures were grown according to the manufacturer's instructions in Luria-Bertani medium and then induced with isopropyl-D-thiogalactoside (final concentration of 1 mM) for 3 h. Pelleted cells were lysed with a French press (16,000 p.s.i.) and re-centrifuged, and the pellet was extracted with B-Per II reagent (Pierce, Rockford, IL). The resulting purified inclusion bodies were solubilized and refolded using the Protein Refolding Kit (Novagen) according to the manufacturer's instructions.

The refolded proteins were dialyzed extensively against 50 mM Tris-HCl, pH 8.5, and 0.1 mM DTT, 2 volumes of buffer A (5 mM imidazole, 1 M NaCl, and 50 mM Tris-HCl, pH 7.9) were added, and the final solution was centrifuged briefly and passed through a 0.45- μ m filter before being applied to a nickel-charged Hi-Trap affinity column (Amersham Pharmacia Biotech, Piscataway, NJ). Proteins were eluted from the column by fast protein liquid chromatography (Amersham Pharmacia) in a gradient of 100% buffer A to 100% buffer B (as for buffer A but with 500 mM imidazole) at 0.5 mL/min. Column fractions were analyzed by SDS-PAGE (12.5% [v/v] acrylamide) and Coomassie brilliant blue G 250 staining. Strips of SDS-polyacrylamide gel containing \sim 2.0 mg of recombinant GIP1 protein were used to generate GIP1 antiserum, as described by Rose et al. (2000).

Surface Plasmon Resonance Analysis

The surface plasmon resonance experiments were performed on a Biacore 3000 instrument (Piscataway, NJ) with a CM5 sensor chip following a protocol similar to that described by Desiderio et al. (1997). After equilibration of the sensor chip with HBS buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, and 0.0005% [v/v] surfactant p20 in distilled water), the sensor surface was activated at a flow rate of 5 μ L/min with a 6-min injection of a mixture of 0.5 M *N*-hydroxysuccinimide and 0.2 M *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide. Native GIP1 protein, purified as described by Ham et al. (1997), in 10 mM sodium acetate buffer, pH 5.0, was used as the ligand and immobilized on the sensor surface. Unreacted esters were blocked by the addition of 1.0 M ethanolamine hydrochloride, pH 8.0. Native EGaseA, purified as described by Ham et al. (1997), was used as the analyte. The interaction analysis between GIP1 and EGaseA was performed by injecting EGaseA in HBS buffer over the immobilized GIP1 surface at a flow rate of 5 μ L/min. The analyte injection time was 1 min followed by 2 min of dissociation, and the surface was regenerated with 100 mM Gly-HCl, pH 2.0.

Soybean Seedling Inoculation

The roots of 3-day-old dark-grown soybean seedlings were dip-inoculated with *P. sojae* zoospores as described previously (Hahn et al., 1985), excised at 14 or 28 h after inoculation, and frozen in liquid nitrogen. The negative control involved incubation of the roots in sterile buffer.

Elicitor Bioassays

Aliquots (2 mL) of a 10 mg/mL suspension of isolated *P. sojae* cell walls in 50 mM sodium acetate, pH 5.5, prepared as described by Hahn et al. (1992), were incubated for 2 h at 37°C with one of the following: (1) 50 mM sodium acetate buffer; (2) 1 μ g of native GIP1 in the acetate buffer; (3) 1 μ g of EGaseA in the acetate buffer; or (4) 1

μ g of EGaseA that had been preincubated with 1 μ g of native GIP1 in the acetate buffer for 1 h at 37°C. After incubation, solubilized glucan elicitors were extracted using a protocol based on that of Yoshikawa et al. (1981). Samples were boiled for 20 min and centrifuged at 12,000g for 30 min, and the supernatant was passed through a 0.45- μ m filter to remove remaining insoluble wall material.

Elicitor activity was determined by adding 0.5 mL of each of the filtered extracts to 25 mL of suspension-cultured soybean cells (growing on a rotary shaker at 25°C) at 5 days after transfer to fresh medium, as described previously (Ebel et al., 1976). The cells were incubated with the elicitor samples for 15 h on a rotary shaker and then collected by filtration through Miracloth (Calbiochem, San Diego, CA), and both the cells and the extracellular media were frozen immediately in liquid N₂. Duplicate samples were prepared for each treatment.

Proteins were extracted from the cellular and extracellular media fractions, as described below, and endo- β -1,3-glucanase and Phe ammonia-lyase activities were measured to determine the elicitor activity of the original 0.5-mL filtered wall extracts. Endo- β -1,3-glucanase activity was assayed as described previously (Ham et al., 1997), and Phe ammonia-lyase activity was determined as described by Zucker (1965).

Protein Extraction, Protein Gel Blot Analysis, and Immunolocalization Studies

Proteins were isolated from the media of *P. sojae* cultures as described by Ham et al. (1997). To extract proteins from the samples generated during the elicitor bioassays, the extracellular medium was adjusted to 80% with respect to ammonium sulfate, stirred at 4°C for 1 h, and centrifuged at 20,000g for 30 min. The pellet was re-suspended in 50 mM Tris-HCl, pH 7.5, and 10 mM NaCl, and the solution was dialyzed extensively against the same buffer and re-centrifuged to remove insoluble material. Proteins were extracted from filtered suspension-cultured cells as described by Ebel et al. (1976).

To extract proteins for the zoospore-inoculated roots, 5 g of frozen roots was powdered in liquid N₂, ground with a pestle and mortar at 4°C with 10 mL of 75 mM sodium acetate, pH 5.2, and 5 mM DTT, and centrifuged at 20,000g for 30 min. The supernatant was clarified by re-centrifugation at 20,000g for 30 min and passed through a 0.45- μ m filter.

All protein samples were quantified using the Bio-Rad protein reagent (Hercules, CA) with BSA as a standard. For the protein interaction studies, 1 μ g of EGaseA or EGaseB was coincubated with 1 μ g of native or recombinant GIP for 1 h at 37°C before gel electrophoresis. One-dimensional gel electrophoresis with denaturing or non-denaturing gels (Invitrogen) and electrotransfer of one-dimensional or two-dimensional gel-separated proteins to membranes were as described previously (Rose et al., 2000). For two-dimensional gel electrophoresis, 100- μ g aliquots of protein were dissolved in solubilization buffer {7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, 0.5% ampholytes, pl 3 to 10 (Bio-Rad), and 100 mM DTT}, incubated overnight with immobilized pH gradient strips (pl range of 3 to 10), and separated in the first dimension in the Protean isoelectric focusing cell (Bio-Rad). The electrofocusing conditions were 500 V for 1 h, 500 to 4000 V (linear gradient) for 4 h, and 4000 V for 5 h.

After electrofocusing, the strips were incubated sequentially for 15 min each in equilibration buffer [50 mM 3-(*N*-morpholino)propane-

sulfonic acid, pH 7.7, 6 M urea, 30% (v/v) glycerol, and 2% (w/v) SDS] containing 65 mM DTT and then for 15 min in equilibration buffer containing 135 mM iodoacetamide. The strips were applied to 10 to 20% acrylamide second-dimension NuPAGE gels (Invitrogen). Protein gel blot analysis using anti-tobacco PR-2c (Ham et al., 1997) or GIP1 polyclonal antisera followed the method described by Rose et al. (2000).

For immunolocalization studies, *P. sojae* mycelia from 2-week-old cultures were washed extensively in distilled water or in 1 M NaCl, shaken for 1 h at room temperature in PBS containing a 1:1500 dilution of GIP1 antiserum or the preimmune antiserum, washed with four changes of PBS, incubated with goat anti-rabbit fluorescein isothiocyanate fluorescent secondary antibodies (Sigma, St. Louis, MO) at a 1:10 dilution, and washed again with four changes in PBS. Samples were imaged using a Zeiss Axioskop microscope with blue excitation filter set number 487,909 (Jena, Germany).

Accession Numbers

The GenBank accession numbers for the *P. sojae* GIP1, GIP2, and GIP3 cDNA sequences are AF406607, AF406608, and AF406609, respectively. The accession numbers for the other sequences mentioned in this article are as follows: ethylene-regulated soybean endo- β -1,3-glucanase that catalyzes the release of glucan elicitors from *P. sojae* cell walls in vitro (M37753); *SGlu5* (AF034110); four other soybean endoglucanase sequences (A26447, AF034106, U41323, and AF034112); a related tobacco endoglucanase (AAA34103); and Ser proteases and orthologs from the following fungi and bacteria: *F. oxysporum* (S63827), *C. carbonum* (U39500), *P. nodorum* (AF092435), *S. erythraea* (D30760), and *A. astaci* (AF355100).

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Molecular Cloning and Characterization of Glucanase Inhibitor Proteins: Coevolution of a Counterdefense Mechanism by Plant Pathogens

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