

# Characterization of the Complex Locus of Bean Encoding Polygalacturonase-Inhibiting Proteins Reveals Subfunctionalization for Defense against Fungi and Insects<sup>1</sup>

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Polygalacturonase-inhibiting proteins (PGIPs) are extracellular plant inhibitors of fungal endopolygalacturonases (PGs) that belong to the superfamily of Leu-rich repeat proteins. We have characterized the full complement of *pgip* genes in the bean (*Phaseolus vulgaris*) genotype BAT93. This comprises four clustered members that span a 50-kb region and, based on their similarity, form two pairs (*Pvpgip1/Pvpgip2* and *Pvpgip3/Pvpgip4*). Characterization of the encoded products revealed both partial redundancy and subfunctionalization against fungal-derived PGs. Notably, the pair PvPGIP3/PvPGIP4 also inhibited PGs of two mirid bugs (*Lygus rugulipennis* and *Adelphocoris lineolatus*). Characterization of *Pvpgip* genes of Pinto bean showed variations limited to single synonymous substitutions or small deletions. A three-amino acid deletion encompassing a residue previously identified as crucial for recognition of PG of *Fusarium moniliforme* was responsible for the inability of BAT93 PvPGIP2 to inhibit this enzyme. Consistent with the large variations observed in the promoter sequences, reverse transcription-PCR expression analysis revealed that the different family members differentially respond to elicitors, wounding, and salicylic acid. We conclude that both biochemical and regulatory redundancy and subfunctionalization of *pgip* genes are important for the adaptation of plants to pathogenic fungi and phytophagous insects.

Molecular diversification is crucial in plant-pathogen interactions. Pathogens encounter a vast number of hosts, against which polymorphic molecular weapons have been evolved (Gassmann et al., 2000; Idnurm and Howlett, 2001). Conversely, plants rely for defense on a finely honed innate surveillance apparatus consisting of highly polymorphic recognition molecules (Bergelson et al., 2001). For example, plants recognize

cell wall-degrading enzymes produced by pathogenic (micro)organisms and inhibit their enzymatic activity to hamper the invasion process and the release of nutrients necessary for pathogen growth. Among the inhibitors of cell wall-degrading enzymes so far identified (Bellincampi et al., 2004, and references therein), polygalacturonase-inhibiting proteins (PGIPs) have been clearly shown to play a role in defense (De Lorenzo and Ferrari, 2002). Endopolygalacturonases (PGs; EC 3.2.1.15) cleave the  $\alpha$ -(1  $\rightarrow$  4) linkages between D-GalUA residues in homogalacturonan and cause separation of cells and maceration of host tissue. The importance of these enzymes in pathogenesis has been demonstrated for fungi and bacteria (De Lorenzo and Ferrari, 2002; Oeser et al., 2002) and has been proposed for nematodes (Jaubert et al., 2002). PGs from salivary glands are considered a main cause of plant damage by phytophagous bugs (Heteroptera: Miridae) and other insects (Girard and Jouanin, 1999; Wheeler, 2001; Boyd et al., 2002). To accommodate pathogenesis in a variety of different conditions and on various hosts, pathogens produce a variety of PG isoenzymes (De Lorenzo et al., 2001), which often exhibit polymorphism in different isolates or races (Caprari et al., 1993; Daroda et al., 2001; Poinssot et al., 2003).

<sup>1</sup> This work was supported by the Giovanni Armenise-Harvard Foundation, by MIUR (Ministero dell'Istruzione, dell'Università e della Ricerca; grants PRIN [Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale] 2002 and FIRB [Fondo per gli Investimenti della Ricerca di Base] 2001), by the Institute Pasteur-Fondazione Cenci Bolognetti, and by European Community Grants (grant nos. ICA4-CT-2000-30033 and QLK1-CT-2000-00811).

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Article, publication date, and citation information can be found at [www.plantphysiol.org/cgi/doi/10.1104/pp.104.044644](http://www.plantphysiol.org/cgi/doi/10.1104/pp.104.044644).

Against the many PGs produced by pathogens, plants have evolved many PGIPs. In addition to reducing the aggressive potential of PGs, PGIPs favor the formation of long-chain oligogalacturonides (OGs) that are able to induce defense responses (De Lorenzo et al., 2001). Consistent with their role in defense, PGIPs are ubiquitous in flowering plants and are up-regulated in response to stress-related signals, wounding, and fungal and insect attack (De Lorenzo et al., 2001, and references therein; Li et al., 2003; Ndimba et al., 2003). The overexpression of PGIPs in transgenic plants limits fungal colonization (Powell et al., 2000; Ferrari et al., 2003), thereby demonstrating the defensive potential of these proteins.

The Leu-rich repeat (LRR) structure of PGIPs is responsible for the molecular interaction with PGs. The LRR structure is shared by many plant proteins involved in recognition, such as the majority of the resistance (*R*) gene products (Martin et al., 2003), and several receptors involved in development, perception of hormones (Becraft, 2002; Szekeres, 2003), or elicitors (Gomez-Gomez et al., 2001), defense responses against insects (Szekeres, 2003), and bacterial and fungal symbiosis (Kistner and Parniske, 2002). The 10 LRRs of PGIPs are of the extracytoplasmic type (LxxLxLxxNxLT/SGxIPxxLxxLxx) and are organized to form two  $\beta$ -sheets, B1 and B2 (Di Matteo et al., 2003). It has been shown that a single amino acid variation in B1 confers the ability to recognize a novel PG (Leckie et al., 1999), implying that diversity in the LRR domain is crucial for determining recognition specificity toward pathogens.

PGIPs are encoded by small gene families in many plant species (De Lorenzo et al., 2001; Li et al., 2003). So far only the Arabidopsis gene family, which comprises two tandemly duplicated genes, has been studied in detail and shown to encode functionally redundant proteins that are up-regulated through different signal transduction pathways in response to fungal infection (Ferrari et al., 2003).

Gene duplication, divergence, and selection pressure are major mechanisms shaping gene families, and family sizes are often the result of selection for useful functions. PGIP families provide an interesting case of molecular evolution and adaptation. Their structure is the result of coevolution with PGs of pathogenic organisms. Moreover, PGIPs, like PGs, are subject to both functional constraints and selection pressure for diversification.

In this study, we have studied the significance of diversity in the bean (*Phaseolus vulgaris*) *pgip* family, which has been mapped in the B2 linkage group of the bean core map (Freyre et al., 1998). We report here that the full complement of *pgip* genes in the bean genotype BAT93 is represented by four clustered genes. We show that diversification of paralogous genes is associated with different modes of regulation, as well as with functional redundancy and subfunctionalization for recognition of PGs of fungi and phytogenous insects.

## RESULTS

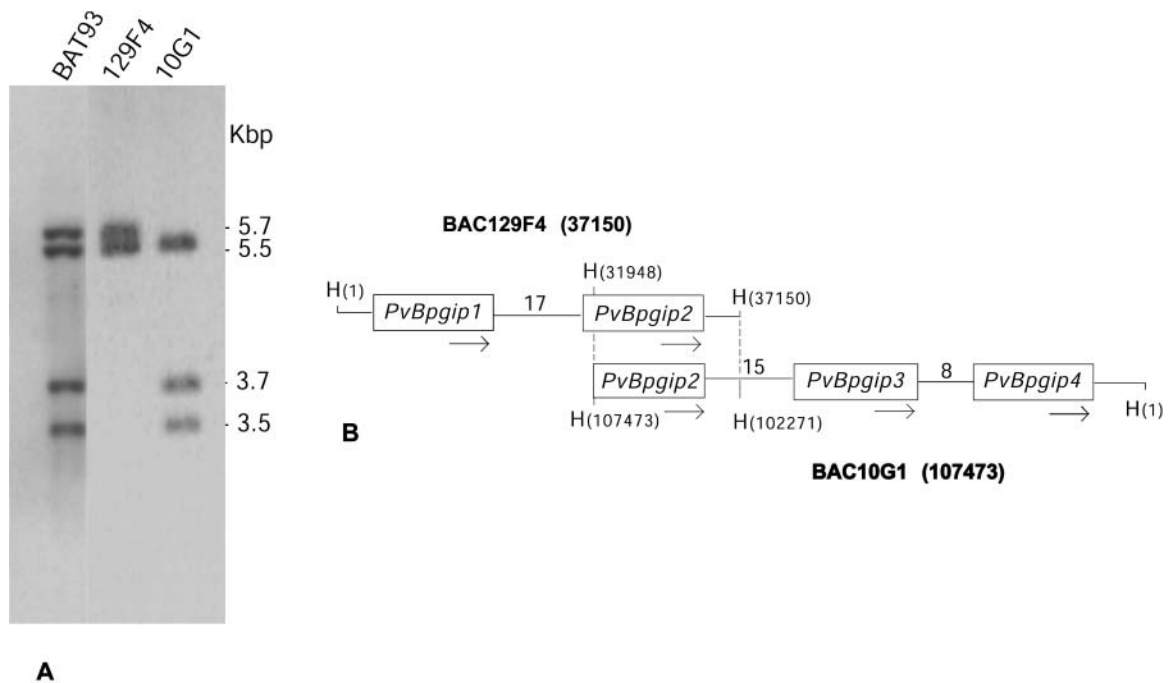
### Isolation of *pgip* Genes of Bean Genotypes BAT93 and Pinto

To investigate intra- and intergenotype variation in bean *pgip* genes, the full complements of *pgip* genes of the Mesoamerican genotypes BAT93 and Pinto were characterized. From a BAT93 bacterial artificial chromosome (BAC) library, two overlapping clones (129F4 and 10G1) were isolated that contained all *pgip* genes, as shown by a Southern blot probed with a fragment corresponding to the portion encoding the mature protein of the previously characterized Pinto *pgip1* gene (Leckie et al., 1999; Fig. 1A). Sequencing of the two clones showed the presence of four intronless *pgip* open reading frames (ORFs) oriented in the same direction and indicated as *PvBpgip1* and *PvBpgip2*, on the basis of their similarity to previously characterized bean *pgip* genes (Fig. 1B), and *PvBpgip3* and *PvBpgip4*. The two latter genes have never been described before. The *Pvpgip* locus spans about 50 kb and does not comprise additional *pgip*-related sequences. Distances between the four genes (about 17, 15, and 8 kb) are much larger than those observed between members of the Arabidopsis and soybean *pgip* families (about 500 bp and 3,000 bp, respectively).

Pinto exhibited a Southern-blot hybridization pattern identical to that of BAT93 (data not shown). Characterization of cDNAs of Pinto *Pgip1* and *Pgip2*, hereon indicated as *PvPpgip1* and *PvPpgip2.1*, has been described previously (Leckie et al., 1999). To isolate additional genes, a phage genomic library was screened with the *pgip*-specific probe described above. Among 34 positive clones analyzed by double digestion with *EcoRI* and *HindIII*, clones Pt38, Pt10, and Pt21 exhibited specific hybridization to three (5.7, 3.7, and 3.5 kb) of the four *EcoRI/HindIII* fragments observed in Southern-blot analysis of genomic DNA. The hybridizing inserts of the three phage clones, individually subcloned and sequenced, each contained a *pgip*-related ORF (*PvPpgip2.2*, *PvPpgip3*, and *PvPpgip4*). *PvPpgip2.2* differed from *PvPpgip2.1* in four synonymous substitutions in the coding region and a 2-nucleotide (nt) insertion (AA) in the 3' untranslated region (UTR), likely due to sequence variation between the Pinto seeds used for the construction of the cDNA and genomic libraries. Pinto is a commercial class of dry bean that includes a number of bean varieties.

### Sequence Diversity of *Pvpgip* Genes and Their Encoded Products

The products encoded by the isolated *Pvpgip* genes share the typical PGIP topology, which includes a signal peptide for secretion (domain A), a 52-amino acid N-terminal domain B, a domain C comprising 10 imperfect LRRs, and a C-terminal 24-amino acid domain D. All exhibit the eight Cys residues, which are conserved in all PGIPs: four are located in domain



**Figure 1.** Genomic organization of the *pgip* gene family in bean genotype BAT93. **A**, Southern-blot analysis of genomic DNA from line BAT93 and plasmid DNA from 129F4 and 10G1 BAC clones; DNA was double digested with *EcoRI-HindIII* and probed with a fragment corresponding to the portion of the *PvPpgip1* ORF encoding the mature protein. The 5.5-kb hybridizing fragment is present in both BAC clones. **B**, Schematic representation of the arrangement of the *PvBpgip* genes as determined by sequencing the 129F4 and 10G1 BAC clones. The two clones overlap for about 5.5 kb; this region includes *PvBpgip2*. Numbers between *pgip* members represent distances in kb. Arrows indicate the direction of the coding region from ATG to stop codon. H, *HindIII* restriction site. Numbers in brackets indicate the position of the terminal *HindIII* sites in each BAC clone, where numbering starts from the *HindIII* site following the T7 promoter sequence of pBeloBAC11. In the overlapping region, the positions of the corresponding *HindIII* sites in the two BAC clones are also indicated.

B, one in the 10th LRR, and three in the C-terminal domain D (Fig. 2). Numbering of *pgip* nucleotide sequences reported in this work differs from that in previous articles, where +1 indicated the A of the first of two in-frame putative translation initiation codons present in the *Pvpgip1* ORF (Toubart et al., 1992; Devoto et al., 1998; Leckie et al., 1999). Since the first Met codon is absent in all the other *Pvpgip* genes, the start of the ORFs is now assigned to the second ATG codon of *Pvpgip1*. Numbering of PGIP amino acid residues has also been changed in this article and starts from the first residue of the mature protein, which corresponds to residue 30 (a Glu) described in a previous article by Leckie et al. (1999).

Nucleotide identity/amino acid divergence among BAT93 and Pinto *pgip* genes and their encoded products are summarized in Table I. On the basis of their similarity, in both bean genotypes the four paralogous genes form two pairs (*Pvpgip1/Pvpgip2* and *Pvpgip3/Pvpgip4*), which may represent functionally distinct classes of PGIPs. The presence of two insertions, one located in the signal peptide and one in the eighth LRR, characterizes the products of the pair *Pvpgip3/Pvpgip4* (Fig. 2). The two deduced proteins lack the first putative N-linked glycosylation site (at position 35), which has been shown to be occupied by a typical

complex N-glycan in Pinto PvPPGIP2 (Mattei et al., 2001). In a comparison with the products of the paralogous *Pvpgip3* genes, both Pinto and BAT93 PvPGIP4 show an additional Cys residue in domain D, as well as two single amino acid deletions in the seventh and eighth LRRs (Fig. 2).

Intergenotype comparison of corresponding ORF sequences of Pinto and BAT93 shows that, whereas *Pvpgip4* genes are identical, *Pvpgip1* and *Pvpgip3* show a single nonsynonymous 1-nt replacement at codons 265 and 9, respectively. Instead, a 9-nt deletion distinguishes BAT93 *PvBpgip2* from Pinto *PvPpgip2.2*; the resulting amino acid deletion includes residue 224 (previously indicated as 253) that has been shown to be crucial for recognition of FmPG (Leckie et al., 1999; Fig. 2).

#### Redundant and Unique Functions of Paralogous *Pvpgip* Genes and Intergenotype Variation

The effect of sequence variation on PGIP function was investigated. The inhibitor activities of Pinto PvPPGIP1 and PvPPGIP2 toward several fungal PGs have been described previously (Desiderio et al., 1997; Leckie et al., 1999). The proteins encoded by the four BAT93 *PvBpgip* genes and the Pinto *PvPpgip3* and



**Figure 2.** Variability of bean PGIPs. Deduced amino acid sequences of BAT93 PGIPs (PvBPGIP1-4) are aligned. Numbering is referred to the PvBPGIP1 sequence and starts from the first residue of the mature protein. Domains are on the basis of crystallographic analysis (Di Matteo et al., 2003). The xxLxLxx region is boxed. Empty spaces indicate gaps. Cys residues are in bold; putative glycosylation sites are underlined and in italics. Sites that vary in Pinto PvPPGIPs are highlighted in gray; corresponding Pinto residues are indicated beyond the vertical line on the right. The 3-amino acid deletion that distinguish BAT93 PvBPGIP2 from Pinto PvPPGIP2 is indicated by dashes and is also highlighted in gray.

*PvPpgip4* were expressed in *Nicotiana benthamiana* using a vector based on potato virus X (PVX; Baulcombe et al., 1995). Western-blot analysis of crude protein extracts from plants inoculated with each construct was carried out to demonstrate the presence of a PGIP-specific signal with a molecular mass of 39 kD, which was absent in extracts from noninfected plants or from plants infected with the empty vector (data not shown).

Because the presence of multiple PGIPs may respond to the need of inhibiting PGs in different micro-environments, such as those created, for example, by altered ion fluxes during the plant defense response, or the need of inhibiting PGs of diverse organisms harmful to plants, we tested the activity of the PvPGIPs purified from *N. benthamiana* protein extracts at two different pH values (4.7 and 7.0), and against PGs of fungi and insects. The two plant bugs *Lygus rugulipennis* Poppius and *Adelphocoris lineolatus* (Goeze; Heteroptera: Miridae) were examined in this study.

The tested PGs differed in their activity: PGs of *Colletotrichum acutatum* (CaPG) and *Botrytis cinerea* (BcPG) showed activity at both pH 4.7 and 7.0; PGs of *Aspergillus niger* (AnPG), *Fusarium moniliforme* (FmPG), and *Stenocarpella maydis* (SmPG) showed activity only at pH 4.7, while PGs of both *L. rugulipennis* and *A. lineolatus* were active only at pH 7.0. Our analyses showed that PGIPs encoded by paralogous genes all exhibited different recognition abilities and that inhibitory activities of all BAT93 PvBPGIPs, except PvBPGIP2, were identical to the corresponding Pinto proteins (Table II). Only PvPGIP3 of both BAT93 and Pinto were unable to inhibit AnPG, and only Pinto PvPPGIP2 inhibited FmPG, because the 3-amino acid deletion encompassing residue 224 abolishes the ability of BAT93 PvBPGIP2 to inhibit this enzyme. All eight PvPGIPs inhibited CaPG, and SmPG, albeit with different strength. Notably, inhibitory activity toward PGs of insects was exhibited only by PvPGIP3 and PvPGIP4, which are weak inhibitors of fungal PGs when compared to PvPGIP1 and PvPGIP2 (Table II).

### The xxLxLxx-Encoding Region Is the Most Variable Portion of *Pvpgip* Genes

Because the structural and functional viability of PGIPs likely derives from a positive diversifying evolution toward the many PGs produced by pathogens, we evaluated the type of selection acting on the bean *pgip* family by using the method that compares the number of nonsynonymous (amino acid changing;  $K_a$ ) and synonymous (silent;  $K_s$ ) substitutions per site. In the absence of selection on codon usage,  $K_s$  represents the frequency of neutral substitutions. With no selection pressure being present,  $K_a/K_s$  ratios are predicted to be 1, whereas in the case of positive diversifying and purifying selection, ratios  $>1$  and  $<1$ , respectively, are obtained (Li, 1997). Pairwise comparisons between all *Pvpgip* members, except *Pvpgip3*/

**Table I.** Nucleotide identities and amino acid variations<sup>a</sup> among *pgip* genes of bean genotypes Pinto and BAT93

	PvPpgip1	PvPpgip2.1	PvPpgip2.2	PvPpgip3	PvPpgip4	PvBpgip1	PvBpgip2	PvBpgip3	PvBpgip4
<i>PvPpgip1</i>	–	<b>8 + 0</b>	<b>8 + 0</b>	<b>66 + 3</b>	<b>79 + 1</b>	<b>1 + 0</b>	<b>7 + 3</b>	<b>67 + 3</b>	<b>79 + 1</b>
<i>PvPpgip2.1</i>	97.4%	–	<b>0 + 0</b>	<b>65 + 3</b>	<b>76 + 1</b>	<b>9 + 0</b>	<b>0 + 3</b>	<b>66 + 3</b>	<b>76 + 1</b>
<i>PvPpgip2.2</i>	97.6%	99.6%	–	<b>65 + 3</b>	<b>76 + 1</b>	<b>9 + 0</b>	<b>0 + 3</b>	<b>66 + 3</b>	<b>76 + 1</b>
<i>PvPpgip3</i>	82.5%	83.2%	82.3%	–	<b>30 + 2</b>	<b>67 + 3</b>	<b>65 + 6</b>	<b>1 + 0</b>	<b>30 + 2</b>
<i>PvPpgip4</i>	79.8%	80.5%	80.3%	94.9%	–	<b>80 + 1</b>	<b>74 + 4</b>	<b>31 + 2</b>	<b>0 + 0</b>
<i>PvBpgip1</i>	99.9%	97.3%	97.7%	82.4%	79.7%	–	<b>8 + 3</b>	<b>68 + 3</b>	<b>80 + 1</b>
<i>PvBpgip2</i>	97.8%	99.6%	100%	83.0%	80.5%	97.7%	–	<b>66 + 6</b>	<b>74 + 4</b>
<i>PvBpgip3</i>	82.4%	83.0%	82.8%	99.9%	94.8%	82.3%	82.9%	–	<b>31 + 2</b>
<i>PvBpgip4</i>	79.8%	80.5%	80.3%	94.9%	100%	79.6%	80.5%	94.8%	–

<sup>a</sup>Only sequences corresponding to mature proteins have been considered. For each comparison, normal characters (bottom left part of the table) indicate nucleotide identities excluding indels. Numbers in bold characters (top right) indicate total amino acid replacements and indels, respectively.

*Pvpgip4*, produced  $K_s$  that significantly exceeded  $K_a$  ( $K_a/K_s < 0.35$ ;  $P < 0.05$ ), indicating that purifying rather than diversifying selection has acted on the gene as a whole. For *Pvpgip3/Pvpgip4* instead  $K_a$  was not significantly different from  $K_s$  and was significantly higher ( $P < 0.05$ ) than that obtained for *Pvpgip1/Pvpgip2* (Table III). We hypothesize that the latter pair has been subject to a stronger purifying selection pressure.

Because different regions in a gene can be subject to different selection pressures,  $K_a/K_s$  ratios were also calculated separately for the regions of the *pgip* ORF encoding the N-terminal domain B (region B), the LRR domain C excluding the xxLxLxx motifs that includes the  $\beta$ -sheet B1 (region C-out), the portion corresponding to the 10 xxLxLxx motifs with and without the conserved hydrophobic residues (xxLxLxx and xx(L)x(L)xx regions, respectively), and the C-terminal domain D (region D). This analysis revealed that patterns of substitutions are not equivalent among the different protein regions (Table III). In regions B and C-out,  $K_a$  values were significantly lower than  $K_s$

values ( $K_a/K_s < 0.30$ ;  $P < 0.05$ ), suggesting purifying selection. In the xx(L)x(L)xx region instead all  $K_a$  values were significantly higher ( $P < 0.01$ ) than those of regions B and C-out and not significantly different from  $K_s$  values ( $K_a/K_s$  ratios approximately 1). Variability in domain D appeared to be intermediate between that of the B and C-out regions and that of the xx(L)x(L)xx region. This analysis therefore shows that the xxLxLxx region is the most variable portion of the *Pvpgip* genes but provides no evidence for positive selection.

#### The Members of the *Pvpgip* Family Are Differentially Regulated

We also investigated whether the presence of multiple genes might respond to the need of a differential expression in different stress situations. Because the high degree of identity at the nucleotide level between the *pgip* genes makes difficult the design of gene-specific probes for northern-blot analysis, a reverse

**Table II.** Inhibitory activities<sup>a</sup> of PGIPs of bean genotypes BAT93 and Pinto against PGs of fungi and insects

Polygalacturonase	pH <sup>b</sup>	Relative Activity <sup>c</sup>	PvBPGIP1	PvPPGIP1	PvBPGIP2	PvPPGIP2	PvBPGIP3	PvPPGIP3	PvBPGIP4	PvPPGIP4
<i>Aspergillus niger</i>	4.7	1		140	10	1	$\infty$			250
	7.0	0		-	-	-	-			-
<i>Fusarium moniliforme</i>	4.7	1		$\infty$	$\infty$	9	$\infty$			$\infty$
	7.0	0		-	-	-	-			-
<i>Stenocarpella maydis</i>	4.7	1		7.5	4.5	5	260			65
	7.0	0		-	-	-	-			-
<i>Colletotrichum acutatum</i>	4.7	1		16	12	12	230			1,200
	7.0	0.8		16	15	13	690			1,400
<i>Botrytis cinerea</i>	4.7	1		200	16	2.5	100			70
	7.0	1.1		$\infty$	17	4	$\infty$			120
<i>Lygus rugulipennis</i>	4.7	0		-	-	-	-			-
	7.0	1		$\infty$	$\infty$	$\infty$	490			1,950
<i>Adelphocoris lineolatus</i>	4.7	0		-	-	-	-			-
	7.0	1		$\infty$	$\infty$	$\infty$	2,100			1,954

<sup>a</sup>Values indicate the amount (in ng) of PGIP that determines 50% inhibition of 1 agarose plate unit (at pH 4.7) of PG. The symbol  $\infty$  indicates  $>6 \mu\text{g}$ . <sup>b</sup>Enzyme activities were assayed in 20 mM Na acetate pH 4.7 or 7.0. <sup>c</sup>Activity at pH 4.7 is taken as a reference (=1).

**Table III.**  $K_a$  and  $K_s$  values in *pgip* genes of bean genotype BAT93

		Complete Gene		Domain B		Region C-Out <sup>a</sup>		xxLxLxx		xx(L)x(L)xx <sup>b</sup>		Domain D	
		Pinto	BAT93	Pinto	BAT93	Pinto	BAT93	Pinto	BAT93	Pinto	BAT93	Pinto	BAT93
<i>Pvpgip1/2</i>	$K_a$	0.010	0.011	0.000	0.000	0.002	0.005	0.023	0.018	0.033	0.026	0.053	0.053
	$K_s$	0.051	0.052	0.040	0.040	0.043	0.043	0.086	0.087	0.127	0.129	0.043	0.043
	$K_a/K_s$	0.196	0.211	0.000	0.000	0.046	0.116	0.267	0.206	0.259	0.201	1.232	1.232
<i>Pvpgip1/3</i>	$K_a$	0.124	0.124	0.098	0.107	0.114	0.112	0.192	0.192	0.233	0.235	0.062	0.062
	$K_s$	0.426	0.431	0.444	0.447	0.383	0.386	0.399	0.405	0.382	0.394	0.641	0.641
	$K_a/K_s$	0.291	0.287	0.220	0.239	0.297	0.290	0.481	0.474	0.609	0.596	0.096	0.096
<i>Pvpgip1/4</i>	$K_a$	0.159	0.156	0.096	0.096	0.130	0.128	0.280	0.276	0.357	0.352	0.177	0.177
	$K_s$	0.484	0.486	0.443	0.443	0.443	0.447	0.519	0.511	0.510	0.497	0.558	0.558
	$K_a/K_s$	0.328	0.320	0.216	0.216	0.293	0.286	0.539	0.540	0.700	0.708	0.317	0.317
<i>Pvpgip2/3</i>	$K_a$	0.124	0.124	0.098	0.107	0.112	0.106	0.192	0.199	0.233	0.246	0.082	0.082
	$K_s$	0.407	0.412	0.363	0.364	0.401	0.404	0.355	0.361	0.320	0.330	1.031	1.031
	$K_a/K_s$	0.304	0.300	0.269	0.293	0.279	0.262	0.540	0.551	0.728	0.745	0.079	0.079
<i>Pvpgip2/4</i>	$K_a$	0.158	0.154	0.096	0.096	0.127	0.121	0.285	0.284	0.363	0.366	0.170	0.170
	$K_s$	0.459	0.465	0.361	0.361	0.462	0.466	0.447	0.461	0.410	0.426	0.983	0.983
	$K_a/K_s$	0.344	0.331	0.265	0.265	0.274	0.259	0.637	0.616	0.885	0.859	0.172	0.172
<i>Pvpgip3/4</i>	$K_a$	0.048	0.048	0.017	0.025	0.022	0.022	0.118	0.113	0.159	0.154	0.107	0.107
	$K_s$	0.073	0.073	0.000	0.000	0.097	0.097	0.071	0.073	0.085	0.087	0.040	0.040
	$K_a/K_s$	0.657	0.657	NA <sup>c</sup>	NA <sup>c</sup>	0.226	0.226	1.661	1.547	1.870	1.770	2.675	2.675
Range $K_a/K_s$	0.196–	0.211–	0.000–	0.000–	0.046–	0.116–	0.267–	0.206–	0.259–	0.201–	0.079–	0.079–	
Average $K_a/K_s$	0.657	0.657	0.269	0.293	0.297	0.290	1.661	1.547	1.870	1.770	2.675	2.675	
		0.353	0.351	0.194	0.202	0.235	0.239	0.687	0.655	0.841	0.813	0.761	0.761

<sup>a</sup>Region C-out represents the region encoding domain C outside of the xxLxLxx. <sup>b</sup>xxLxLxx-encoding region without the triplets for the conserved aliphatic residues. <sup>c</sup>NA, Not applicable.

transcription (RT)-PCR based approach was used to study their regulation in Pinto. Primers specific for *PvPpgip1*, *PvPpgip2* (both *PvPpgip2.1* and *PvPpgip2.2*), *PvPpgip3*, and *PvPpgip4* were synthesized (see "Materials and Methods"); they included, as the last base at the 3' end, a mismatched nucleotide specific for the individual gene members.

Analyses were carried out on RNA extracted from bean hypocotyls at different times after wounding, or treatment with salicylic acid (SA) or water (control), and from suspension-cultured cells after treatment with OGs or glucan from *Phytophthora megasperma* f. sp. *glycinea* or water (control). PCR amplification followed the RT of total RNA using the appropriate antisense primer. Conditions for PCR were optimized to show induction relative to basal levels (time 0).

Transcripts of *PvPpgip1* accumulated 3 h after wounding and returned to basal levels after 6 h; the other treatments had no effects. Even with a high number of amplification cycles and at an RNA template concentration higher than that used for the analysis of the other *PvPpgip* genes, amplification of *PvPpgip1* never occurred in RNA samples extracted at time 0 from both untreated tissues and suspension-cultured cells. Transcripts of *PvPpgip2* were induced by all the treatments examined. *PvPpgip3* transcripts were induced in suspension-cultured cells in response to OGs but not to glucan, and did not vary in hypocotyls upon wounding or SA treatment, whereas *PvPpgip4* transcripts were not induced by any treatment (Fig. 3).

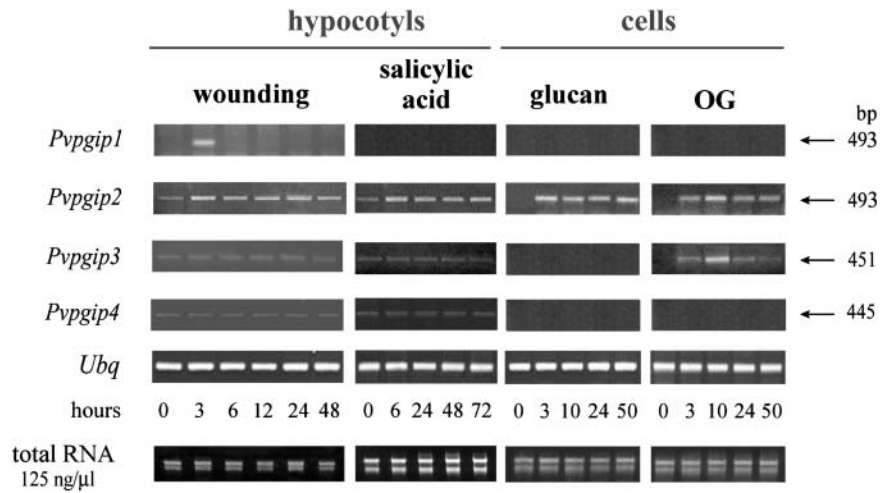
#### Analysis of Sequences Upstream and Downstream of the *pgip*-Coding Regions

The observed difference in their regulation of expression reflects differences in the sequence of the *Pvpgip* regulatory regions. *PvBpgip1* and *PvBpgip2* share 93.4% identity in 500 bp of the 5' region proximal to the ATG, with several insertions/deletions (indels; Fig. 4), and 97% identity in the proximal 400-bp region downstream of the stop codon. Putative 3' UTR, deduced by comparison with the Pinto cDNA sequences, are of about 100 bp and differ by only 3 nt. The 5' region proximal to the ATG of *PvBpgip1* shares about 99% identity with the corresponding region of the Saxa *Pvpgip1* (Toubart et al., 1992), with two deletions of 27 bp and 48 bp at positions -1,109 and -928, while the 3' UTRs differ by 2 nt.

Regions sharing sequence similarity between *PvBpgip3* and *PvBpgip4* span about 2,500 bp upstream of the translation initiation codon (95.1% identity), with an indel of 569 bp and a few additional short ones, and about 600 bp at the 3' of the translation stop codon (96% identity). No similarity was observed in the flanking regions between the pairs *PvBpgip1/PvBpgip2* and *PvBpgip3/PvBpgip4*. *PvBpgip3* and *PvBpgip4* abruptly diverge from *PvBpgip1* and *PvBpgip2* upstream of nt -21 and immediately downstream of the translation stop codon, with only a few short nucleotide stretches conserved up to 150 nt after the TAA.

A number of sequences with significant similarity to known cis-acting elements are present in the 1.5-kb

**Figure 3.** Differential expression of Pinto *pgip* genes in response to stress-related stimuli. RT-PCR was performed on total RNA extracted from bean tissues or cells at different times after the indicated treatments, using the gene-specific primers described in "Materials and Methods." Amplification products were analyzed by electrophoresis on agarose gel; the expected size of amplified fragment for each experiment is indicated by the arrow on the right. Conditions for PCR were optimized to show induction relative to basal levels (time 0). Comparison of band intensities is meaningful only within a single data set. Analyses of the water-treated controls showed no induction (data not shown).



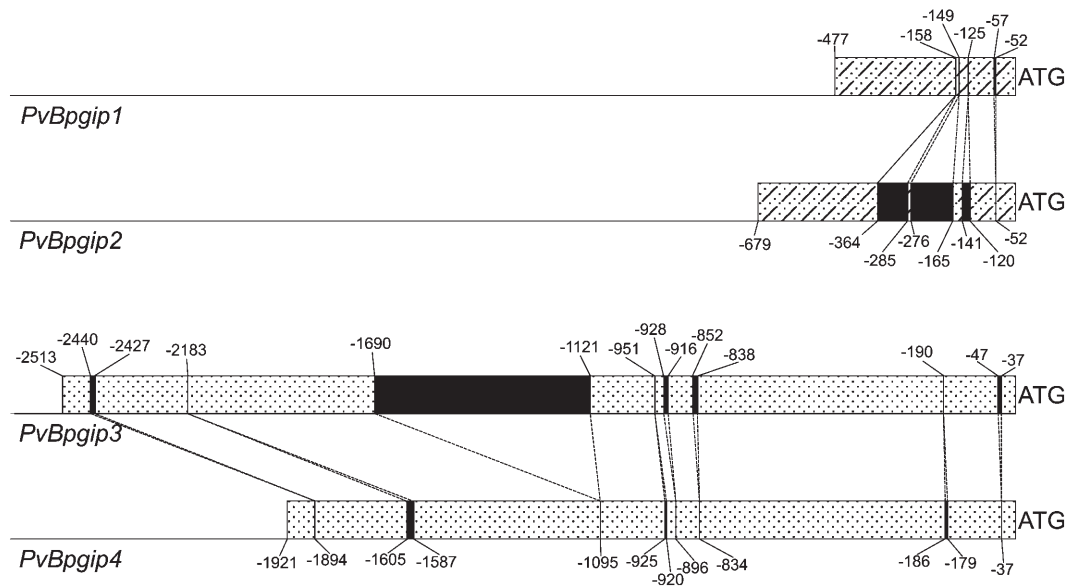
region upstream from the start codon of all four *PvBqip* genes. In particular, several W-box elements, which have been identified in the promoters of several defense genes and are known to mediate transcriptional responses to pathogen-derived elicitors (Eulgem et al., 2000), can be observed. Notably, *PvBqip1* and *PvBqip2* contain six (-84, -97, -332, -352, -486, -502) and five (-79, -538, -727, -736, -764) W-boxes in the proximal 500 bp and 800 bp from the start codon, respectively, whereas *PvBqip3* contains a single W-box at -1,220 and *PvBqip4* contains none.

Regions immediately upstream of the ATG and downstream of the TAA of the cloned Pinto *pgip* genes were analyzed and compared to the corresponding regions of the BAT93 genes. We sequenced 1,607 bp, 1,786 bp, and 357 bp of the 5' regions, and 581 bp, 896

bp, and 131 bp of 3' regions of *PvPqip2.2*, *PvPqip3*, and *PvPqip4*, respectively. In the 5' regions, *PvPqip2.2* and *PvPqip4* were identical to the corresponding BAT93 genes, while *PvPqip3* showed a few substitutions (98.6% identity). In the 3' region *PvPqip2* showed one single base deletion and one substitution, *PvPqip3* had three substitutions and two small deletions (1 nt and 11 nt), and *PvPqip4* showed no variations.

**DISCUSSION**

In this study, we have structurally and functionally characterized the *pgip* gene family of bean. The family consists of four clustered genes; the arrangement and



**Figure 4.** Schematic representation of the 5' flanking region of the four BAT93 *pgip* genes. Regions sharing sequence similarity are indicated by dots-dashes in *PvBqip1* and *PvBqip2* or dots in *PvBqip3* and *PvBqip4*. Black boxes indicate insertions. The A of the ATG codon corresponds to +1.

the similarities among them suggest that they derive from a common ancestor by a sequence of duplication-divergence-duplication events. Duplication was at close proximity to the parent sequence, rather than to ectopic chromosomal location, similar to what was observed for nucleotide binding site-LRR genes (Baumgarten et al., 2003). The first duplication event might have generated the ancestors of the two pairs *Pvpgip1/Pvpgip2* and *Pvpgip3/Pvpgip4*, each of which independently underwent diversification, with limited genetic rearrangement within the coding regions represented by point mutations and small insertions/deletions. Subsequently, duplication of genomic segments of about 2,000 bp and 4,000 bp may have generated *Pvpgip1* and *Pvpgip2*, and *Pvpgip3* and *Pvpgip4*, respectively. The extent of nucleotide identity within the two gene pairs, including the regulatory regions (95% to 97.6%), suggests that the two recent duplications occurred approximately at the same time. The observation that *Gmpgip3* of soybean (*Glycine max*), a species of the Phaseoleae tribe very close to bean, groups with *Pvpgip1* and *Pvpgip2* (De Lorenzo et al., 2001), suggests that these two genes are probably closer to the ancestral gene.

The finding that a large number of families of genes have arisen in eukaryotes during evolution through duplication and have persisted for longer periods of time than expected by classical models (Lynch et al., 2001) is drawing attention into the dynamics of duplicate genes. Only a few studies on gene families having a known function have been carried out. A key question is whether duplicate genes are elements of developmental stability and conservation of function or of evolutionary innovation. Once structural genomic information about a family is available, an understanding of whether changes in gene numbers and structure have adaptive significance generally requires information about function and phenotypes. In many cases, genetics is of little help in the analysis of many gene families because null mutation or deletion of one copy may have no phenotype due to compensation by duplicated genes. The difficult task of designing appropriate experiments to test the adaptive role of each gene relies mainly on biochemical and regulatory information (Brenner, 2000; Meyer, 2003). The results presented in this article show that partial regulatory and functional redundancy, as well as recruitment into diverse biochemical functions and regulatory patterns (biochemical and regulatory subfunctionalization), may explain why four bean *pgip* genes have passed through the effective sieve of natural selection.

Functional redundancy is apparent for recognition of BcPG, CaPG, and SmPG, while evidence for subfunctionalization emerges when a wider spectrum of PGs is considered. For example, in the Pinto *pgip* family, only PvPPGIP2 inhibits FmPG. Moreover, in both Pinto and BAT93, only PvPGIP3 is unable to inhibit AnPG, only PvPGIP2 and PvPGIP4 inhibit BcPG at pH 7.0, and, remarkably, only PvPGIP3 and PvPGIP4 inhibit PGs of insects. Sequence diversifica-

tion between the gene pairs *Pvpgip1/Pvpgip2* and *Pvpgip3/Pvpgip4* therefore corresponds to functionally distinct classes of PGIPs, one devoted to high-affinity recognition of fungal PGs, the other exerting a weak activity against fungal PGs but showing inhibition of PGs of phytophagous insects. The biochemical functions of these two gene pairs therefore are not interchangeable. Inhibition of PGs by PGIPs is here reported for sap-sucking insects such as mirids and was previously reported for a weevil by a PGIP from orange (*Citrus sinensis*) exocarp (flavedo; Doostdar et al., 1997). Inhibition of PGs of *L. rugulipennis* and *A. lineolatus* however is not exerted by Arabidopsis PGIPs (our unpublished data), suggesting that the function of inhibiting PGs of insect has been acquired (or lost) more than once during evolution. Since PGIP expression is induced in response to insect feeding (Li et al., 2003), the possibility exists that the weak inhibitory activity of PGIPs against PGs of insects has a relevance in defense. The possible adaptive significance of the presence of PvPGIP1 is not obvious. Although its weak inhibitory activity may be due to relaxation of purifying selection, it is possible that the limited and simplified experimental conditions examined were not suitable to reveal its main function.

Our studies may help understand how the ability to recognize a pathogenicity factor that continually varies to escape recognition is maintained in a plant. No evidence for adaptive sequence evolution in *pgips* was obtained by determining  $K_a/K_s$  ratios, which are considered as suitable indicators to evaluate whether variability is the result of random drift ( $K_a/K_s = 1$ ) or adaptive evolution ( $K_a/K_s > 1$ ).  $K_a/K_s$  ratios estimated for the different regions of the *pgip* ORFs and the whole genes were either lower than 1 (for regions B, C-out, and D) or around 1 (for the xxLxLxx-encoding region that includes the  $\beta$ -sheet B1). However, frequency of nonsynonymous substitutions significantly higher than those in the other portion of the ORF indicated a higher variability of the xxLxLxx region, consistent with the hypervariability observed in many *R* genes (Meyers et al., 1998; Hulbert et al., 2001; Lehmann, 2002) and the role of this region in ligand recognition (Warren et al., 1998; Leckie et al., 1999). Maximum likelihood models of codon evolution in protein-coding DNA sequences (Nielsen and Yang, 1998) have been applied to *pgip* genes from several dicots to propose that positive selection acting at a handful of sites is responsible for the evolution of these genes (Stotz et al., 2000). Unfortunately, this model cannot be applied at this stage to analyze the intraspecific evolution of the bean *pgip* family, due to the low number of members and the low degree of variability of paralogs and homologs so far characterized.

However, positive selection has likely acted during evolution of bean *pgip* genes, as in most cases the limited variation between PvPGIPs results in significant changes of their recognition ability. A gain of



function is associated with the difference between Pinto PvPPGIP1 and PvPPGIP2 (recognition of FmPG), between PvPGIP3 and PvPGIP4 (recognition of AnPG), and between the paralogous pairs PvPGIP1/PvPGIP2 and PvPGIP3/PvPGIP4 (recognition of PGs of insects).

The characteristics of the PGIP-PG interaction may explain why limited changes can drastically modify PGIP specificity. A stable interaction between the two proteins likely requires a network of multiple and relatively weak contacts, which may be maintained during the evolution, and only one or very few strong contacts that lock the complex (Di Matteo et al., 2003). This limited number of locking contacts may be different in different PGIP-PG interactions and involve any of the solvent-exposed side chains of the non-conserved and nonstructural residues located in or close to the concave surface of PGIP. Different though overlapping subsets of residues may therefore be critical for binding different PGs (Federici et al., 2001; Di Matteo et al., 2003). The LRR structure of PGIP is particularly amenable to a tuning of the surface interaction properties with a very limited number of amino acid changes.

The amino acid sites 224 and 271 differ in all four paralogous *Pvpgip* genes; the former shows intergenotype variation in PvPGIP2. Replacements at position 271 do not significantly change the ability to recognize FmPG and AnPG (Leckie et al., 1999). Instead, replacements at 224 determine gain/loss of recognition of FmPG both between paralogous genes (PvPPGIP1 versus PvPPGIP2; Leckie et al., 1999) and between corresponding genes of different genotypes (PvBPGIP2 versus PvPPGIP2). Surprisingly, neither position is among those that are proposed to evolve adaptively in PGIPs, according to models of codon evolution (Stotz et al., 2000).

Duplication and diversification of *Pvpgip* genes result not only in a diversification of their biochemical function, but also in a diversification of their regulation. *PvPpgip3* responds to OGs but not to fungal glucan or SA or wounding, while *PvPpgip4* responds to none of these treatments. *PvPpgip1* responds only to wounding, in agreement with the results of the functional analysis of its promoter in *Nicotiana tabacum* (Devoto et al., 1998). Remarkably, *PvPpgip2*, which encodes the most efficient inhibitor of fungal PGs so far characterized, is the only family member that is up-regulated by all the stress stimuli examined. The presence of at least one W-box element in the 5' regulatory regions correlates with the inducibility of *Pvpgip1*, *Pvpgip2*, and *Pvpgip3* by stress-related stimuli.

Strategies of exploitation of *pgip* genes appear to differ in different plant species; for example, SA-dependent up-regulation of *pgip* expression occurs in bean but not in *Arabidopsis* or *Brassica napus* (Ferrari et al., 2003; Li et al., 2003). Interestingly, in both bean and *Arabidopsis*, OGs are elicitors of the expression of *pgip* genes in a specific manner because they induce only some of the family members (*Atpgip1*, *Pvpgip2*,

and *Pvpgip3*). The ability to switch on different *pgip* members in response to different stress-related signals therefore appears to be a common feature in plants and, like the biochemical subfunctionalization, is likely to have adaptive significance because it ensures the expression of at least one PGIP if a pathogen blocks or avoids the activation of a particular defense transduction pathway.

In conclusion, by using both a structural and functional genomic approach, we have shown that the presence of multiple *pgip* genes in bean likely reflects the need to adapt both their regulation and recognition features to combat more efficiently pathogenic fungi and phytophagous insects. Our observations will help to define the adaptive role of the multiple PGIPs present in bean. Furthermore, they pave the way to molecular anatomy studies to identify the amino acid replacements with specific adaptive significance and, therefore, to shed light into the basis of the recognition properties of plant LRR proteins.

## MATERIALS AND METHODS

### Plant Material

Seeds of *Phaseolus vulgaris* genotypes BAT93 and Pinto were obtained from Dr. V. Geffroy (Institut de Biotechnologie des Plantes, Orsay, France) and from a local merchant, respectively. Seeds were germinated and grown for 4 to 5 d in moist sterile vermiculite and maintained at 24°C with a 16-h-light period. Wounding and treatment with SA were performed as described previously (Bergmann et al., 1994).

Calli of Pinto bean were obtained by incubation of axenic bean hypocotyls as described previously (Salvi et al., 1990). Suspension-cultured cells were established and grown in Schenk and Hildebrand liquid medium containing 17 g L<sup>-1</sup> Suc, 1 g L<sup>-1</sup> naphthylacetic acid, 0.2 g L<sup>-1</sup> kinetin, in the dark at 26°C, under continuous agitation. When the packed cell volume (obtained by sedimenting cells for 30 min at RT) of 5 mL of suspension-cultured cells was 1.2 mL, cells were subcultured (packed cell volume = 0.4 mL). Four days after subculture, OGs (degree of polymerization = 9–18) or glucan were added to final concentration of 50 µg mL<sup>-1</sup>. Control cells were treated with distilled water. Cells were collected 0, 4, 10, 24, and 50 h after treatments. All tissues and cells were frozen at -70°C prior to RNA extraction. Three independent experiments were carried out for each treatment.

### Nucleic Acid Manipulation and Sequence Analysis

DNA manipulation, PCR, and cloning were performed according to standard procedures (Sambrook et al., 1989). For DNA sequencing, the Dye Terminators cycle sequencing kit with AmpliTaq DNA polymerase (Applied Biosystems, Monza, Italy) was used. Sequence reactions were analyzed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Nucleotide and amino acid sequence analysis was carried out using the programs in the GCG package (Devereux et al., 1984) and DNAMAN (Lynnon BioSoft, Vaudreuil, Quebec). Scans of promoter sequences for putative regulatory elements were performed using the PlantCARE (<http://sphinx.rug.ac.be:8080/PlantCARE/>) database algorithm (Rombauts et al., 1999).  $K_a$  and  $K_s$  nucleotide substitutions per site were estimated using the program K-estimator 5.5 (Comeron, 1999). A 2 × 2 contingency table G test was used to test for the significance of differences in  $K_a$  and  $K_s$  values (Zhang et al., 1997). The G-test was also applied to verify the homogeneity of the distribution for the different values observed between the six pairwise comparisons and between the different sequence regions. The expected values of the  $K_a$  and  $K_s$  were computed considering the sequence of the gene coding for the mature protein.

### Screening of Genomic Libraries

A λ phage and a BAC library were screened using as a probe a radiolabeled 732-bp DNA fragment, corresponding to most of the *PvPpgip1* coding region

(Toubart et al., 1992). The BAC library was prepared from bean genotype BAT93 into the *Hind*III site of pBelobAC11. Screening was performed using the same conditions used for the  $\lambda$  phage library. The phage genomic library, constructed in Lambda FIX II vector from Pinto bean, was obtained from Stratagene (La Jolla, CA). The library was plated, and a total of approximately  $1 \times 10^6$  recombinant plaque-forming units were screened. Plaque hybridization was carried out at high stringency as described in Sambrook et al. (1989). BAC DNA fragments containing *pgip* gene sequences were subcloned into plasmid vectors for subsequent analysis. Similarly, phage DNA from selected positive clones was prepared, and *Eco*RI fragments showing positive signals in Southern-blot experiments were subcloned in pBlueScript II SK+ for further analysis.

### Preparation of Vectors for Expression Using PVX

The coding regions of *pgip* genes from BAT93 and Pinto were amplified by PCR using sequence-specific oligonucleotides, including restriction sites for *Clal* and *Sall* at 5' and 3' ends, respectively, to facilitate cloning. The amplified fragments were digested with *Clal* and *Sall* and cloned into the pPVX201 expression vector. The plasmids obtained were used to inoculate *Nicotiana benthamiana* plants directly using 30  $\mu$ g of DNA/plant as described by Baulcombe et al. (1995).

### Preparation of PGIPs

PGIPs were purified from leaves of PVX-infected *N. benthamiana* plants. Leaves were homogenized in 1 M NaCl (2 mL/g), incubated with gentle shaking for 1 h at 4°C, and centrifuged 20 min at 10,000g. Supernatants were filtered through Miracloth (Calbiochem, San Diego). PvPGIP1 and PvPGIP2 of Pinto or BAT93 were purified as described by Leckie et al. (1999). For purification of PvPGIP3 and PvPGIP4, samples were brought to 25%  $(\text{NH}_4)_2\text{SO}_4$ , incubated at 4°C, and centrifuged. Proteins were precipitated from supernatant with 85%  $(\text{NH}_4)_2\text{SO}_4$ , recovered by centrifugation, and redissolved in 0.5 M NaCl, 20 mM Na acetate, pH 4.7. For PvPGIP3 purification, protein samples were subjected to chromatography on a desalting Sephadex G-25 Superfine column equilibrated with 100 mM sodium acetate, pH 4.7, and subsequently on a cation exchange (SP-Sepharose) column. Bound proteins were eluted with a 40-min linear gradient of 0 to 0.5 M NaCl in 100 mM sodium acetate, pH 4.7, at a flow rate of 1 mL min<sup>-1</sup>. For PvPGIP4 purification, the dissolved proteins were diluted about 10-fold and loaded on a diethylaminoethyl cellulose (DE52; Whatman, Kent, UK) column pre-equilibrated with 20 mM acetate, pH 4.7. The nonabsorbed proteins were loaded on a Sepharose-Aspergillus niger PGII column pre-equilibrated with 20 mM acetate, pH 4.7; bound PvPGIP4 was eluted with 1  $\times$  phosphate-buffered saline (10 mM sodium phosphate, 150 mM NaCl, pH 7.4). SDS-PAGE and immunoblotting were performed as described previously (Desiderio et al., 1997). Polyclonal antibodies raised against PGIP purified from bean pods were used for immunoblotting experiments.

### Preparation and Assay of Polygalacturonases and PGIPs

*Colletotrichum acutatum* isolate SHK788 (from lupin) and *Stenocarpella maydis* isolate PPRI #6353 (from maize) were a kind gift of Dr. Berger (University of Pretoria, South Africa). These are the same strains used in a previous work (Ferrari et al., 2003), where *C. acutatum* was indicated as *Colletotrichum gleosporioides* according to Yang and Sweetingham (1998); this fungal isolate has been now renamed by Dr. Berger based on the study of Talhinhas et al. (2002). *Botrytis cinerea* strain B05-10 was a kind gift of Dr. P. Tudzynski (Institut für Botanik Westfälische Wilhelms-Universität, Münster, Germany). Fungi were grown for 20 d on potato dextrose agar (Oxoid, Milan) at 24°C under constant light. Conidia of *C. acutatum* ( $5 \times 10^{-5}$  mL<sup>-1</sup>) or mycelium of *S. maydis* (1 cm<sup>2</sup>) were harvested, used to inoculate Czapek-Dox medium (NaNO<sub>3</sub> 2 g L<sup>-1</sup>, K<sub>2</sub>HPO<sub>4</sub> 1 g L<sup>-1</sup>, MgSO<sub>4</sub> 0.5 g L<sup>-1</sup>, KCl 0.5 g L<sup>-1</sup>, FeSO<sub>4</sub> 10 mg L<sup>-1</sup>, pH 7.0), and supplemented with 1% pectin. Cultures were incubated in a rotary shaker at 180 rpm and 21°C for 12 d, and filtrates were used for the PG activity assay. PGII of *A. niger* was prepared as described by Cervone et al. (1987), and PG of *Fusarium moniliforme* expressed in *Saccharomyces cerevisiae* was prepared as described previously (Caprari et al., 1996). *Lygus rugulipennis* Poppius and *Adelphocoris lineolatus* (Goeze) were field collected from alfalfa (*Medicago sativa*) and laboratory reared on fresh green beans and sunflower kernels (25  $\pm$  2°C, 70%  $\pm$  10% relative humidity, 14-h-light:10-h-dark photoperiod). For total protein extracts, males and females of

either species were homogenized in 1 M NaCl, 25 mM Na acetate, pH 4.7 (2 mL/g) in a mortar; the homogenate was incubated with gentle shaking for 1 h at 4°C and centrifuged 20 min at 10,000g. Supernatants were filtered through Miracloth and frozen.

Enzymatic activity of PGs (expressed in agarose plate units) and inhibitory activity of PGIPs were measured using a modified agarose diffusion assay in the presence of 20 mM Na acetate, pH 4.7 or 7.0, as described by Ferrari et al. (2003).

### Elicitor Preparation

Glucan, defined as those oligosaccharides, prepared by partial acid hydrolysis of isolated mycelia walls of *Phytophthora megasperma* f. sp. *glycinea*, that elute at the void volume of a low-resolution P-2 column (void  $\beta$ -glucan), was kindly provided by Dr. M. Hahn (University of Georgia, Athens). Elicitor-active OGs were prepared as described by Bellincampi et al. (1996).

### RT-PCR Analysis

Total RNA was isolated using RNeasy kit (Qiagen USA, Valencia, CA) according to manufacturer's instructions. After extraction, RNA samples were treated with RNase-free DNase I by incubation at 37°C for 30 min, extracted with one volume of phenol/chloroform, and the RNA precipitated by adding 0.1 volume 3 M sodium acetate and 2.5 volumes of 100% ethanol and stored at -70°C. Prior to each experiment RNA aliquots were centrifuged, washed with 70% ethanol, and resuspended in sterile diethylpyrocabonate-treated water. RNA concentration was determined both spectrophotometrically and by densitometric analysis of RNA bands following agarose gel electrophoresis. RT-PCR was carried out on total RNA (300 ng) using Ready-To-Go RT-PCR beads (Amersham Biosciences, Buckinghamshire, UK), according to manufacturer's instructions, in a Perkin Elmer 9600 thermal cycler (Perkin-Elmer Applied Biosystems, Foster City, CA). Extensive trials indicated that the number of amplification cycles and the initial amount of RNA template were critical parameters to avoid reaction plateau. Oligonucleotide primers (sense and antisense, respectively) were as follows: 5'-TCTTTGAGAACTGCACT and 5'-CGTCGAATGTGATTCCCT for *Pvpgip1*, 5'-TCTTTGAGCACTGCACA and 5'-CGTCGAATGTGATTCCGA for *Pvpgip2.1/2.2*, 5'-CCAAACTCCGTTTTCTCTAC and 5'-CCCTCTAAGTCTCTCGACT for *Pvpgip3*, and 5'-CCAAA-CTCGGTTTTATCCGA and 5'-CCCTCTAAGTCTCTCGACC for *Pvpgip4*.

The PCR conditions consisted of 35 cycles as follows: 94°C 1 min, 60°C 1 min, 72°C 1 min. The specificity of the primers was assessed in separate PCR experiments using, as a template, recombinant plasmid DNA containing the appropriate *pgip* cDNAs. A dilution series, ranging from 2 pg to 20 ng, confirmed that each oligonucleotide pair specifically amplified the correct *pgip* gene at 58°C and 60°C (data not shown). Negative control experiments, where the reverse transcriptase step was omitted, showed no amplification, demonstrating that specific amplification was due to initial RNA templates and not to contaminating DNA fragments. For each treatment, three independent experiments were performed.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AJ786408 to AJ786411.

### ACKNOWLEDGMENTS

We thank F. Cervone for helpful discussion and S. Benedetelli for statistical analysis of  $K_a$  and  $K_s$  values.

Received April 15, 2004; returned for revision June 10, 2004; accepted June 14, 2004.

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