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Tandemly Duplicated Arabidopsis Genes That Encode Polygalacturonase-Inhibiting Proteins Are Regulated Coordinately by Different Signal Transduction Pathways in Response to Fungal Infection

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Polygalacturonase-inhibiting proteins (PGIPs) are plant proteins that counteract fungal polygalacturonases, which are important virulence factors. Like many other plant defense proteins, PGIPs are encoded by gene families, but the roles of individual genes in these families are poorly understood. Here, we show that in Arabidopsis, two tandemly duplicated *PGIP* genes are upregulated coordinately in response to *Botrytis cinerea* infection, but through separate signal transduction pathways. *AtPGIP2* expression is mediated by jasmonate and requires *COI1* and *JAR1*, whereas *AtPGIP1* expression is upregulated strongly by oligogalacturonides but is unaffected by salicylic acid, jasmonate, or ethylene. Both *AtPGIP1* and *AtPGIP2* encode functional inhibitors of polygalacturonase from Botrytis, and their overexpression in Arabidopsis significantly reduces Botrytis disease symptoms. Therefore, gene duplication followed by the divergence of promoter regions may result in different modes of regulation of similar defensive proteins, thereby enhancing the likelihood of defense gene activation during pathogen infection.

INTRODUCTION

Plants need to coordinate the expression of a large number of defense-related antimicrobial proteins to restrict pathogen infections. The biochemical roles in the plant defense response of some of these proteins, such as chitinases and glucanases, have been defined, whereas others are poorly characterized (van Loon and van Strien, 1999). It has been shown that different signaling molecules regulate the induction of different defense proteins during infection. For example, the induction of PR1 in Arabidopsis requires salicylic acid (SA) and is mediated by the *NPR1* gene product (Cao et al., 1994). On the other hand, the expression of defensin requires the concomitant activation of transduction pathways mediated by both jasmonic acid (JA) and ethylene (Penninckx et al., 1996). Usually, defense proteins are encoded by families of closely related genes, and individual members of a family often exhibit different patterns of expression (Penninckx et al., 1996; Tornero et al., 1997). Nevertheless, detailed studies have not yet been performed that clearly show how specific structural, functional, and regulatory characteristics of individual members of these protein families contribute to defense against specific pathogens.

An important family of defense proteins are the polygalacturonase-inhibiting proteins (PGIPs). PGIPs belong to the large superfamily of Leu-rich repeat (LRR) proteins (Toubart et al., 1992), which also includes the products of several plant resistance (*R*) genes (Hammond-Kosack and Jones, 1997), the receptor kinase FLS2 that responds to bacterial flagellin (Gomez-Gomez and Boller, 2000), and receptor kinases involved in hormone perception (Li and Chory, 1997), development (Clark et al., 1997), insect defense responses (Scheer and Ryan, 2002), or bacterial and fungal symbiosis (Endre et al., 2002; Stracke et al., 2002). These proteins all contain LRRs of the extracytoplasmic type, which are characterized by the consensus sequence GxIPxxLGxLxxLxxLx-LxxNxLx (Kajava, 1998). PGIPs are present in the cell walls of all plants examined to date and specifically inhibit endopolygalacturonases (PGs) of fungi, but not those of plants or bacteria. PGs cleave the α-(1$\rightarrow$4) linkages between D-galacturonic acid residues in nonmethylated homogalacturonan polymers of the plant cell wall and cause the separation of cells from each other and the maceration of host tissue, thereby playing a key role in the development of soft-rot symptoms. The requirement of PG activity for full virulence

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has been demonstrated for the fungi *Botrytis cinerea* (ten Have et al., 1998), *Alternaria citri* (Isshiki et al., 2001), and *Claviceps purpurea* (Oeser et al., 2002) as well as for the bacterial pathogens *Agrobacterium tumefaciens* (Rodriguez-Palenzuela et al., 1991) and *Ralstonia solanacearum* (Huang and Allen, 2000), suggesting that PGs are important pathogenicity factors in a wide range of plant pathogens. The inhibition of PGs by PGIPs also is thought to cause the accumulation in the plant apoplast of oligogalacturonide (OG) fragments, which serve as elicitors of a wide range of defense responses (Cervone et al., 1989). A direct role for PGIPs in plant defense was demonstrated recently by showing that transgenic tomato plants overexpressing a pear *PGIP* gene exhibit enhanced resistance to Botrytis (Powell et al., 2000). However, no additional evidence for an in vivo protective role of PGIPs is available at present.

Not only do PGIPs from different plants differ in their inhibitory activity, but PGIPs from a single plant often exhibit different inhibitory activities against PGs from different fungi or different PGs from the same fungus (Desiderio et al., 1997). The presence of small families of *PGIP* genes accounts for the different inhibiting activities found in plant tissues (Frediani et al., 1993; Stotz et al., 1993, 1994). In *Phaseolus vulgaris*, for instance, at least four genes encode PGIPs; the protein encoded by *PvPGIP2* inhibits PGs from both *Fusarium moniliforme* and *Aspergillus niger*, whereas the product of *PvPGIP1* is effective only against the *A. niger* enzyme (Leckie et al., 1999).

The expression of PGIPs, taken as the sum of the expression of individual family members, is induced in response to several stress stimuli, such as wounding, pathogen infection, and elicitor treatments (Bergmann et al., 1994; Yao et al., 1999; Favaron et al., 2000). However, nothing is known about the regulation of expression or about the role of particular *PGIP* genes during pathogen infection. Because the biochemistry of PGIPs is well defined, it is possible to assess the actual contribution of individual family members to defense against a particular pathogen by determining how specific functional characteristics and modes of expression of PGIP proteins account for the effect on pathogen growth. The characterization of all members of a *PGIP* gene family is facilitated by the recent completion of the Arabidopsis genome sequence (Arabidopsis Genome Initiative, 2000).

Here, we show that two tandemly duplicated Arabidopsis genes, *AtPGIP1* and *AtPGIP2*, encode functional inhibitors of similar activity, both of which are able to enhance resistance against Botrytis when they are overexpressed in transgenic plants. Importantly, although *AtPGIP1* and *AtPGIP2* are induced coordinately during fungal infection, they are regulated by independent signal transduction pathways. These results suggest that the expression of proteins with similar defensive functions is not simply a reflection of functional redundancy. Rather, the differential regulation of functionally redundant proteins may ensure the expression of important defense activities regardless of the defense-related transduction pathway activated by a particular pathogen. Thus, these studies shed new light on the evolution of plant defense strategies against pathogens.

RESULTS

Two Closely Related *PGIP* Genes Are Located in Tandem on Chromosome 5 in Arabidopsis

A 21–amino acid sequence (5′-FDXSYFHNKCLCGAPLPS-CK-3′) conserved in the C terminus of all previously characterized PGIPs (De Lorenzo et al., 2001) was used as a virtual probe to search for putative *PGIP* genes in the Arabidopsis Database for EST Assemblies (http://www.tigr.org/tdb/agi). Two closely related tentative consensus assemblies of EST sequences, TC88249 and TC101150, showing 85 and 90% identity, respectively, to the probe, correspond to two distinct proposed genes, *AtPGIP1* (At5g06860) and *AtPGIP2* (At5g06870), located in direct tandem on chromosome 5 (Figure 1A). The distance between the predicted *AtPGIP1* and *AtPGIP2* open reading frames is 507 bp. No additional genes with significant nucleotide similarity to *AtPGIP1* or *AtPGIP2* could be identified in the Arabidopsis genome. Comparison of the sequences of the EST clones and of the genomic region harboring *AtPGIP1* and *AtPGIP2* indicated that both genes are interrupted at the same position by introns of 69 and 83 bp, respectively. The coding regions of *AtPGIP1* and *AtPGIP2* share 77.9 and 76.1% identity at the nucleotide and amino acid levels, respectively. Similarity also is high in the 3′ untranslated region (61% in the 100 bp downstream of the predicted stop codons), as deduced from the sequences of the available cDNA clones, whereas no significant conservation exists upstream of the translation start and in intron sequences.

The promoter regions of *AtPGIP1* and *AtPGIP2* (p*AtPGIP1* and p*AtPGIP2*) were analyzed for the presence of putative *cis*-acting regulatory elements (Table 1). Several elements containing the type II MYB consensus sequence (MBSII), a binding site for Myb-related transcription factors, are present in both promoters. Myb factors have been shown to regulate the transcription of several plant genes in response to a wide range of environmental cues, including wounding and elicitors (Jin and Martin, 2000; Sugimoto et al., 2000), and MBSII sites often are found upstream of pathogen-inducible genes (Rushton and Somssich, 1998). In addition, three sequences identical to the *LS4* element, TTGACT, which acts as a negative regulator of Arabidopsis *PR1* expression and as an elicitor-responsive element (W box) in parsley *PR1* (Eulgem et al., 1999, 2000), are located in p*AtPGIP1*, whereas only one is located in p*AtPGIP2*. Additional putative *cis*-acting regulatory elements are present in p*AtPGIP1* but not in p*AtPGIP2*. A sequence at position −188 shows high similarity to the sequence 5′-AAGCGTAAGT-3′ found at position −165 of the potato proteinase inhibitor II K promoter, in which it is required for wound-induced expression

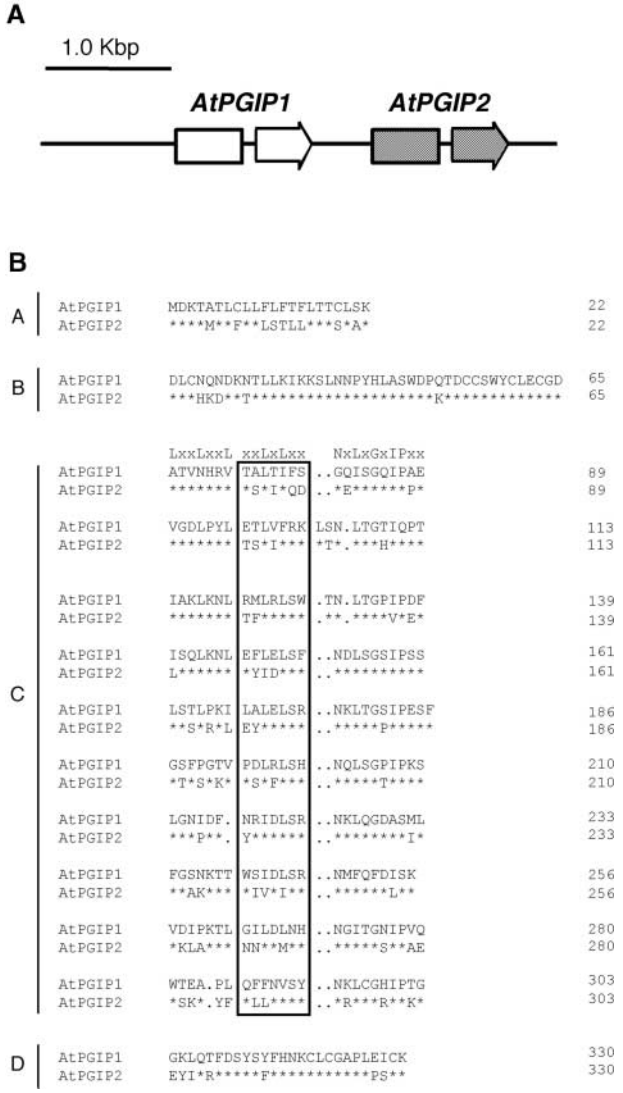

Figure 1. Genomic Organization of *AtPGIP1* and *AtPGIP2* and Comparison of the Predicted Amino Acid Sequences.

(A) Genomic organization of *AtPGIP1* and *AtPGIP2*. Arrows represent *AtPGIP1* and *AtPGIP2* open reading frames, each interrupted by an intron.

(B) Sequence alignment of AtPGIP1 and AtPGIP2. The predicted amino acid sequences were aligned using the CLUSTAL W method (Higgins and Sharp, 1988). Typical PGIP domains are indicated (A, signal peptide; B, presumed N terminus of the mature protein; C, 10 LRR modules; D, C terminus). The consensus sequence for extracytoplasmic LRR is indicated above the first LRR module. Residues in the boxed region are predicted to form a β-sheet/β-turn motif (xxLxLxx). Asterisks indicate invariant amino acids in AtPGIP1 and AtPGIP2. Dots represent gaps inserted in the sequences for the optimal alignment of LRR modules.

(Palm et al., 1990). Three putative HSRE elements, which are AT-rich motifs conserved in the promoters of several tobacco pathogen-induced genes (Pontier et al., 2001), also are present in p*AtPGIP1*. Finally, two putative low temperature–responsive elements (5′-CCGAC-3′), similar to that required for cold induction of the Arabidopsis *cor15a* gene (Baker et al., 1994), are present in p*AtPGIP1* but not in p*AtPGIP2*.

AtPGIP1 and AtPGIP2 Are Functional Inhibitors Capable of Restricting Fungal Infection

The predicted proteins encoded by *AtPGIP1* and *AtPGIP2* both consist of 330 amino acids and display the typical topology of all previously described PGIPs, including a 22–amino acid signal peptide for secretion (domain A), an N-terminal domain (domain B), a LRR domain composed of 10 imperfect modules and characterized by the extracytoplasmic-type LRR consensus sequence (domain C), and a C-terminal domain (domain D) (Figure 1B). The mature AtPGIP1 and AtPGIP2 polypeptides have a predicted molecular mass of ~34 kD and pI values of 7.8 and 8.7, respectively. A high degree of amino acid identity, ranging from 60 to 64%, was observed with PGIPs from kiwifruit, orange, apple (Yao et al., 1999), and pear (Stotz et al., 1993). There are 70 amino acid substitutions between mature AtPGIP1 and AtPGIP2, mostly in the first five LRRs, suggesting that AtPGIP1 and AtPGIP2 might differ in their inhibiting activities.

To study their biochemical properties, AtPGIP1 and AtPGIP2 were overexpressed stably in Arabidopsis as described in Methods. Several of these transgenic plants exhibited very high levels of inhibitory activity against a PG activity isolated from Botrytis (>15,000 inhibitory units/mg total protein; see below), whereas extracts from control plants exhibited a much lower inhibitory activity (150 to 200 inhibitory units/mg). No obvious morphological differences between transgenic plants and nontransformed controls were observed. T2 plants of the high-expressing transgenic lines 1-5E1 and 2-4A3 (see below) were used to purify AtPGIP1 and AtPGIP2, respectively, to near homogeneity. Both AtPGIP1 and AtPGIP2 bound to a cation-exchange chromatography column (see Methods) and were eluted with 0.35 and 0.52 M NaCl, respectively, whereas extracts from untransformed plants showed very low levels of an inhibitory activity eluting at the same ionic strength as AtPGIP1 (Figure 2A). When subjected to SDS-PAGE, purified AtPGIP1 and AtPGIP2 migrated as broad protein bands with mobility corresponding to a molecular mass of ~38 kD for AtPGIP1 and 36 kD for AtPGIP2 (Figure 2B, left). These bands reacted with polyclonal antibody prepared against PGIP from *P. vulgaris* (Figure 2B, right). The inhibitory activities of purified AtPGIP1 and AtPGIP2 were measured against several fungal PGs (Table 2). Both AtPGIPs exhibited comparable inhibitory activity against PG of Botrytis but failed to inhibit PGs of *A. niger* or *F. moniliforme*. However, AtPGIP1

Table 1. Notable Putative *cis*-Acting Elements in the *AtPGIP1* and *AtPGIP2* Promoters

		AtPGIP1		*AtPGIP2*	
Category	*cis* Element[a]	Sequence[b]	Position[c]	Sequence[b]	Position[c]
Myb sites	MBSII	ttgggtt	−314/−307[d]	tcctacc	−350/−357
	[a(a/c)c(a/t)a(a/c)c]	aaccaac	−876/−883	aaccaaa	−414/−421
		accaac	−1088/−1095	ttggggtt	−274/−266[d]
Pathogen response	HSRE (taaaatnttng)	taaatctcttc	−436/−447		
		tttaatattta	−1165/−1154[d]		
		taaaatgtgt	−1240/−1250		
	LS4 (ttgact)	agtcaa	−1190/−1184[d]	agtcaa	−468/−462[d]
		ttgact	−146/−152		
		ttgact	−128/−134		
Wound induction	PINIIK (aagcgtaagt)	aacgcgtaatt	−188/−199		
Cold induction	LTRE (gccgac)	gccgacat	−155/−163		
		gtcgg	−420/−415[d]		

[a] For additional details and references, see text. The consensus sequence is indicated in parentheses.
[b] Sequence is indicated from the 5′ to the 3′ end.
[c] Position of the *cis* element with respect to the translation start (5′ end/3′ end).
[d] Sequence on the complementary strand.

had ~10-fold and 7-fold more inhibitory activity than AtPGIP2 against PGs from *Colletotrichum gloeosporioides* and *Stenocarpella maydis*, respectively. Thus, comparative analysis of the inhibitory activities against PGs from Botrytis and *C. gloeosporioides* can be used to help discriminate between AtPGIP1 and AtPGIP2 in Arabidopsis extracts.

Because both AtPGIP1 and AtPGIP2 are very efficient inhibitors of Botrytis PG, we assessed whether the overexpression of AtPGIP1 or AtPGIP2 in transgenic Arabidopsis resulted in enhanced Botrytis resistance. Three transgenic lines corresponding to AtPGIP1 (1-5E1, 1-5E2, and 1-4E2) and three transgenic lines corresponding to AtPGIP2 (2-4A3, 2-4B1, and 2-3F3) were selected for analysis. Lines 1-5E1 and 1-5E2, expressing high levels of *AtPGIP1* transcripts, and lines 2-4A3 and 2-4B1, expressing high levels of *AtPGIP2* transcripts (Figure 3A), also exhibited very high levels of inhibitory activity against PG of Botrytis (>15,000 inhibitory units/mg total protein; data not shown). Lines 1-4E2 and 2-3F3, which failed to express detectable levels of *AtPGIP1* or *AtPGIP2* mRNA, respectively (Figure 3A), exhibited levels of inhibitory activity comparable to those of untransformed plants. Leaves of wild-type and transgenic plants were inoculated with Botrytis, and the extent of disease symptoms was determined after 3 days. No significant differences were observed between the average diameters of the lesions formed by Botrytis on the leaves of untransformed plants or of 1-4E2 or 2-3F3 plants (Figures 3B and 3C). By contrast, lesion size was reduced by ~30% in leaves overexpressing AtPGIP1 (1-5E1 and 1-5E2 plants) (Figure 3B) and in leaves overexpressing AtPGIP2 (2-4A3 and 2-4B1 plants) (Figure 3C). Therefore, overexpression of AtPGIP1 and AtPGIP2 conferred similar levels of protection against Botrytis.

AtPGIP1 and *AtPGIP2* Are Induced in Response to Botrytis Infection through Separate Transduction Pathways

The expression of *AtPGIP1* and *AtPGIP2* in untransformed plants was investigated by RNA gel blot analysis using specific probes corresponding to the 3′ untranslated region sequence of each gene. Levels of both *AtPGIP1* and *AtPGIP2* transcripts increased in a similar manner in infected Arabidopsis rosette leaves after inoculation with Botrytis or mechanical damage but not in uninfected or nonwounded leaves (Figures 4A and 4B). Analysis of transgenic Arabidopsis plants transformed with the *uidA* reporter gene under the control of the *AtPGIP1* or *AtPGIP2* promoter (*AtPGIP1*::*GUS* [β-glucuronidase] and *AtPGIP2*::*GUS*) confirmed that both promoters are activated upon infection and wounding (Figure 4C). PGIP expression has been shown to be induced in bean by plant cell wall–derived elicitors (Bergmann et al., 1994). Incubation of Arabidopsis seedlings with elicitor-active OGs caused a transient increase of *AtPGIP1* but not *AtPGIP2* transcripts, detectable as early as 90 min after treatment and sustained for at least 6 h (Figure 5A). By 24 h, *AtPGIP1* transcripts returned to basal levels. OGs also induced an increase of GUS activity in *AtPGIP1*::*GUS* seedlings (Figure 5B) but not in *AtPGIP2*::*GUS* seedlings (data not shown). In agreement with the presence of low temperature–responsive elements in the *AtPGIP1* promoter, incubation of seedlings at 4°C also elicited a significant accumulation of *AtPGIP1* but not of *AtPGIP2* transcripts (Figure 5C) and an increase of GUS activity in the leaves of *AtPGIP1*::*GUS* but not *AtPGIP2*::*GUS* plants (Figure 5D).

Many defense genes active against necrotrophic fungi appear to be regulated primarily by signal transduction path-

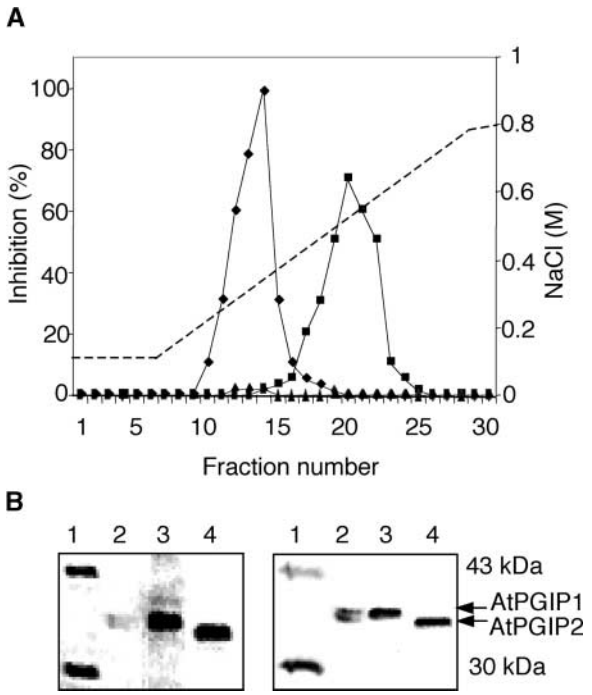

Figure 2. Purification of AtPGIP1 and AtPGIP2.

(A) Inhibitory activity of purified AtPGIP1 and AtPGIP2. Desalted total protein extracts from 1-5E1 (squares) and 2-4A3 (diamonds) plants, overexpressing *AtPGIP1* and *AtPGIP2*, respectively, and from untransformed plants (triangles) were subjected to chromatography on a cation-exchange column (SP-Sepharose). Inhibitory activity of the collected fractions was tested against PG of Botrytis.
(B) SDS-PAGE of AtPGIPs purified from overexpressing transgenic plants (left) and immunodetection using a polyclonal antibody against bean PGIP (right). Lanes 1, molecular mass markers; lanes 2, PGIP purified from bean pods; lanes 3, AtPGIP1; lanes 4, AtPGIP2. The sizes of the marker bands shown in lanes 1 are indicated at right.

ways that use ethylene and/or jasmonate as secondary messengers, but not by SA (Thomma et al., 1998, 1999). We determined the role of SA and methyl jasmonate (MeJA) on the expression of *AtPGIP1* and *AtPGIP2*. Exogenous SA had no significant effect on the expression of either gene but, as expected, induced high levels of *PR1* mRNA (Figure 6A). Instead, MeJA activated the expression of *AtPGIP2* but not of *AtPGIP1* (Figure 6A) and resulted in high GUS activity in *AtPGIP2*::*GUS* but not *AtPGIP1*::*GUS* plants (Figure 6B). As reported previously (Penninckx et al., 1996), *PDF1.2* expression increased dramatically in response to MeJA treatments (Figure 6A).

To further define the role of SA, MeJA, and ethylene in AtPGIP expression during infection, Arabidopsis wild-type, *nahG*, *npr1*, *ein2*, *coi1*, and *jar1* plants were inoculated with Botrytis. Transgenic *nahG* Arabidopsis plants, in which SA is degraded by the product of the bacterial *nahG* gene (Gaffney et al., 1993), and the Arabidopsis *npr1* mutant, which is unable to respond to exogenous SA and is blocked in *PR1* induction (Cao et al., 1994), are more susceptible to infection with biotrophic fungi such as *Peronospora parasitica* (Cao et al., 1994; Delaney et al., 1994) and *Erisyphe orontii* (Reuber et al., 1998). The *ein2* mutant is insensitive to ethylene (Guzman and Ecker, 1990), whereas *coi1* and *jar1* are insensitive to jasmonates (Staswick et al., 1992; Feys et al., 1994). In previously published work, both *ein2* and *coi1* mutants failed to express the defensin gene *PDF1.2* and showed enhanced symptoms after Botrytis infection (Thomma et al., 1998, 1999). We found that 48 h after Botrytis infection, expression of *AtPGIP1* was induced to a similar extent in wild-type plants and in all of the mutants tested, whereas *AtPGIP2* mRNA accumulation was reduced strongly in the *coi1* and *jar1* mutants (Figure 6C). In agreement with previous reports (Zimmerli et al., 2001), *PDF1.2* expression in response to Botrytis infection was impaired in the *ein2*, *coi1*, and *jar1* mutants, whereas *PR1* expression was reduced severely in the *nahG* and *npr1* plants (Figure 6C). *AtPGIP1* expression in response to OGs also was unaffected in *nahG*, *npr1*, *ein2*, *jar1*, and *coi1* seedlings (Figure 6D), suggesting that *AtPGIP1* expression is induced by OGs independently of SA, ethylene, or JA.

To determine if the accumulation of *AtPGIP1* and *AtPGIP2* transcripts is followed by an increase of the corresponding inhibitory activities in the plant tissue, total protein extracts from Arabidopsis seedlings were assayed after different treatments for their ability to inhibit PG from Botrytis and *C. gloeosporioides.* Wild-type seedlings treated with OGs or incubated at low temperature showed an increase in inhibitory activity against both PGs (Figures 7A and 7B), with kinetics similar to that of the accumulation of *AtPGIP1* transcripts (Figures 5A and 5C). By contrast, extracts from seedlings treated with MeJA were more efficient against PG from Botrytis than against PG from *C. gloeosporioides* (Figure

Table 2. Inhibitory Activities of PGIPs against Fungal *endo*-PGs

	Bean	Arabidopsis	
Endo-PG	PvPGIP2[a] (ng)[b]	AtPGIP1 (ng)[b]	AtPGIP2 (ng)[b]
Aspergillus niger	1.0	∞	∞
Fusarium moniliforme	9.0	∞	∞
Colletotrichum gloeosporioides	12	1.7	16.5
Stenocarpella maydis	5.0	1.7	11.7
Botrytis cinerea	2.5	2.0	2.9

[a] Purified PvPGIP2 of *P. vulgaris* cv Pinto expressed in *Nicotiana benthamiana* using a modified *Potato virus X*–based vector (Leckie et al., 1999).
[b] The amount of PGIP that determines 50% inhibition of 1 agarose diffusion unit of PG. The symbol ∞ indicates no inhibition.

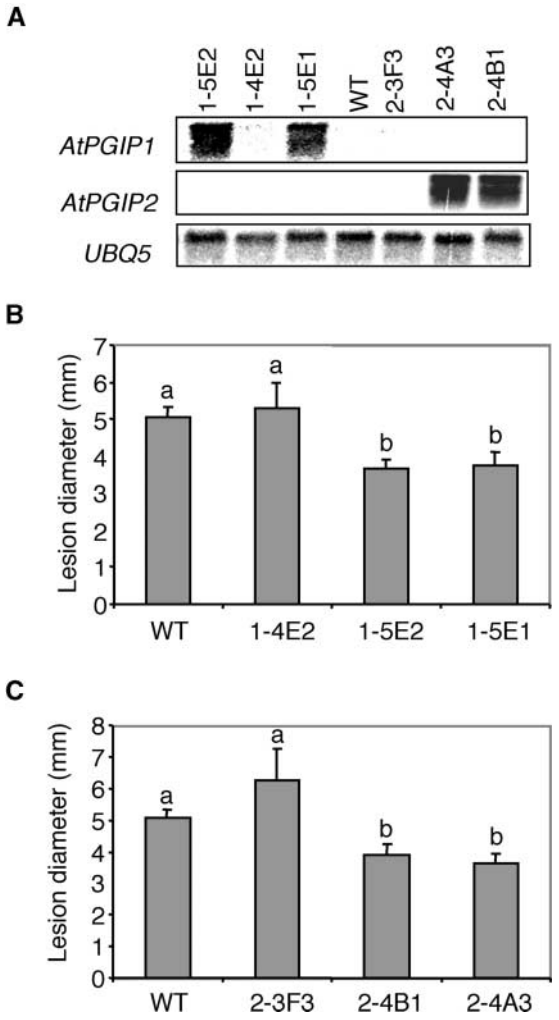

Figure 3. Reduction of Botrytis Symptoms in Arabidopsis Plants Overexpressing *AtPGIP1* or *AtPGIP2*.

(A) RNA gel blot of independent lines transformed with either *35S*::*AtPGIP1* (1-4E2, 1-5E1, and 1-5E2) or *35S*::*AtPGIP2* (2-3F3, 2-4A3, and 2-4B1). WT, untransformed wild-type plants (Col-0).
(B) and **(C)** Botrytis symptoms in *35S*::*AtPGIP1* **(B)** and *35S*::*AtPGIP2* **(C)** transgenic plants. Detached leaves from wild-type (WT) or T2 lines transformed with *35S*::*AtPGIP1* (1-4E2, 1-5E1, and 1-5E2) or *35S*::*AtPGIP2* (2-3F3, 2-4A3, and 2-4B1) were inoculated with Botrytis. The diameter of the necrotic lesions was measured 3 days after infection. The experiment was repeated twice with similar results. Bars represent the average of at least 30 samples ± SE. Different letters indicate data sets significantly different according to Tukey's Student range test ($P > 0.95$).

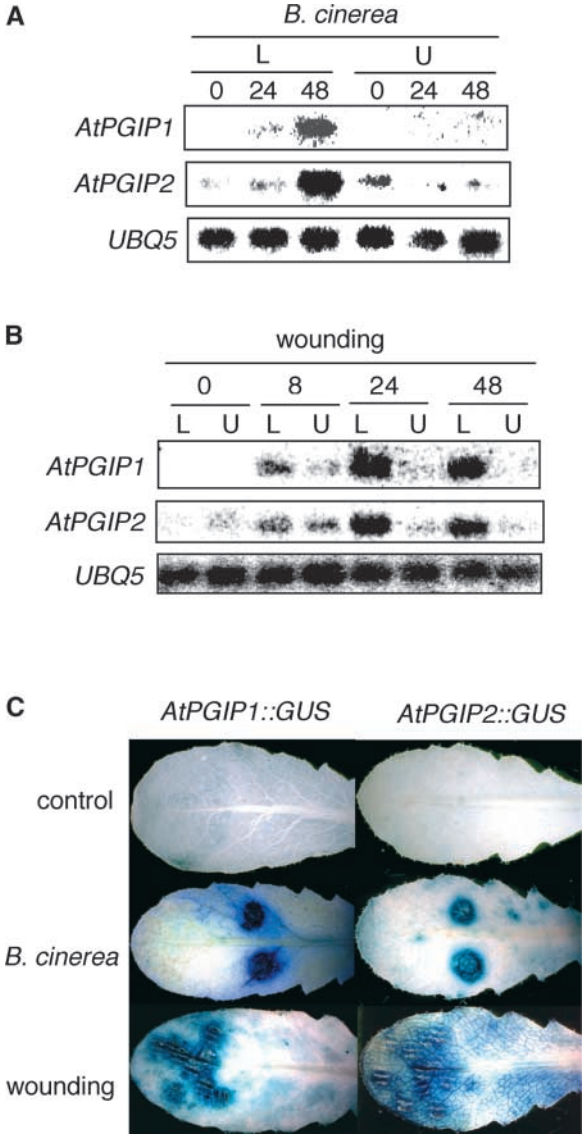

Figure 4. Induction of *AtPGIP1* and *AtPGIP2* in Response to Botrytis Infection and Wounding.

(A) and **(B)** RNA gel blot of wild-type leaves inoculated with Botrytis **(A)** or mechanically wounded **(B)** and harvested at the indicated times (in hours). L, treated leaves; U, upper, untreated leaves.
(C) Leaves from transgenic *AtPGIP1*::*GUS* and *AtPGIP2*::*GUS* plants harvested at 48 h after inoculation with sterile medium (top leaves) or Botrytis (middle leaves) or at 24 h after wounding (bottom leaves) and stained for GUS activity. A representative sample is shown for each treatment.

7C). This result is consistent with the induction of *AtPGIP2*, but not of *AtPGIP1*, mRNA observed after MeJA treatment (Figure 6A). Therefore, the increased expression of *AtPGIP1* or *AtPGIP2* in Arabidopsis seedlings is accompanied by the accumulation of the expected inhibitory activities.

These results indicate that *AtPGIP1* and *AtPGIP2* are regulated differentially in response to defense-related signals. The induction of *AtPGIP2* is independent of SA or ethylene but appears to be mediated by JA, because it requires the

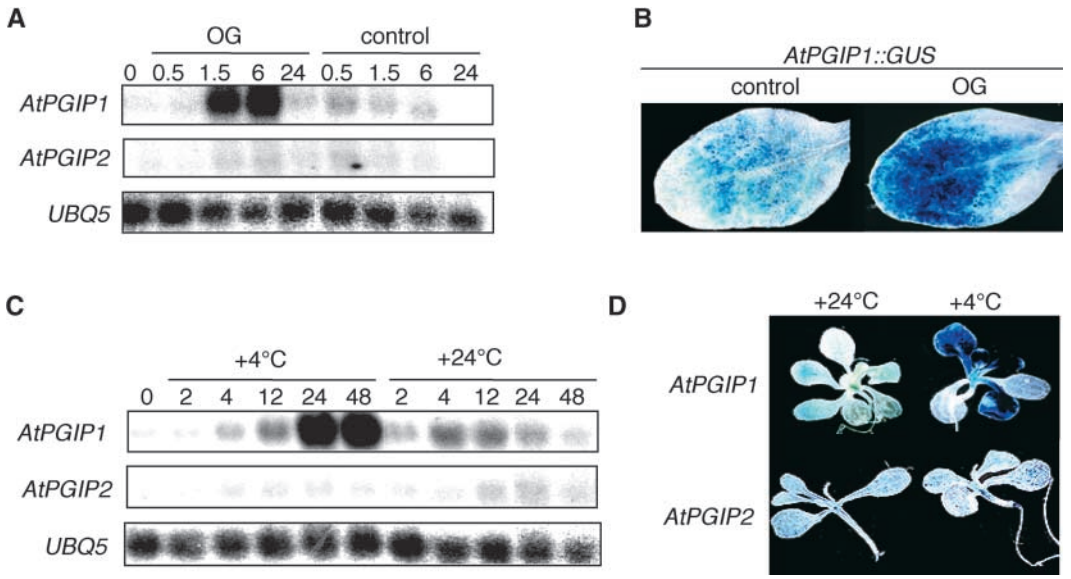

Figure 5. Induction of *AtPGIP1* in Response to OGs and Low Temperature.

(A) RNA gel blot of seedlings incubated in the presence of 100 μg/mL OGs or in sterile medium (control) and harvested at the indicated times (in hours).

(B) *AtPGIP1*::*GUS* seedlings were treated as in **(A)** and stained for GUS activity after 6 h of treatment. The image shows the first leaf of a representative seedling treated with sterile medium (control) or OGs.

(C) RNA gel blot of Arabidopsis seedlings incubated at 4 or 24°C and harvested at the indicated times (in hours).

(D) *AtPGIP1*::*GUS* and *AtPGIP2*::*GUS* seedlings were incubated at 4 or 24°C for 48 h and stained for GUS activity. The image shows a representative seedling for each treatment.

COI1 and *JAR1* gene products. By contrast, the induction of *AtPGIP1* is independent of all of these effectors and is likely to be mediated by OGs. We conclude that the regulation of *AtPGIP1* and *AtPGIP2* during pathogen infection takes place through distinct signal transduction pathways.

DISCUSSION

Most plant defense proteins are encoded by families of closely related genes that usually display specific structural and regulatory features. The presence of multiple genes may reflect the evolutionary advantage of functional redundancy, which is likely to ensure a higher level of protection and confer a selective advantage, or it may be a consequence of the acquisition of new recognition specificity or subfunctionalization, such as the partitioning of the task of an ancestral protein into separate gene products (Lynch et al., 2001). Alternatively, the advantage of having multiple genes may derive from their different modes of expression. These possibilities have been considered in this study, which was designed to elucidate the role of a small Arabidopsis *PGIP* gene family comprising two adjacent genes. We have shown that *AtPGIP1* and *AtPGIP2* encode functional PGIPs with comparable inhibitory activities toward a PG from Botrytis that was shown previously to be an important virulence factor (ten Have et al., 1998). We also have shown that both *PGIP* genes are activated by Botrytis infection with similar kinetics and limit Botrytis tissue colonization to a similar extent when they are overexpressed in transgenic plants. However, *AtPGIP1* and *AtPGIP2* are responsive to different defense-related signals, and their expression during infection is mediated through separate transduction pathways. These results suggest that one consequence of gene duplication can be the regulation of proteins with similar function and defensive potential by independent signal transduction pathways.

Our experiments show that no apparent loss or gain of recognition ability, but only a different ability to inhibit several fungal PGs, is associated with the divergence of AtPGIP1 and AtPGIP2: the latter is less efficient toward PGs of *C. gloeosporioides* and *S. maydis*. Seventy amino acids distinguish the two proteins, a number much higher than that observed in other cases. Between PvPGIP1 and PvPGIP2 of *P. vulgaris*, for example, only eight amino acids are different; nevertheless, these latter proteins exhibit different recognition specificities (Leckie et al., 1999). Furthermore, a single variation introduced in the xxLxLxx motif of the *P. vulgaris* PvPGIP1 LRR, which is predicted to form a β-strand/β-turn

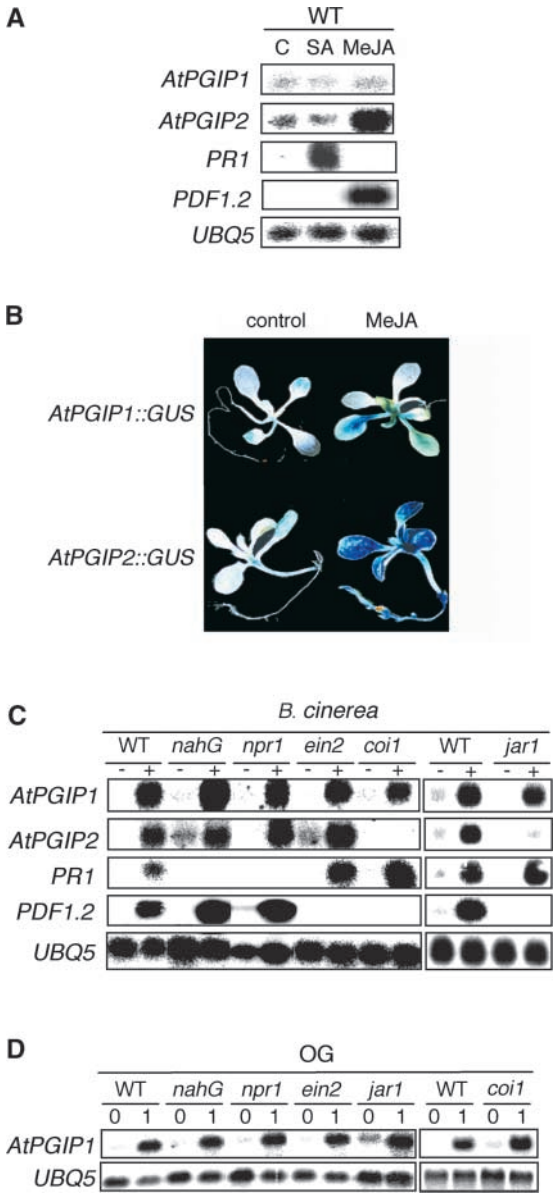

Figure 6. Effect of Disease-Related Signals on *AtPGIP1* and *AtPGIP2* Expression.

(A) RNA gel blot of leaves harvested at 48 h after treatment with 5 mM SA, 100 μM MeJA, or control solution (C). WT, wild type.

(B) *AtPGIP1*::*GUS* and *AtPGIP2*::*GUS* seedlings were incubated for 48 h in the presence of 50 μM MeJA or in control medium and stained for GUS activity. The image shows a representative seedling for each treatment.

(C) RNA gel blot of wild-type, *nahG*, *npr1*, *ein2*, *coi1*, and *jar1* leaves inoculated with Botrytis (+) or with sterile medium (−) and harvested after 48 h.

(D) RNA gel blot of wild-type, *nahG*, *npr1*, *ein2*, *jar1*, and *coi1* seedlings treated with 100 μg/mL OG and harvested at the indicated times (in hours).

structure in which the x residues are exposed to solvent and involved in ligand binding (Kobe and Deisenhofer, 1995), is critical for affinity and specificity for the PG ligands and confers the ability to recognize a novel PG (Leckie et al., 1999). The evolution of the interactive properties of PGIPs and their cognate fungal PGs is complicated by the fact that PGIPs likely require the maintenance of features necessary for the recognition of the basic PG structure while at the same time maintaining a high degree of variability in those surface residues that establish specific contacts with each PG. The observation that the two Arabidopsis PGIPs show similar inhibitory activities against PG of Botrytis but not of *C. gloeosporioides* or *S. maydis* indicates that PGIP residues that are important for the interaction with a certain PG may not be involved in the interaction with other PGs and suggests that different but overlapping subsets of residues may be critical for the binding of different ligands.

Interestingly, although *AtPGIP1* and *AtPGIP2* show an apparently identical pattern of induction in response to wounding and Botrytis infection (Figures 4A to 4C), they respond differentially to several signals known to regulate these responses. The induction of both *PGIP* genes during Botrytis infection is independent of the SA and ethylene pathways, but only the expression of *AtPGIP2* appears to be mediated by JA, because it is induced by exogenous MeJA and its induction during infection is impaired in the *coi1* and *jar1* mutants (Figures 6A and 6C). In Arabidopsis, JA mediates the accumulation of several defense proteins in response to pathogen infection (Epple et al., 1995; Penninckx et al., 1996) and is required for resistance to some fungal pathogens, including Botrytis (Thomma et al., 1998). Other pathogen-induced genes, such as *LOX2* and *VSP* (Reymond and Farmer, 1998), are known to be regulated by JA but not by ethylene; the antifungal gene *PDF1.2* requires the activation of both pathways (Penninckx et al., 1998). In contrast to *AtPGIP2*, *AtPGIP1* is induced by exogenously added OGs (Figures 5A and 5B), a signal known to be involved in the wound response (Doares et al., 1995). Interestingly, *AtPGIP1* expression in response to OGs is independent of SA, ethylene, or JA (Figure 6D). An OG-dependent, JA- and ethylene-independent pathway for the induction of gene expression has been described, but only for response to wounding (Rojo et al., 1999). Our data suggest that this pathway also may be activated in response to fungal infection and is responsible for the expression of *AtPGIP1*. Thus, the *AtPGIP1* promoter represents a useful tool with which to gain further insight into the mechanisms of perception and transduction of OG signals as well as into SA-, ethylene-, and JA-independent signal transduction pathways.

The data presented here indicate that the regulation of two closely related defensive proteins with similar biochemical activity can occur through different signaling pathways in response to pathogen attack. Different modes of regulation of PGIPs during pathogen attack may confer a selective advantage and help explain the maintenance of duplicated *PGIP* genes in Arabidopsis. Although it is known that mem-

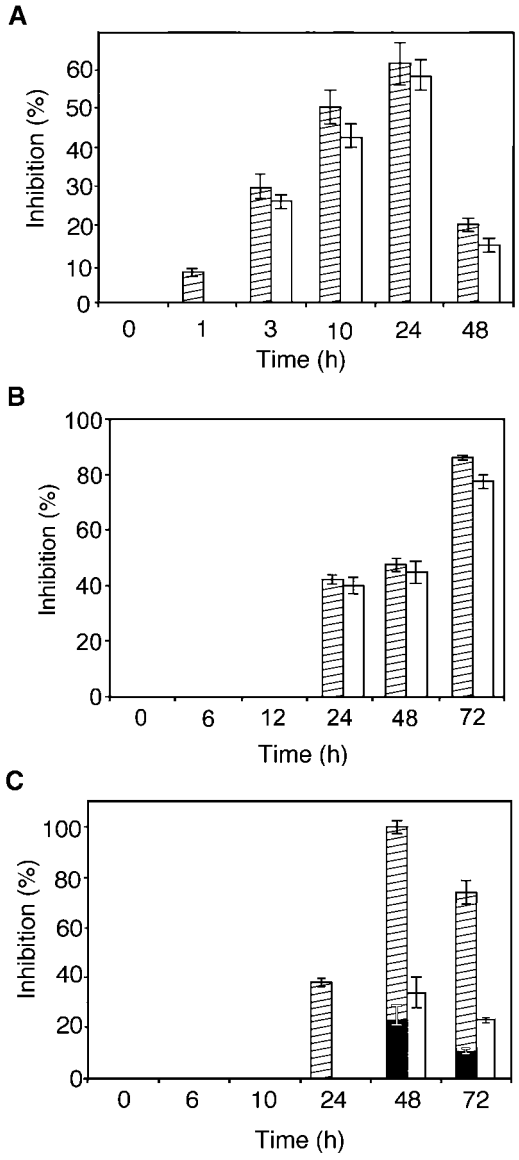

Figure 7. Accumulation of PGIP Activity in Response to OGs, Cold, and MeJA.

Inhibition of Botrytis (striped bars) and *C. gloeosporioides* (open bars) endo-PGs by protein extracts from seedlings treated with OGs **(A)**, low temperature **(B)**, or MeJA **(C)**.

(A) Seedlings were treated with OGs (100 μg/mL) and harvested at the indicated times. Bars represent the average inhibition of PG activity ± SD of three independent experiments, using 8 μg of total protein. The comparable ability to inhibit these two enzymes indicates that the induced PGIP is represented mainly by AtPGIP1. No inhibitory activity was detected in untreated control plants.

(B) Seedlings were incubated at 4°C and harvested at the indicated times. Bars represent the average inhibition of PG activity ± SD of three independent experiments, using 3 μg of total protein. The comparable ability to inhibit these two enzymes indicates that the induced PGIP is represented mainly by AtPGIP1. No inhibitory activity was detected in untreated control plants.

(C) Seedlings were treated with 50 μM MeJA and harvested at the indicated times. Bars represent the average inhibition of PG activity ± SD of three independent experiments, using 3 μg of total protein. Superimposed black bars indicate the activity against Botrytis PG detected in control plants. The lower inhibition of *C. gloeosporioides* PG indicates that the induced PGIP is represented mainly by AtPGIP2.

bers of a defense gene family can be regulated differentially, the independent activation of more than one family member during infection has not been described. For example, in the cases of both the tobacco genes *PR1a2* and *PR1b1* (Tornero et al., 1997) and the Arabidopsis genes *PDF1.1* and *PDF1.2* (Penninckx et al., 1996), only one gene in each pair is induced by infection, whereas the other copy is expressed constitutively in a tissue-specific manner or at a specific developmental stage. Moreover, there is no evidence that the constitutively expressed gene in either case contributes to defense. The observation that two members of the peanut class II chitinase gene family, *A.h.Chi2;1* and *A.h.Chi2;2*, are induced upon inoculation with fungal spores in suspension cultured cells, but that only *A.h.Chi2;2* responds to exogenous ethylene or SA, suggests that different signaling pathways activate the expression of the two genes (Kellmann et al., 1996). However, it is not known whether the encoded chitinases play similar roles in defense or whether this differential regulation also occurs in planta during infection.

The differential regulation of the Arabidopsis *PGIP* genes also is reflected in the observation that cold induces the expression of *AtPGIP1* but not *AtPGIP2* (Figures 5C and 5D). The accumulation of *PGIP* transcripts after storage in the cold also has been shown in apple (Yao et al., 1999), and interestingly, a carrot antifreeze protein shows a high degree of identity to PGIPs (Worrall et al., 1998). A dual role for PGIPs in protection against pathogens and cold stress is conceivable. Because low temperatures, like other stressful conditions, can increase susceptibility to diseases, cold induction of defensive proteins might provide protection from infections. Furthermore, several additional antimicrobial proteins possess antifreeze properties. For example, proteins with β-glucanase or chitinase activity highly homologous with PR proteins accumulate in the leaf apoplast of winter rye after cold exposure (Hon et al., 1995) and display antifreeze activity (Hiilovaara-Teijo and Palva, 1999).

The facts that *AtPGIP1* and *AtPGIP2* show completely overlapping expression patterns and kinetics after Botrytis infection, that they encode proteins with similar activity against PG of the same fungus, and that their overexpression confers comparable levels of protection suggest that they play similar protective roles against Botrytis. Therefore, the finding that the expression of *AtPGIP1* and *AtPGIP2* during fungal infection is mediated by separate signals is

intriguing. Extensive cross-talk occurs between separate defense-related pathways, and it has been proposed that some pathogens may exploit the antagonistic interaction between different host signals to avoid the activation of specific defense responses (Kunkel and Brooks, 2002). For instance, the induction of SA-dependent defense responses by Botrytis appears to be repressed by the activation of the JA-dependent pathway (Zimmerli et al., 2001). It is potentially advantageous for the plant to activate the expression of defense proteins through separate pathways, and it is likely that genes belonging to families other than the PGIP family may be regulated independently in a similar manner. If a pathogen is able to block or avoid the activation of the pathway required for the induction of one copy, the other copy still will be expressed. This is compatible with the recent duplication-degeneration-complementation model of gene evolution, according to which the loss of regulatory subfunctions after gene duplication may facilitate, rather than hinder, the preservation of duplicated genes (Force et al., 1999).

In conclusion, once again the model plant Arabidopsis has proven to be an invaluable tool with which to investigate in detail the biochemistry and molecular biology of a plant defense mechanism. We have shown that gene duplication allows the expression of proteins with similar inhibitory activity through independent signaling pathways, resulting in more flexible regulation of an important defense response.

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana accession Columbia (Col-0) was obtained from G. Redei and A.R. Kranz (Arabidopsis Information Service, Frankfurt, Germany). Generation of the Col-0 *nahG* transgenic line was as described (Reuber et al., 1998). Seeds of the *ein2-1* (Guzman and Ecker, 1990) and *jar1-1* (Staswick et al., 1992) lines were obtained from the ABRC (Columbus, OH). The isolation of the *npr1-1* line has been described by Cao et al. (1994). Heterozygous *coi1-1/COI1-1* seeds were a kind gift from J. Turner (University of East Anglia, Norwich, UK).

Plants were grown in a greenhouse as described previously (Reuber et al., 1998). Alternatively, seeds were sterilized and grown at 22°C with a 16-h photoperiod on agar plates containing sterile Murashige and Skoog (1962) (MS) medium (Life Technologies, Rockville, MD) and 1% Suc.

DNA Manipulation and Sequence Analysis

Standard techniques were used for DNA preparation (Sambrook et al., 1989). EST database searches were performed using Basic Local Alignment Search Tool (BLAST; Altschul et al., 1990). Sequence analysis was performed using the Genetics Computer Group (Madison, WI) and DNAStar (Lasergene, Madison, WI) software packages. Scans of promoter sequences for putative regulatory elements were performed using the PlantCARE (http://sphinx.rug.ac.be:8080/PlantCARE/) (Rombauts et al., 1999) and PLACE (www.dna.affrc.go.jp/htdocs/PLACE/) (Higo et al., 1999) database algorithms.

Cloning of *AtPGIP1* and *AtPGIP2* cDNA

Approximately 5×10^6 phage plaques of a cDNA library of Arabidopsis Col-0 (a gift of I. Ruberti, Università di Roma "La Sapienza") were screened using standard techniques (Ausubel et al., 2002). The insert of the EST 179F6T7, corresponding to *AtPGIP1*, was labeled by random priming (Amersham) and used as a probe. Because no complete *AtPGIP2* open reading frame could be retrieved from the library screening, the 5′ region was amplified from seedling RNA by reverse transcriptase–mediated PCR using the GeneAmp kit (Perkin-Elmer Applied Biosystems, Foster City, CA). Primers were AT2R1 (5′-CGCCGTCTTGTATGATTAGGGAA-3′) and AT2U (5′-ATAGCCTATATGTATATCAATCATAGTTTCC-3′). The fragment obtained was ligated with the EST FAFK96 to reconstruct a full-length *AtPGIP2* cDNA. The constructs obtained, containing a full-length *AtPGIP1* or *AtPGIP2* cDNA, were named pBSAt1 and pBSAt2, respectively, and sequenced.

Generation of Transgenic Plants

For transgenic expression in Arabidopsis, the coding sequences of *AtPGIP1* and *AtPGIP2* were amplified from pBSAt1 and pBSAt2 using the Expand High Fidelity PCR System (Boehringer Mannheim, Indianapolis, IN). The primer pairs used were PGIPX1S (5′-TGACACCATGGATAAGACAGC-3′) and X1AORF (5′-CTGAGAGCTCCTTGGTTTACTTGCAAATTTC-3′) for *AtPGIP1* and PGIPX2S (5′-CTGACCATGGATAAGACAATGACAC-3′) and X2AORF (5′-CTGAGAGCTCAATCTTCACTTGCAACTAGG-3′) for *AtPGIP2*. The products were sequenced and subcloned between the NcoI and SacI sites in the pJD301 plasmid (Luehrsen and Walbot, 1991). The cassettes, comprising the 35S promoter of *Cauliflower mosaic virus*, the Ω leader of *Tobacco mosaic virus*, the *AtPGIP* open reading frame, and the *nopaline synthase* 3′ sequence, were inserted in pCAMBIA 3300 (Cambia, Canberra, Australia).

To generate promoter–β-glucuronidase (GUS) fusions, fragments corresponding to 1213 and 509 nucleotides upstream of the predicted translation start of *AtPGIP1* and *AtPGIP2*, respectively, were amplified from genomic DNA. The primer pairs used were ATP1S (5′-GCAAATGAGCTCTCATGAGG-3′) and ATP1A (5′-TGTCTTATCCATGGTGTTGG-3′) for *AtPGIP1* and ATP2S (5′-TAAACCAAGCTTATCTCTAGG-3′) and ATP2A (5′-CCATCCATGGTGTTTTTGGTGTTTG-3′) for *AtPGIP2*. The promoter fragments were sequenced and cloned in pCAMBIA 3301 (Cambia) upstream of the *uidA* gene.

Transgenic Arabidopsis Col-0 plants were generated by *Agrobacterium tumefaciens*–mediated transformation as described previously (Clough and Bent, 1998). T2 lines showing a segregation ratio of 3:1 for resistance to the herbicide Basta were selected for subsequent analysis.

Protein Purification and Analysis

Total proteins were prepared by homogenization in the presence of 1 M NaCl and 20 mM sodium acetate, pH 4.7 (2 mL/g tissue). Homogenates were incubated for 1 h at 4°C and centrifuged for 15 min at 15,000*g*, and the supernatant was filtered through Miracloth (Calbiochem). Total proteins from Arabidopsis leaves were subjected to chromatography on a desalting Sephadex G-25 Superfine column (Pharmacia, Uppsala, Sweden) equilibrated with 1 M NaCl and 20 mM sodium acetate, pH 4.7, and subsequently on a cation-exchange column (SP-Sepharose) equilibrated with 20 mM sodium acetate, pH 4.7, containing 100 mM NaCl. Bound proteins were eluted with a

15-min linear gradient of 100 mM-1 M NaCl at a flow rate of 1 mL/min. Fractions (500 μL) were collected and assayed for inhibitory activity against *Botrytis cinerea* polygalacturonase (PG; see below).

SDS-PAGE and immunoblot analysis were performed as described previously (Desiderio et al., 1997). Polyclonal antibodies against PGIP from *Phaseolus vulgaris* pods were used for immunoblot experiments.

Preparation and Assay of Fungal PGs

Botrytis strain B05.10 was grown for 10 days on malt extract agar (Oxoid, Basingstone, UK) at 20°C in constant light. Conidia were harvested and used to inoculate Gamborg's B5 medium (Duchefa Biochemie BV, Haarleem, The Netherlands) supplemented with 1% Glc and 0.05% yeast extract in 10 mM $NH_4H_2PO_4$, pH 6.0. Cultures were incubated in a rotary shaker at 180 rpm and 20°C in constant light for 16 h. *Colletotrichum gloeosporioides* isolate PCASHK 188 and *Stenocarpella maydis* isolate PCASHK 1033 were grown for 20 days on potato dextrose agar (Oxoid) at 24°C in constant light. Conidia (5×10^{-5}/mL) of *C. gloeosporioides* or 1 cm^2 of mycelium of *S. maydis* were harvested and used to inoculate Czapek-Dox medium (2 g/L $NaNO_3$, 1 g/L K_2HPO_4, 0.5 g/L $MgSO_4$, 0.5 g/L KCl, and 10 mg/L $FeSO_4$, pH 7.0) supplemented with 1% pectin. Cultures were incubated in a rotary shaker at 180 rpm and 21°C for 12 days, and culture filtrates were used for PG activity assay. PG II from *Aspergillus niger* was prepared as described (Armand et al., 2000), and PG of *Fusarium moniliforme* also was prepared as described (Caprari et al., 1996).

PG activity was measured using a modified agarose diffusion assay (Taylor and Secor, 1988). A solution containing PG or culture filtrates was added to 0.5-cm wells on plates containing 100 mM sodium acetate, pH 4.6, 0.5% polygalacturonic acid, and 0.8% agarose. Plates were incubated for 12 h at 30°C, and the halo caused by enzyme activity was visualized after 5 min of treatment with 6 N HCl. PG activity was expressed as agarose diffusion units, with 1 agarose diffusion unit defined as the amount of enzyme that produced a halo of 0.5 cm radius (external to the inoculation well) after 12 h at 30°C. Inhibitory activity was expressed as inhibitory units, with 1 inhibitory unit defined as the amount of PGIP that inhibited 1 agarose diffusion unit of PG by 50%.

Plant Inoculation and Lesion Size Determination

Botrytis was grown on potato dextrose agar for 7 to 10 days at 24°C with a 12-h photoperiod before spore collection. Rosette leaves from 4-week-old soil-grown Arabidopsis plants were inoculated with two 5-μL droplets of a suspension of 5×10^5 conidiospores/mL in 12 g/L potato dextrose broth (Difco, Detroit, MI). Plants were incubated at 22 to 24°C with a 12-h photoperiod at high humidity. Homozygous *coi1-1/coi1-1* plants were identified subsequent to fungal infection by their sterile phenotype (Feys et al., 1994). For lesion size determination, leaves were detached before inoculation and put into a Petri dish with the petiole embedded in 0.6% agarose. Lesion diameter was measured after 3 days. Statistical analysis of the results was performed with one-way analysis of variance.

Plant Treatments

Wounding experiments were conducted by crushing the distal half of four lower rosette leaves with flat-tip forceps and incubating the damaged plants at 22°C. Damaged leaves and upper, unwounded leaves were harvested for analysis. Salicylic acid (SA) and methyl jasmonate (MeJA) were obtained by Sigma-Aldrich (St. Louis, MO) and dissolved in water or methanol, respectively. Adult plants were sprayed evenly with a solution containing 0.01% Silwet L-77 (OSi Specialties, Sistersville, WV) and 5 mM SA or 100 μM MeJA. Control plants were sprayed with a solution of 0.01% methanol and 0.01% Silwet L-77.

For oligogalacturonide (OG) and MeJA treatments, ~20 seeds were added to each well of a 24-well plate containing MS medium supplemented with 0.5% Suc. Plates were incubated for 10 days, and medium was replaced with 1 mL of fresh medium containing 50 μM MeJA or 100 μg/mL OGs with a degree of polymerization of 10 to 15 (a gift of D. Bellincampi, Università di Roma "La Sapienza"). Plates were incubated under the conditions mentioned above before harvesting. Heterozygous *COI1/coi1* seeds were germinated on agar plates containing 30 μM MeJA, and after 8 days, resistant homozygous *coi1* seedlings were transferred to liquid MS medium 2 days before OG treatment. As a control, Col-0 seedlings were grown for 8 days on agar plates and then transferred to liquid MS medium.

Cold treatment was conducted on 10-day-old seedlings grown on agar plates as described above. Plates were wrapped in aluminum foil and placed at 4 or 22°C for different times before harvesting.

RNA Gel Blot Analysis

Total RNA was prepared using the Trizol reagent (Life Technologies). RNA gel blots were prepared and hybridized with single-stranded radioactive probes. Blots were washed twice with 1% SDS and 2 × SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 65°C for 45 min, and images were taken with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) after overnight exposure.

Templates for *AtPGIP1* and *AtPGIP2* were prepared from the pBSAt1 and pBSAt2 plasmids by PCR. The following primers were used to prepare probes for the analysis of overexpressing plants: RRA1A (5′-CGATCCGGTTAAAGTCGATG-3′) and RRA1S (5′-TTACCGCCTTAACCATATTCTC-3′) for *AtPGIP1* and RRA2A (5′-CGATGCGGTAAAAGTCGGG-3′) and RRA2S (5′-GTCACTTCCCTAATCATACAAG-3′) for *AtPGIP2*. Templates for the probes corresponding to the 3′ untranslated region were amplified using the following primers: U1F (5′-TTGAAATTTGCAAGTAAACC-3′) and U1R (5′-ATTAATCAATCCGAATAACATT-3′) for *AtPGIP1* and U2F (5′-CCTAGTTGCAAGTGAAGATTCC-3′) and U2R (5′-AACATTGGTTCATGCTTTTATTA-3′) for *AtPGIP1*. Specific *PR1*, *UBQ5*, and *PDF1.2* probes were prepared as described previously (Penninckx et al., 1996; Rogers and Ausubel, 1997).

GUS Histochemical Analysis

Histochemical staining for GUS activity was performed by vacuum infiltration of the samples with a solution containing 5-bromo-4-chloro-3-indolyl glucuronide, as described previously (Jefferson et al., 1987). The samples were incubated overnight at 37°C and cleared with 70% ethanol before photography.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

Accession Numbers

The Genbank accession numbers for the sequences cited in this work are the following: kiwifruit PGIP protein, CAA88846; orange

PGIP protein, CAA69910; Arabidopsis EST clone 179F6, H36821; and Arabidopsis EST clone FAFK96, Z33878.

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