Polygalacturonase-Inhibiting Proteins (PGIPs) with Different Specificities Are Expressed in *Phaseolus vulgaris*

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The *pgip-1* gene of *Phaseolus vulgaris*, encoding a polygalacturonase-inhibiting protein (PGIP), PGIP-1 (P. Toubart, A. Desiderio, G. Salvi, F. Cervone, L. Daroda, G. De Lorenzo, C. Bergmann, A. G. Darvill, and P. Albersheim, Plant J. 2:367-373, 1992), was expressed under control of the cauliflower mosaic virus 35S promoter in tomato plants via *Agrobacterium tumefaciens*-mediated transformation. Transgenic tomato plants with different expression levels of PGIP-1 were used in infection experiments with the pathogenic fungi *Fusarium oxysporum* f. sp. *lycopersici*, *Botrytis cinerea*, and *Alternaria solani*. No evident enhanced resistance, compared with the resistance of untransformed plants, was observed. The *pgip-1* gene was also transiently expressed in *Nicotiana benthamiana* with potato virus X (PVX) as a vector. PGIP-1 purified from transgenic tomatoes and PGIP-1 in crude protein extracts of PVX-infected *N. benthamiana* plants were tested with several fungal polygalacturonases (PGs). PGIP-1 from both plant sources exhibited a specificity different from that of PGIP purified from *P. vulgaris* (bulk bean PGIP). Notably, PGIP-1 was unable to interact with a homogeneous PG from *Fusarium moniliforme*, as determined by surface plasmon resonance analysis, while the bulk bean PGIP interacted with and inhibited this enzyme. Moreover, PGIP-1 expressed in tomato and *N. benthamiana* had only a limited capacity to inhibit crude PG preparations from *F. oxysporum* f. sp. *lycopersici*, *B. cinerea*, and *A. solani*. Differential affinity chromatography was used to separate PGIP proteins present in *P. vulgaris* extracts. A PGIP-A with specificity similar to that of PGIP-1 was separated from a PGIP-B able to interact with both *Aspergillus niger* and *F. moniliforme* PGs. Our data show that PGIPs with different specificities are expressed in *P. vulgaris* and that the high-level expression of one member (*pgip-1*) of the PGIP gene family in transgenic plants is not sufficient to confer general, enhanced resistance to fungi.

Additional keywords: fungal pathogens, leucine-rich repeat (LRR) proteins.

Polygalacturonase-inhibiting proteins (PGIPs), present in the cell wall of many dicotyledonous plants (Cervone et al. 1997), inhibit the activity of fungal endopolygalacturonases and in vitro favor the accumulation of elicitor-active oligogalacturonides (De Lorenzo and Cervone 1997). PGIPs are proteins structurally related to several resistance gene products recently cloned in plants (Bent 1996), and belong to a superfamily of leucine-rich repeat (LRR) proteins specialized for recognition of non-self molecules and rejection of pathogens. It has been proposed that PGIPs and resistance gene products may function as integrated components of a cell surface apparatus, part of the plant "immune system," in which the role of each component is defined by both its structure and regulation (De Lorenzo and Cervone 1997).

Bulk bean PGIP interacts with a broad spectrum of fungal polygalacturonases (PGs) (Cervone et al. 1990). In *P. vulgaris*, PGIP is encoded by a family of genes (Frediani et al. 1993), and the possibility exists that different PGIP members encode PGIPs with distinct regulation and distinct specificity, i.e., ability to interact with and inhibit PGs from different fungal sources. In this study we show that the product of one *pgip* gene, now named PGIP-1 (Toubart et al. 1992), is unable to inhibit a PG from *Fusarium moniliforme*, and only partially inhibits PGs from *Fusarium oxysporum* f. sp. *lycopersici*, *Botrytis cinerea*, and *Alternaria solani*, thus exhibiting a limited specificity in comparison with the bulk bean PGIP. Consistent with this observation, transgenic tomato plants expressing high levels of PGIP-1 do not exhibit enhanced resistance to these fungi. Also, consistent with the observed peculiar specificity of PGIP-1, we show that the bulk bean PGIP is composed of multiple forms distinguishable in terms of interaction with different PGs.

RESULTS

Expression of *pgip-1* in tomato does not confer enhanced resistance to fungal infections.

The complete coding sequence of the *pgip-1* gene isolated by Toubart et al. (1992) was inserted downstream of the cauli-

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flower mosaic virus (CaMV) 35S promoter and the alfalfa mosaic virus RNA4 leader sequence (Sijmons et al. 1990) (Fig. 1). The construct was introduced into tomato cv. Mon-eymaker via *Agrobacterium tumefaciens*-mediated gene transfer (Van Roekel et al. 1993). Total proteins were extracted from leaves and stems of independent transgenic plants with a diploid genome and the ability of plant extracts to inhibit *Aspergillus niger* PG activity in vitro was determined. The PG-inhibiting activity present in each independent transgenic plant varied from a low level, comparable to that of the untransformed tomato plants, to very high expression levels (up to 120-fold higher than the control) in plants no. 3, 6, 10, 14, 22, 35, and 51 (Table 1). The low PGIP levels observed in tis-

sues of untransformed plants were consistent with the observation that most of the endogenous PGIP in tomato is expressed in fruits (Stotz et al. 1994).

A Western blot (immunoblot) analysis was performed to confirm the transgene expression levels measured by activity in some of the transgenic plants. A PGIP-specific signal was present in extracts with high activity but not in extracts with low activity or in extracts of untransformed tomato plants (Fig. 2). Steady state levels of *pgip* mRNA in transgenic plants no. 3, 7, 9, 10, 11, and 35 were determined by Northern (RNA) blotting analysis of total RNA extracts at high stringency conditions. A single hybridization signal, corresponding to a 1.2-kb transcript, was detected in all plants, except for the untransformed plants and the transgenic plant no. 7 with low PGIP activity (data not shown). In both Western and Northern blot analyses, the intensity of the signal correlated with the measured levels of activity.

Genomic DNA, extracted from the transgenic plants no. 3, 6, 9, 10, 11, 14, 16, 18, 19, 21, 22, 25, and 35, was independently digested with *Eco*RI and *Bgl*III, and separately subjected to Southern blot analysis with either a *pgip* probe or an *nptII* probe at high stringency conditions. More than one copy of the transgene was present in plants no. 3, 10, 11, and 21, whereas only one copy of the *pgip* gene was detected in all the other plants. In genomic DNA from untransformed tomatoes, no signal was detected under the high stringency conditions used (data not shown).

Expression of the transgene was analyzed in S_1 progeny of the transgenic plant lines no. 3 and 6. Segregation of the transgene was determined from the proportion of seeds able to germinate on kanamycin: S_1 progeny of plant no. 6, containing a single copy of transgene, showed a typical Mendelian segregation (ratio 3:1), while nearly all the seeds of the transgenic plant no. 3, containing at least 3 copies of the transgene, germinated on the selective medium. Levels of PGIP activity were tested in the S_1 and S_2 progenies of lines no. 3 and 6. In all S_1 plants PGIP levels were lower than in the parental transgenic tomatoes and an evident variability was present among the plants of the same line. Levels of PGIP activity similar to those of the S_1 progeny were found in the S_2 progeny of the lines tested (Table 2).

S_1 progenies of transgenic plant lines no. 3, 6, 9, 11, 14, 15, 16, 18, 21, 22, and 51 were infected with three fungal pathogens: *F. oxysporum* f. sp. *lycopersici*, *B. cinerea*, and *A. solani*. The severity of the symptoms caused by these pathogens was compared with that of the symptoms caused on untrans-

Fig. 1. Preparation of chimeric *pgip-1* construct and cloning in the binary plasmid for *Agrobacterium tumefaciens* transformation. **A**, Oligonucleotides with *Bam*HI tails used as polymerase chain reaction (PCR) primers to amplify a DNA fragment corresponding to the entire open reading frame of the *pgip-1* gene and the 3' untranslated transcribed region plus 143 nucleotides of the 3' flanking region of the gene *pgip-1*. O-N1 was a 26-mer sense primer synthesized from the first of the two ATGs of *pgip-1* sequence; O-C2 was a 33-mer constructed in antisense orientation on the basis of a 20-bp sequence (from nucleotide 1269) downstream the polyadenylation site. Underlined regions indicate the *Bam*HI restriction site. **B**, Cloning of *pgip-1* PCR product in the *Bam*HI site of pMOG464 and following transfer of the expression cassette (35S promoter - polygalacturonase-inhibiting protein (PGIP) encoding region - 3' nopaline synthase terminator) in *Eco*RI-*Sac*I sites (E - S) of the binary vector pMOG402. The resulting recombinant plasmid, called pMOG636, was used for *Agrobacterium* transformation. Abbreviations: LB/RB = left/right T-DNA border sequence; 5' nos/3' nos = nopaline synthase promoter/terminator sequence.

Table 1. Polygalacturonase-inhibiting protein (PGIP) activity in protein extracts of transgenic tomato plants^a

Plants ^b	PGIP activity ^c
Untransformed	25
5, 7, 8, 12, 13, 15, 16, 17, 19, 24, 27, 29-33, 39, 41, 50	<50
2, 20, 23, 26, 28, 34, 36, 37, 38	50 to 250
9, 49	500 to 750
11, 21, 25, 48	800 to 1,000
10, 14, 51	1,400 to 1,700
3, 22, 35	1,900 to 2,200
6	3,191

^a Extracts were prepared from leaves and stems.

^b Numbers identify each independent transgenic plant tested.

^c PGIP activity against PG purified from *Aspergillus niger* is expressed as units per mg of total protein.

formed tomato plants. No enhanced resistance was observed in the transgenic plants analyzed (Table 3).

PGIP-1 exhibits a specificity different from that of PGIP purified from *P. vulgaris*.

PGIP-1 was purified from stems and leaves of S₁ progeny plants of line no. 3 by the non-affinity-based purification procedure reported in Materials and Methods and summarized in Table 4. The purified PGIP-1, analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), consisted of a broad protein band with molecular mass of about 39 kDa, identical to the molecular mass of PGIP purified from *P. vulgaris* pods. This band reacted with polyclonal antibodies

Fig. 2. Western blot (immunoblot) analysis of protein extracts from tomato transgenic plants. A polyclonal antibody, that recognized the N-terminal polygalacturonase-inhibiting protein (PGIP) peptide, was used. Total protein extracts (1 µg) from the transgenic plants with high PGIP levels (no. 3, lane B; no. 6, lane C; no. 10, lane D; no. 9, lane E) were compared to a transgenic plant extract with a very low PGIP level (no. 7, lane F) and to untransformed tomato plant extract (lane A). Lane G contained 5 ng of bulk bean PGIP (indicated by the arrow).

Table 2. Polygalacturonase-inhibiting protein (PGIP) specific activity in S₁ and S₂ progeny plants, compared with the activity measured in parental transgenic tomato plants^a

Plants ^b	PGIP activity ^c
Parental	
3	2,188
6	3,191
S ₁ progeny	
3.2	1,875
3.6	771
3.7	448
6.2	405
6.4	371
6.6	1,112
6.7	1,818
6.15	757
S ₂ progeny	
3.2.3	672
3.2.4	1,546
3.2.14	1,400
3.2.60	1,635
6.7.7	1,326
6.7.12	2,015
6.7.14	1,576

^a Extracts were prepared from leaves and stems (approximately 250 µg of total protein per gram of fresh tissue).

^b Numbers identify the different plants tested.

^c PGIP activity against PG purified from *Aspergillus niger* is expressed as units per mg of total protein.

prepared against the N-terminal peptide of *P. vulgaris* PGIP-1 (Fig. 3), and corresponded to two forms slightly differing in their electrophoretic mobility, probably due to different glycosylation. The amino terminal sequence (first 20 amino acids) of the purified protein was also identical to that reported for the *P. vulgaris* PGIP (Toubart et al. 1992) and different from that reported for the endogenous PGIP of tomato (Stotz et al. 1994).

The ability of PGIP-1 from transgenic tomato plants to inhibit PGs from different fungi was investigated and compared with the inhibitory activity of the bulk bean PGIP. Bulk bean PGIP (20 ng) was able to inhibit almost completely homogeneous PGs from *A. niger* and *F. moniliforme*, and a crude PG preparation from *B. cinerea*; moreover, the same quantity of bulk bean PGIP inhibited by 40 and 10%, respectively, the activity of crude PG preparations from *F. oxysporum* f. sp. *lycopersici* and *A. solani* (Fig. 4A). PGIP-1 from transgenic tomato plants (20 ng) inhibited the *A. niger* enzyme to the same extent, but did not inhibit the *F. moniliforme* enzyme and only partially inhibited PG preparations from *B. cinerea* (30% inhibition), *F. oxysporum* f. sp. *lycopersici* (10% inhibition), and *A. solani* (10% inhibition) (Fig. 4B).

Table 3. Response of S₁ progeny of different transgenic tomato plant lines to infection with *Fusarium oxysporum*, *Alternaria solani*, and *Botrytis cinerea*

Plant line	Infection source		
	<i>F. oxysporum</i> ^a	<i>A. solani</i> ^b	<i>B. cinerea</i> ^c
3	4.2 ± 0.8	ND ^d	ND
6	3.8 ± 0.9	0.24 ± 0.04	15.0 ± 7.1
9	4.7 ± 1.5	0.28 ± 0.13	12.5 ± 3.5
11	5.2 ± 1.1	0.22 ± 0.09	22.5 ± 3.5
14	5.3 ± 0.8	0.21 ± 0.14	30.0 ± 14.1
15	ND	0.19 ± 0.08	ND
16	5.1 ± 1.1	ND	ND
18	4.6 ± 0.3	0.16 ± 0.09	25.0 ± 7.1
21	6.9 ± 1.2	0.21 ± 0.13	22.5 ± 3.5
22	5.7 ± 1.4	0.15 ± 0.07	15.0 ± 7.1
51	ND	ND	30.0 ± 14.1
Untransformed	5.2 ± 0.2	0.23 ± 0.20	17.5 ± 3.5
Uninfected	0 ± 0	0 ± 0	0 ± 0

^a Disease scale: 0 (no symptoms) to 9 (severe wilting, dead plant).

^b Defoliation: number of detached leaves/total leaves.

^c Percentage of infected leaf area.

^d Not determined.

Table 4. Purification of polygalacturonase-inhibiting protein (PGIP) PGIP-1 from transgenic tomato plants^a

Fraction	Volume (ml)	Protein (mg)	Total activity ^b (units)	Specific activity (units/mg)	Yield (%)
Crude extract	400	372	245,200	659	...
Diethylaminoethyl	550	198	156,200	789	64
Isofocusing	8	36	70,000	1,944	28
Sephadex G-75	40	18	70,000	3,889	28
MonoS	3	3.3	31,000	9,394	13

^a PGIP-1 was purified from tissues of S₁ progeny plants of the transgenic line no. 3.

^b One unit of PGIP is the amount of protein required to reduce the activity of 0.01 RGU (see text for definition) of *Aspergillus niger* PG by 50%.

Expression of the *P. vulgaris* *pgip-1* in *N. benthamiana* produces a protein with the same specificity as PGIP-1 from transgenic tomato plants.

The complete coding sequence of the PGIP-1 gene was also introduced into the expression cassette of the virus vector based on potato virus X (PVX) (Baulcombe et al. 1995) for transient expression in *N. benthamiana*. Western blot analysis of crude protein extracts from symptomatic leaves demonstrated the presence of a PGIP-specific signal that was absent in wild-type extracts and extracts from leaves inoculated with transcripts containing the coding sequence of an unrelated gene (*gusA*) (Chapman et al. 1992). A smaller protein (about 27 kDa) that also cross-reacted with the PGIP antibody was present in all extracts from inoculated plants (Fig. 3, lane C) but absent in extracts from mock-inoculated plants. In control assays the 27-kDa protein did not exhibit any inhibitory activity against *A. niger* PG.

The specificity of PGIP-1 in the *N. benthamiana* extracts was similar to the specificity of PGIP-1 purified from tomato, and differed from that of the bulk bean PGIP: in particular, it was unable to inhibit the homogeneous *F. moniliforme* PG (Fig. 4C).

Inability of PGIP-1 to inhibit the *F. moniliforme* PG is due to its inability to interact with the enzyme.

The interaction between the homogeneous PGs of *A. niger* and *F. moniliforme* with PGIP-1 from transgenic tomato plants and PGIP from *P. vulgaris* was studied with a biosensor based on surface plasmon resonance (SPR) (Granzow and Reed 1992; Schuster et al. 1993). PGIPs were immobilized as ligands on the sensor surfaces while PGs were passed in solution as analyte over the surface. The interactions between the two PGIPs with increasing amounts of the two PGs were analyzed kinetically. The analysis showed that PGIP-1 from transgenic tomato plants interacted with the *A. niger* PG and did not interact with the *F. moniliforme* enzyme. On the other hand, the bulk bean PGIP interacted with both enzymes (Fig. 5; the kinetic parameters of the interactions are reported in the caption).

SPR studies were also performed with extracts from PVX-infected *N. benthamiana* leaves and pure PGs from *A. niger* and *F. moniliforme* immobilized as ligands on the sensor sur-

Fig. 3. Western blot (immunoblot) analysis of protein extracts from potato virus X-infected *Nicotiana benthamiana* plants (5 µg, lane C) compared with the bulk polygalacturonase-inhibiting protein (PGIP) purified from *Phaseolus vulgaris* pods (10 ng, lane A) and PGIP-1 purified from transgenic tomato plants (15 ng, lane B).

Fig. 4. Inhibition curves of various fungal polygalacturonases (PGs) by increasing amounts of purified bulk bean polygalacturonase-inhibiting protein (PGIP) (A), purified PGIP-1 from transgenic tomato plants (B), and crude PGIP-1 extracts from potato virus X-infected *Nicotiana benthamiana* plants (C). Enzymes used: 0.015 units of homogeneous *Aspergillus niger* PG (■), 0.008 units of PG purified from *Fusarium moniliforme* (×), 0.01 units of a crude PG preparation from *F. oxysporum* f. sp. *lycopersici* (●), 0.011 units of a crude PG preparation from *Alternaria solani* (▲), and 0.004 units of a crude PG preparation from *Botrytis cinerea* (◆). One unit of PG is the enzyme amount producing 1 microequivalent of reducing groups per min at 30°C.

faces. The kinetic data obtained confirmed that the product of the *pgip-1* gene interacts with the *A. niger* enzyme but not with the *F. moniliforme* enzyme (data not shown).

A PGIP with specificity similar to that of PGIP-1 is present in *P. vulgaris* and can be separated from other PGIPs.

The peculiar specificity characteristics of PGIP-1 prompted us to investigate whether a PGIP with a similar specificity was present in *P. vulgaris* tissues. We used a differential affinity chromatography strategy. Extracts of *P. vulgaris* pods were passed through a *F. moniliforme* PG-Sepharose affinity chromatography column under conditions suitable for the PG-PGIP interaction (50 mM acetate buffer pH 5); the column was then eluted with phosphate-buffered saline (PBS), which favors dissociation of the PG-PGIP complex (Cervone et al. 1987). Two fractions were obtained: Fraction A, the flow-through, contained proteins not absorbed; the second fraction, eluted with PBS and named PGIP-B, contained proteins absorbed by *F. moniliforme* PG (Fig. 6A).

Fraction A, passed through an *A. niger* PG-Sepharose column, contained a protein (PGIP-A) capable of binding *A. niger* PG (Fig. 6B). Fraction A, PGIP-A, and PGIP-B were separately tested for inhibitory activity against *F. moniliforme* and *A. niger* PGs: fraction A and PGIP-A inhibited *A. niger* PG but not *F. moniliforme* PG, while PGIP-B inhibited both *A. niger* PG and *F. moniliforme* PG (Table 5). Based on the observed specificity, PGIP-A may contain the product of the *pgip-1* gene characterized in this study, while PGIP-B contains one or more PGIP forms with specificity different from those of both PGIP-1 and PGIP-A.

DISCUSSION

In this study the *pgip-1* gene cloned from *P. vulgaris* (Toubart et al. 1992) has been constitutively expressed in tomato plants and transiently expressed in *N. benthamiana* plants. The product of this gene, PGIP-1, has been characterized in terms of ability to interact with and inhibit several fungal PGs. Both inhibition studies and SPR measurements clearly show that the specificity of PGIP-1 from transgenic tomato and *N. benthamiana* plants is different from that of the bulk bean PGIP. Notably, PGIP-1 is unable to inhibit PG from *F. moniliforme* and shows a limited ability to inhibit PGs from *F. oxysporum* f. sp. *lycopersici*, *B. cinerea*, and *A. solani*; in comparison, the bulk bean PGIP has a broader spectrum of interactions and completely inhibits PGs from *F. moniliforme* and *B. cinerea*. Differences in processing or post-translational modifications, for example glycosylation, of the PGIP-1 polypeptide in the heterologous hosts are unlikely to explain the observed discrepancy between the specificity of PGIP-1 and that of the bulk bean PGIP, since tomato- and *N. benthamiana*-expressed PGIP-1s exhibit the same specificity.

PGIP-1 is likely to be only one of several PGIPs with different specificities expressed in *P. vulgaris*. This is supported by the observation that, after removing by affinity chromatography a PGIP capable of binding to *F. moniliforme* PG, a form of PGIP unable to inhibit the *F. moniliforme* PG can be isolated from *P. vulgaris* extracts. This form may be the product of the *pgip-1* gene. The presence in the same tissue of a second PGIP with a distinct specificity is strong evidence that multiple PGIP forms (at least two) with different functions are

Fig. 5. Interactions between the bulk bean polygalacturonase-inhibiting protein (PGIP) and PGIP-1 from transgenic tomato plants with homogeneous polygalacturonases (PGs) from *Aspergillus niger* and *Fusarium moniliforme*. Different panels show the surface plasmon resonance sensorgrams. **A**, Bulk bean PGIP and increasing concentrations of *A. niger* PG (from bottom to top curve 6.25, 12.5, 25, 50, 87.5 nM, calculated $K_a = 8.9 \times 10^7 \text{ M}^{-1}$). **B**, PGIP-1 and increasing concentrations of *A. niger* PG (from bottom to top curve 50, 125, 175, 250, 364 nM, calculated $K_a = 2.5 \times 10^7 \text{ M}^{-1}$). **C**, Bulk bean PGIP and increasing concentrations of *F. moniliforme* PG (from bottom to top curve 8.5, 12.5, 25, 50, 100 nM, calculated $K_a = 9.7 \times 10^7 \text{ M}^{-1}$). **D**, PGIP-1 and increasing concentrations of *F. moniliforme* PG (from bottom to top curve 125, 250, 500, 750 nM).

present in *P. vulgaris*. Recently, evidence of two PGIPs differing in their amino terminal sequence, both present in *P. vulgaris* cv. Blue Lake, has been published (Pressey 1996). Therefore, the bulk bean PGIP may comprise polypeptides encoded by different PGIP genes. The different PGIPs, however, are likely to be nearly indistinguishable in biochemical terms, due to similarity in molecular weight and amino acid sequence; this explains the difficulty so far encountered in their identification and separation. The narrow specificity observed for PGIP-1 raises the hypothesis that the ability of the bulk bean PGIP to inhibit a wide spectrum of fungal PGs may be the sum of the abilities of PGIPs with different specificities present in the preparation, each PGIP contributing in part to confer a broad range of inhibitory activities to *P. vulgaris*. Interestingly, PGIPs purified from tomato and pear also show differential specificity toward several fungal PGs (Powell et al. 1994).

PGIPs encoded by different members of the PGIP gene family of *P. vulgaris* may differ not only in specificity but also in regulation, as suggested by the observation that the activity of the *pgip-1* promoter does not mirror the pattern of expression of the whole gene family, and does not respond to

elicitors or infection (A. Devoto, F. Leckie, E. Lupotto, F. Cervone, and G. De Lorenzo, *unpublished*). Differential specificity and regulation may explain the presence of multiple PGIP genes in *P. vulgaris* (Frediani et al. 1993).

Given the limited ability of PGIP-1 to inhibit PGs from *F. oxysporum* f. sp. *lycopersici*, *B. cinerea*, and *A. solani*, it is not surprising that, in comparison with the untransformed plants, the transgenic tomato plants overexpressing PGIP-1 do not exhibit enhanced resistance to these pathogenic fungi. Expression of

Table 5. Inhibition (%) of *Aspergillus niger* polygalacturonase (PG) and *F. moniliforme* PG by Fraction A and polygalacturonase-inhibiting protein (PGIP) PGIP-B and PGIP-A fractions from the affinity chromatography experiments reported in Figure 6

PGIP	Total proteins (µg)	Inhibition (%)	
		<i>A. niger</i> PG	<i>F. moniliforme</i> PG
Fraction A	8	45	0
	32	100	0
PGIP-B	0.85	48	30
	2.38	100	90
PGIP-A	2.7	42	0
	13.5	100	0

Fig. 6. Elution profiles of extracts from *Phaseolus vulgaris* pods subjected to affinity chromatography. **A**, Extract (70 ml) was applied to a Sepharose–*Fusarium moniliforme* polygalacturonase (PG) affinity column. Elution buffer was 50 mM Na-acetate. Two fractions were obtained: Fraction A, the flow-through, and a second fraction eluted with phosphate-buffered saline (polygalacturonase-inhibiting protein [PGIP] PGIP-B), which contained proteins absorbed by the *F. moniliforme* PG. **B**, Fraction A, in turn passed through a Sepharose–*Aspergillus niger* PG affinity column, contained proteins (PGIP-A) absorbed by the *A. niger* PG.

multiple *pgip* genes encoding proteins with different specificities may therefore be necessary to obtain resistance.

In conclusion, our data show that PGIPs with different specificities are expressed in *P. vulgaris*. The apparent broad activity of PGIP in *P. vulgaris* may result from the presence of different molecules with narrow specificities. To gain information about the number of PGIPs with different specificity existing in *P. vulgaris*, it is now important to isolate other members (possibly all) of the PGIP gene family. These studies will also provide information about the structural requirement of PG-PGIP interaction. The molecular basis of the PGIP specificities may provide a model for how plant LRR proteins with narrow specificities like the R gene products have evolved to accommodate the recognition of many non-self molecules.

MATERIALS AND METHODS

Chimeric gene construction and tomato transformation.

The pPT-2 plasmid, containing the genomic clone of *pgip* (Toubart et al. 1992), was used as template in a polymerase chain reaction (PCR) at 0.1 ng μl^{-1} to amplify a DNA fragment corresponding to the entire open reading frame and the 3' untranslated transcribed region plus 143 nucleotides of the 3' flanking region of the gene. Oligonucleotide primers, synthesized with a *Bam*HI tail at 3' end (Fig. 1A), were used at a final concentration of 1 μM each. PCR was performed in a Perkin-Elmer/Cetus automated temperature cyclor as followed: (i) 3 cycles of 2 min at 94°C, 1 min at 47°C and 1 min 30 s at 72°C; (ii) 30 cycles of 1 min 30 s at 94°C, 1 min at 52°C and 1 min 30 s at 72°C; and (iii) 7 min at 72°C. One unit of AmpliTaq DNA polymerase (Perkin-Elmer, Monza, Italy) was used per 100 μl of reaction mixture.

The fragment was inserted into the *Bam*HI sites of Bluescript SK(+) (Stratagene, La Jolla, CA) plasmid (pAD 16). After sequencing, the *pgip* insert was recloned into the *Bam*HI site of pMOG464 to place the gene under control of the CaMV 35S promoter (Fig. 1B). The correct orientation of the *pgip* encoding region in the expression cassette was checked by *Sac*I-*Hind*III digestion and the entire 35S-*pgip* construct was transferred into the *Eco*RI-*Sac*I sites of pMOG402, containing the *ntpIII* gene as a selection marker. The recombinant vector was named pMOG 636. *Escherichia coli* DH5 α containing pMOG 636 was used in triparental mating with *Agrobacterium tumefaciens* strain EHA105 and *E. coli* HB101 containing the helper plasmid pRK2013, according to reported protocol (Hood et al. 1993). *A. tumefaciens* strain EHA105 (pMOG636) was subsequently used to infect cotyledons of *Lycopersicon esculentum* cv. Moneymaker as described (Van Roekel et al. 1993).

Isolation and analysis of nucleic acids.

Standard techniques were used for recombinant DNA and RNA work (Sambrook et al. 1989). Hybridization probes were prepared by random oligonucleotide priming (Hodgson and Fisk 1987). For the *pgip* probe a PCR-amplified, 732-bp fragment, consisting of nucleotides 167 through 898 of the *pgip* gene (Toubart et al. 1992), was used. The *ntpIII* probe was an 883-bp restriction fragment of nucleotides 177 through 1060, prepared by *Apa*I-*Pst*I digestion of the *ntpIII* gene (Yenofsky et al. 1990).

Analysis of transgenic plants.

Ploidy levels of transgenic plants were analyzed by counting chloroplasts in stomata cells according to the method described by Koornneef et al. (1989).

For determination of PGIP levels, leaves and stems were homogenized in 1 M NaCl (2 ml/g), incubated under gentle shaking for 1 h at 4°C, and centrifuged 20 min at $8,000 \times g$. The supernatant was filtered and proteins were precipitated in 75% $(\text{NH}_4)_2\text{SO}_4$ overnight at 4°C. The pellet obtained from centrifugation at $8,000 \times g$ for 20 min was dissolved in 1 M NaCl, 20 mM Na acetate pH 5 and dialyzed overnight against 20 mM Na acetate pH 5.

The ability of plant extracts to inhibit *A. niger* PG activity in vitro was measured by the parahydroxybenzoic acid hydrazide (PAHBAH) assay (Cervone et al. 1989). One PG activity unit (RGU) was defined as the amount of the enzyme producing one microequivalent of reducing groups per min at 30°C with 0.5% (wt/vol) polygalacturonic acid as substrate. One unit of inhibitor was defined as the amount of PGIP required to reduce the activity of 0.01 RGU of PG by 50%.

Protein concentration was determined by the method of Bradford (Bradford 1976) with Bio-Rad (Milan, Italy) reagents and bovine serum albumin as a standard.

SDS-PAGE was performed as described by Laemmli (1970). Gels were stained with silver as described by Blum et al. (1987).

For immunoblotting experiments, the proteins were electrophoretically transferred to nitrocellulose after SDS-PAGE according to Towbin et al. (1979) and a polyclonal antibody that recognizes an N-terminal peptide of mature PGIP (Bergmann et al. 1994) was used to detect the antigen-antibody complex with the ECL kit (Amersham, Rome, Italy).

Fungal infection assays.

Three fungal pathogens were used to infect tomato transgenic plants: *F. oxysporum* f. sp. *lycopersici*, *B. cinerea*, and *A. solani*. Plants were selected for kanamycin resistance first, with a nondestructive kanamycin spraying assay (Weide et al. 1989).

The *F. oxysporum* infection and assay were performed exactly as described previously (Jongedijk et al. 1995). Seven plants per line (corresponding to S_1 progeny) in three replicates were used. Tomato roots were rinsed with water, dipped into spore suspension (10^6 spores per ml), and planted into pots at 18°C, relative humidity 80%, 40 lx. Thirty days after inoculation, plants were scored for wilting symptoms, and chlorosis.

For the *A. solani* assay, S_1 seedlings were planted in 21 pots (60% black peat/40% white peat containing 6 g of CaCO_3 per liter and 19 g of NPK 12/14/24 per liter). Three replicates of pots, each containing three plants, were placed under a condition of continuous leaf wetness in a greenhouse at 21°C and 16-h daylength with a light intensity of 4000 lx, in a randomized complete block design. Inoculum of *A. solani* was harvested from fungal mycelium freshly grown on potato dextrose agar (PDA) plates for 21 days (25°C, 16 h light, light intensity 300 lx) by rinsing off spores with nanopure water. Plants were inoculated by spraying a spore suspension (10^4 spores per ml, 3 ml per plant). Fifteen days after inoculation, plants were scored for degree of defoliation (number detached leaves/total leaves).

For *B. cinerea*, the same procedure was followed until inoculation. Inoculum of *B. cinerea* was harvested from fungal mycelium freshly grown on PDA plates for 12 days (18°C, 12 h daylength, 300 lx UV/white light) by rinsing off the spores with nanopure water. Plants were inoculated by spraying a spore suspension (10^4 spores per ml, 3 ml per plant) after adding 2 g of sucrose per liter and 6 g of KH_2PO_4 per liter to the spore suspension. Fifteen days after inoculation, disease severity was evaluated as a percentage of infested leaf area.

Vector construction for PVX: Transcription and plant inoculation.

A PCR-generated fragment identical to the fragment-cloned *Bam*HI in pAD16, except for the cloning sites, was, after sequencing, inserted into the *Cla*I and *Sal*I sites of the pPC2S PVX expression vector to generate PVX.PGIP-1 (Baulcombe et al. 1995). RNA transcripts were prepared and used to inoculate *N. benthamiana* plants as previously described (Chapman et al. 1992). Leaf material was harvested upon appearance of viral symptoms (15 to 20 days after inoculation). Leaf protein extracts were prepared and analyzed as described above for the transgenic tomato plants.

Preparation and assay of PGs.

PG from *A. niger* was a commercial preparation (Sigma, Sigma Italia, Milano, Italy). PG from *F. moniliforme* was prepared according to De Lorenzo et al. (1987). Crude PG preparations were obtained from *F. oxysporum* f. sp. *lycopersici*, *B. cinerea*, and *A. solani* grown in Czapek medium (Nothnagel et al. 1983) containing 2% (wt/vol) glucose and 1.5% (wt/vol) pectin from citrus fruit (Sigma). Cultures of *F. oxysporum* and *B. cinerea* were incubated on a rotary shaker at 25°C for 2 weeks. *A. solani* cultures were incubated standing at room temperature for 3 weeks. Culture media were filtered through Miracloth (Calbiochem, Milano, Italy) and precipitated with 75% $(\text{NH}_4)_2\text{SO}_4$ overnight at 4°C. The pellet obtained after centrifugation at $10,000 \times g$ for 10 min was resuspended in the minimal volume possible of 20 mM Na acetate and dialyzed against 20 mM Na acetate pH 5. PG activity was determined by the PAHBAH procedure (Cervone et al. 1989).

PGIP purification and assay.

PGIP-1 was purified from transgenic tomato no. 3 by a non-affinity-based purification procedure. Total proteins were extracted from leaves and stems of transgenic tomato plants as described above (paragraph title: "Analysis of transgenic plants") and dialyzed against 20 mM Na acetate pH 5. The dialyzed proteins were mixed with a suspension of diethyl-aminoethyl (DEAE) cellulose (DE52, Whatman, Kent, U.K.) pre-equilibrated with 20 mM Na acetate pH 5. The nonabsorbed proteins were precipitated with 70% $(\text{NH}_4)_2\text{SO}_4$, resuspended in a small volume of water, dialyzed against 0.5% glycine, and subjected to isoelectric focusing at 4°C with an LKB 8100 column (Pharmacia, Milan, Italy) containing 4.5 ml of 40% ampholine, pH 8 to 10.5, in a linear sucrose gradient (0 to 40% wt/vol). Fractions corresponding to a range of pH between 9.5 and 10.5 that contained the highest inhibitory activity were pooled. Aliquots of 4 ml were loaded on a Sephadex G-75 column pre-equilibrated with 20 mM Na acetate pH 5. Flow-rate was 0.5 ml/min; fractions of 1 ml were collected. One peak of activity was detected within fractions 53

to 72. The peaks of the two columns were pooled and chromatographed on a Mono-S column (Pharmacia, Milan, Italy) and eluted with a linear gradient of 0 to 0.5 M NaCl in 20 mM Na acetate. About 3.3 mg of pure PGIP was obtained in this way from 700 g of fresh tissue (Table 4).

PGIP was purified from pods of *P. vulgaris* cv. Cannellino by an affinity-based procedure on a Sepharose-A. *niger* PG column as described previously (Cervone et al. 1989). Inhibition was measured with the standard PAHBAH assay for PG.

SPR determinations.

This analytical system allows for the direct visualization of biomolecular interactions in real time. One molecule (the ligand) is covalently attached to the dextran matrix of a sensor chip while the other molecule (the analyte) is introduced in a flow passing over the surface. As the two molecules bind, the refractive index in the vicinity of the surface is altered, and the angle at which plasmon resonance occurs is correspondingly modified. This change, measured in resonance units (RU), directly correlates with the amount of protein interacting with the surface (1,000 RU = 1 ng of bound protein per mm^2). Results from BIA are presented as a sensorgram, which is a plot of changes in the resonance signal as a function of time.

BIAlite system, sensor chip CM5, HBS buffer (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], pH 7.4, 150 mM NaCl, 0.005% vol/vol surfactant p20 in distilled water), and amine coupling kit were from Pharmacia Biosensor (Uppsala, Sweden). PGIPs, purified from *P. vulgaris* pods or from tomato transgenic plants, were immobilized on sensor chips via amine coupling as reported (Caprari et al. 1996). The amount of PGIP immobilized was 1,500 to 2,000 RU for the *P. vulgaris* PGIP and 700 to 1,000 RU for the transgenically expressed PGIP-1. Solutions of PGs in MES (2-(*N*-morpholino)ethanesulfonic acid) 25 mM, pH 5.0, were injected into the flow cell and passed over the PGIP surfaces at flow rate of 5 $\mu\text{l}/\text{min}$ at different analyte concentrations. Sensorgrams were analyzed by nonlinear least squares curve fitting with BIAevaluation 2.0 software (O'Shannessy et al. 1993).

Homogeneous PGs from *A. niger* and *F. moniliforme* were also immobilized as ligands on the sensor surfaces with amine-coupling chemistry. Surfaces containing densities of 700 to 2,000 RU were used for the analysis of PVX extracts.

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