

## TRANSCRIPT ACCUMULATION OF POLYGALACTURONASE INHIBITING PROTEIN (PGIP) FOLLOWING PATHOGEN INFECTIONS IN SOYBEAN

F. Favaron<sup>1</sup>, T. Destro<sup>1</sup> and R. D'Ovidio<sup>2</sup>

<sup>1</sup> Istituto di Patologia Vegetale, Università degli Studi di Padova, Strada Romea 16, I-35020 Legnaro, Padova, Italy

<sup>2</sup> Dipartimento di Agrobiologia e Agrochimica, Università degli Studi della Tuscia, Via S. Camillo de Lellis, I-01100 Viterbo, Italy

### SUMMARY

Proteins inhibiting fungal endo-polygalacturonase (PGIP) are constitutively expressed and localized on cell walls of most plant species. Induction of *pgip* transcripts following pathogen infection would demonstrate a role of PGIP in active plant defence mechanisms. We investigated *pgip* expression in hypocotyls of soybean seedlings (cvs 'Sapporo' and 'Kure') infected with the fungal pathogens *Diaporthe phaseolorum* var. *caulivora*, *Sclerotinia sclerotiorum* and an avirulent (race 1) and virulent (race 20) races of *Phytophthora sojae*. Two *pgip* transcripts were transiently expressed in all types of interaction with similar timing and maximum accumulation at 16-24 h after infection. However, soybean seedlings of both cvs infected with the virulent race 20 of *P. sojae* showed higher levels of *pgip* expression than seedlings infected with the avirulent race 1. A delay of *pgip* accumulation was observed when plants were inoculated with zoospores in place of mycelium of *P. sojae*.

The presence of endo-polygalacturonase (endo-PG) in infected tissue was monitored both by immunological detection and by determining PG activity. PG was detected only in soybean seedlings infected with *S. sclerotiorum* and not in those infected with *D. phaseolorum* var. *caulivora* or *P. sojae*. The apparent inability in vivo of the virulent and avirulent races of *P. sojae* to produce PG suggests that PG-PGIP interaction is not required for the resistance response of soybean against this pathogen.

*Key words:* defence genes, fungal pathogens, *Glycine max*, *Phytophthora sojae*.

### INTRODUCTION

The breakage of plant cell walls is one of earliest events during plant infection by a number of plant pathogens (Cooper, 1983), and occurs after production of enzymes capable of depolymerizing components of the wall (Walton, 1994). Among cell wall degrading enzymes, pectinases have received considerable attention as major determinants of plant cell wall breakdown and as putative pathogenicity factors (Collmer and Keen, 1986; Cooper, 1987).

To counter cell wall degradation and pathogen infection, plants have evolved mechanisms to reinforce the cell wall. These include cross-linking of hydroxyproline-rich wall glycoproteins (Bradley *et al.*, 1992), formation of cell wall papillae (Kovats *et al.*, 1991) and lignification (Ride, 1980). In addition plants contain pectinase inhibitors, and protein inhibitors of fungal endo-polygalacturonase (PGIPs) are especially widespread among plants (De Lorenzo *et al.*, 1997). The constitutive expression and localization on plant cell walls support a role of PGIP as a preformed defence molecule against pathogen invasion. Increase of PGIP transcript after fungal infection has been observed in apple and common bean (Nuss *et al.*, 1996; Devoto *et al.*, 1997; Yao *et al.*, 1999). Furthermore the finding that in common bean PGIP accumulates earlier and at a greater extent with incompatible interaction also indicate a role of PGIP in active resistance mechanisms (Nuss *et al.*, 1996; Devoto *et al.*, 1997).

To assess whether PGIP accumulation is a general phenomenon in plants following pathogen infection, we investigated PGIP mRNA accumulation in soybean infected with different plant pathogens. In particular, we used the fungal pathogens *Diaporthe phaseolorum* var. *caulivora*, *Sclerotinia sclerotiorum* and avirulent and virulent races of *Phytophthora sojae*. In order to establish if PGIP expression was related to fungal PG accumulation, we also monitored the presence of endo-PG in infected tissue both by immunological detection and by determining PG activity.

## MATERIAL AND METHODS

**Pathogens, plant materials and infection experiments.** *D. phaseolorum* var. *caulivora* Athow and Caldwell isolate 62 k was provided by Dr. M.M. Kulik (Plant Sciences Institute, ARS-USDA, Beltsville, MD, USA). *S. sclerotiorum* Lib. De Bary isolate B24 was from the Institute collection. *P. sojae* Kaufmann and Gerdemann race 1 was from the Max-Planck-Institut für Züchtungsforschung, Köln. An isolate of *P. sojae* provided by Dr. B.M. Tyler (Department of Plant Pathology, University of California, Davis) was determined to belong to race 20 by establishing virulence against the following soybean cvs containing specific resistance genes (*Rps*): 'Williams' (no *Rps* gene), 'Williams 82' (*Rps1k*), 'Harosoy' (*Rps7*), 'Harosoy 63' (*Rps1a*, *Rps7*), 'L83-570' (*Rps3a*).

*D. phaseolorum* var. *caulivora* and *S. sclerotiorum* were grown in potato dextrose agar. *P. sojae* was grown in Lima bean agar (Difco, USA).

Soybean plants (*Glycine max* [L.] Merr.) cvs 'Sapporo' and 'Kure' (Asgrow, Lodi Italy) were grown in the dark at 25°C in sterilized moist vermiculite. After 5 days, seedlings were placed horizontally in plastic trays. Roots were covered with a layer of paper towel soaked in sterilized water.

Inoculation was performed by placing slices (2 x 3 mm) of mycelium-colonized agar, cut from marginal zones of colonies, onto the middle region of hypocotyls, above longitudinal wounds (approx. 4 mm long) made by a sterile syringe needle (0.50 x 16 mm), basically according to the wound method (Keeling, 1976). Seedlings were also inoculated with zoospores of *P. sojae*. Zoospores were produced according to the method of Eye *et al.* (1978). A 10 µl droplet of zoospore suspension adjusted to 1 x 10<sup>5</sup> ml<sup>-1</sup> with sterile tap water, was placed on each seedling in the middle region of the unwounded hypocotyl. The trays containing inoculated seedlings were sealed with plastic film and incubated at 25°C in the dark.

**RNA extraction, Northern blot analysis and digoxigenin-labelling of probe.** Tissue for RNA analysis was excised by transversely cutting the infected hypocotyls exactly at the border between the healthy and injured tissue. Total RNA was extracted using RNeasy Plant Minikit (Qiagen, Germany) following the manufacturer's procedure.

Fifteen µg of total RNA from infected and control tissue were fractionated on a 1.2% agarose gel containing 6.7% formaldehyde and blotted onto nylon membrane (Hybond N, Amersham Pharmacia, Sweden) with 10x SSC (1.5 M NaCl, 0.15 M trisodium citrate pH 7.0).

Pre-hybridization and hybridization reactions were carried out at 42°C in a solution containing 50% formamide, 3x SSC, 60 mM phosphate buffer (pH 6.8), 10 mM EDTA (pH 7.2), 0.1% SDS and 5x Denhardt (Sambrook *et al.*, 1989). Filters were pre-hybridized for 4 h and hybridized for 12-16 h. After hybridization, filters were washed twice for 5 min at room temperature in 2x SSC, 0.1% SDS and twice for 10 min at 46°C in 0.1x SSC, 0.1% SDS. Filters were then soaked for 5 min in blocking buffer containing 0.2% w/v blocking reagent (Roche, Germany), 1x PBS (0.058 M Na<sub>2</sub>HPO<sub>4</sub>, 0.019 M NaH<sub>2</sub>PO<sub>4</sub>, 0.068 M NaCl) and 0.1% v/v Tween 20, and then incubated in the same solution for 30 min. Filters were incubated for 1 h with anti-Digoxigenin-AP antibody (Roche) diluted 1:5000 in blocking buffer without Tween 20, washed briefly in the same buffer and then washed 4 times for 5 min with 1x PBS containing 0.3% Tween 20. Filters were equilibrated twice for 5 min in assay buffer (0.1 M diethanolamine and 1 mM MgCl<sub>2</sub>, pH 9.5) and incubated for 20 min in CDP-Star (Roche) diluted 1:100 in assay buffer. Filters were finally wrapped in Saran Wrap and exposed to X-ray film (X-Omat AR, Kodak, USA).

The probe used to detect *pgip* mRNA was the *pGM7* clone (Favaron *et al.*, 1994), labelled with digoxigenin (Digoxigenin-11-uridine-5'-triphosphate, Roche) by PCR following the procedure of D'Ovidio and Anderson (1994).

**PG extraction and assays.** Hypocotyl samples (0.5-0.8 g fresh wt) comparable to those collected for RNA extraction, were ground at 4°C with a mortar and pestle using 2 ml g<sup>-1</sup> fresh wt of 50 mM Tris-HCl buffer pH 7.8 containing 1 M NaCl and 2.5 mM dithiothreitol. The slurry was centrifuged at 3000 g for 30 min. The supernatant was dialyzed overnight against deionized water. The dialyzed extract was centrifuged at 3000 g for 30 min.

Protein contained in the samples was determined by the Bradford method (1976) using BSA as a standard.

PG activity was determined as an increase of reducing end-groups over time. Reducing end-groups were measured using the method of Milner and Avigad (1967) using D-galacturonic acid as a standard. Incubation mixtures contained 20 µl of sample and 200 µl of 0.5 % polygalacturonic acid (Sigma, USA) in 100 mM sodium acetate, pH 4.8. Mixtures were incubated at 30°C from 15 min to 24 h. One enzyme unit was defined as that producing at 30°C 1 µmol of reducing groups min<sup>-1</sup>.

Immunodetection of endo-PG was performed on sample extracts containing 100 µg of protein. Samples were air-dried under vacuum. Protein was redissolved in sample buffer and run in SDS-PAGE according to Laemmli (1970). After electrophoresis the resolved pro-

teins were transferred to an Immobilon-P membrane (Millipore, USA) by an SD Electrophoretic Transfer Cell (Biorad, USA). The membrane was soaked in 100% methanol for 10 s and subsequently air dried. Primary (anti-PG antibody) and secondary antibodies (alkaline phosphatase conjugate of sheep anti-rabbit IgG, Roche) were diluted 1:300 and 1:2000, respectively, in blocking buffer (1% BSA, 0.05% Tween-20 in PBS). The membrane was incubated with primary antibody solution for 1 h, washed twice for 10 s with blocking buffer and incubated with secondary antibody solution for 30 min. After two washing with the blocking buffer the membrane was washed twice for 2 min with the assay buffer (20 mM Tris base and 1 mM MgCl<sub>2</sub>) and incubated with CDP-Star diluted 1:100 with the assay buffer. The membrane was wrapped in Saran Wrap film and exposed overnight to X-ray film.

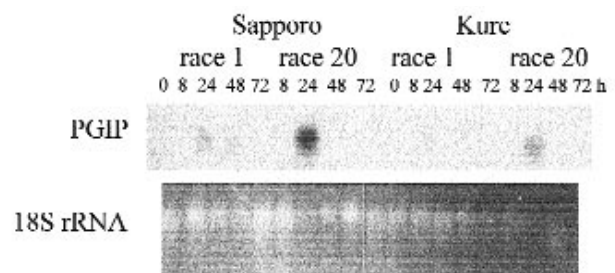
## RESULTS

**Disease symptoms.** Soybean seedlings of cvs 'Kure' and 'Sapporo' were inoculated with mycelium of race 1 and race 20 of *P. sojae* according to the method described by Keeling (1976). Lesions in all interactions appeared similar in size up to 24 h after inoculation. In the next 48 h, plants inoculated with race 1 displayed dark brown necrotic lesions extending along hypocotyls for a length of 0.5-1.0 cm. Plants infected by race 20, instead, showed yellow soft rotting lesions spreading on hypocotyls and, at 72 h from inoculation, these plants appeared completely collapsed. Therefore, both cvs appeared as resistant to race 1 and susceptible to race 20. cv. 'Sapporo' was also inoculated by *P. sojae* zoospores of both races. Symptoms similar to those obtained with mycelium inoculation were observed, but on hypocotyls infected with race 1, hypersensitive necrotic lesions appeared more limited in size (3-4 mm long).

Sapporo plants infected with *S. sclerotiorum* mycelium showed characteristic soft rot disease spreading with time, whereas infection with *D. phaseolorum* var. *caulivora* produced necrotic slightly sunken lesions slowly enlarging with time.

**Pgip expression.** Northern blots of total RNA extracted from soybean cvs 'Kure' and 'Sapporo' each infected with compatible (race 20) and incompatible (race 1) *P. sojae* races, showed similar *pgip* expression patterns (Fig. 1). Signals of *pgip* transcripts (of about 1.3 Kb) were clearly visible only 24 h after infection and were much higher in compatible interactions (with race 20). The weaker signal level showed by cv. 'Kure' was due to the lower amount of total RNA loaded, as indicated in the

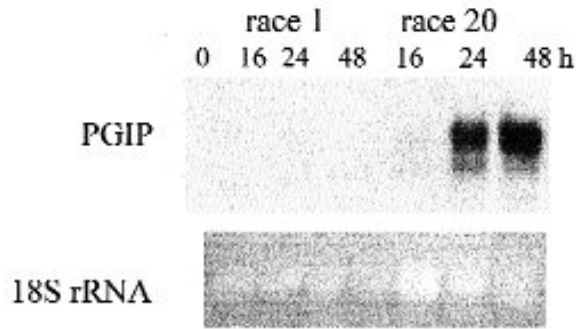
18S rRNA panel (Fig. 1). No *pgip* mRNA was detected in control seedlings mock inoculated with non-colonized agar medium (not shown). Expression of *pgip* mRNA was further analyzed at 16, 24 and 32 h after pathogen inoculation by performing two additional experiments on cvs 'Sapporo' and 'Kure'. The results confirmed in both cvs that plants infected with race 20 showed maximum *pgip* transcript accumulation at 24 h. Absence of *pgip* transcript or weaker signals were observed when plants were infected with race 1 (data not shown).



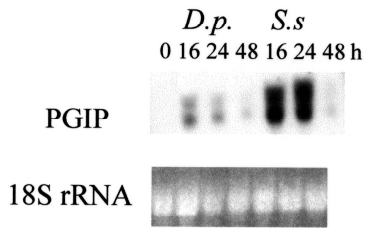
**Fig. 1.** Northern blot analysis using the *pGM7* clone of total RNA (approx. 15 µg per lane) from hypocotyls of soybean cvs 'Sapporo' and 'Kure'. RNA was extracted at 0, 8, 24, 48 and 72 h from inoculation with mycelium of race 1 and 20 of *P. sojae*. To evaluate differences on total RNA loading among samples, the ethidium bromide staining of 18S rRNA is shown in the lower panel.

Analyses performed on total RNA extracted from 'Sapporo' plants inoculated with *P. sojae* zoospores revealed *pgip* transcript only in the compatible interaction (with race 20) (Fig. 2). In particular, a strong *pgip* signal was observed at 24 and 48 h after infection with race 20. The absence of *pgip* transcript observed with soybean seedlings infected with race 1 seems unlikely to be due to lower amount of total RNA loaded onto the gel (Fig. 2). In fact, at 48 h, where RNA loaded was comparable to that obtained from interaction with race 1, a *pgip* signal was still not observed. Differently from results obtained with mycelium inoculation, *pgip* transcript was still increasing from 24 to 48 h after inoculation. We also noted the appearance of a second weaker transcript signal of lower molecular weight (of about 1.1 Kb) not previously visible during the mycelium infection.

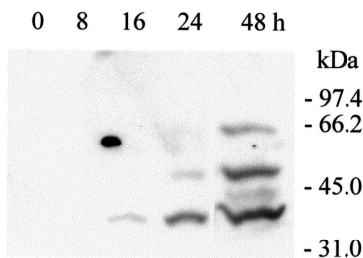
Northern blot experiments were also performed on total RNA extracted from soybean seedlings infected with *S. sclerotiorum* and *D. phaseolorum* var. *caulivora*. The results of these analyses showed induction of *pgip* transcripts at 16-24 h after inoculation, with a stronger expression in plants inoculated with *S. sclerotiorum* (Fig. 3). In both cases the presence of two hybridizing *pgip* transcripts was still more evident than those observed in cv. 'Sapporo' infected with *P. sojae* zoospores (Fig. 2).



**Fig. 2.** Northern blot analysis using the *pGM7* clone of total RNA (approx. 15 µg per lane) from hypocotyls of the soybean cv. 'Sapporo'. RNA was extracted at 0, 16, 24 and 48 h from inoculation with zoospores of race 1 and 20 of *P. sojae*. To evaluate differences on total RNA loading among samples, the ethidium bromide staining of 18S rRNA is shown in the lower panel.



**Fig. 3.** Northern blot analysis using the *pGM7* clone of total RNA (approx. 15 µg per lane) from hypocotyls of soybean cv. 'Sapporo'. RNA was extracted at 0, 16, 24, and 48 h from inoculation with mycelium of *D. phaseolorum* var. *caulivora* (*D.p.*) and *S. sclerotiorum* (*S.s.*). To evaluate differences on total RNA loading among samples, the ethidium bromide staining of 18S rRNA is shown in the lower panel.



**Fig. 4.** Western blot analysis using an anti-PG polyclonal antibody of total protein (100 µg per lane) extracted from hypocotyls of soybean cv. 'Sapporo' at different times from inoculation with mycelium of *S. sclerotiorum*. Enzyme units loaded onto the gel were 0, 0.003, 0.026, 0.080 and 0.64, respectively, as determined at 0, 8, 16, 24 and 48 h after inoculation.

**PG production in soybean infected hypocotyls.** PG activity determined on extracts of infected hypocotyls was detectable from 8 h after inoculation with *S. sclerotiorum* (see legend to Fig. 4). On the contrary no PG activity was detected on soybean hypocotyls infected with *D. phaseolorum* var. *caulivora* or with both races of *P. sojae*. The presence of endo-PG in infected tissue was monitored by Western blot analysis of hypocotyl extracts by using a polyclonal antibody raised against an endo-PG from *F. moniliforme* (De Lorenzo *et al.*, 1988). Time course analysis on 100 µg protein extracted from infected hypocotyls (equivalent approx. to 100 mg of fresh wt) showed cross-reaction bands only on samples from hypocotyls infected with *S. sclerotiorum*. A 37 kDa band appeared at 16 h after inoculation and two additional major bands at about 50 and 62 kDa, respectively, appeared later (Fig. 4). The 37 kDa band corresponds to a previously characterized endo-PG from *S. sclerotiorum* (Favaron *et al.*, 1992, 1993) and its amount at 16 h after infection was estimated to be less than 1 pmol.

**DISCUSSION**

Our results show that *pgip* transcripts accumulate after infection of soybean plants with all 3 pathogens tested. In all interactions, the *pgip* transcript was transiently expressed with similar timing and maximum accumulation at 16-24 h after infection by mycelium inoculation. The delay in reaching maximum accumulation of *pgip* transcripts in soybean plants inoculated with *P. sojae* zoospores (48 h) in comparison to plants inoculated with *P. sojae* mycelium is likely due to the time needed for zoospore germination and plant penetration.

Differences in the level of expression of *pgip* genes were observed among plants infected with different pathogens. Higher levels of *pgip* seem apparently expressed by plants infected by *S. sclerotiorum* or compatible race of *P. sojae*. In particular, plants infected with compatible *P. sojae* race 20 showed higher levels of *pgip* expression in comparison to plants infected with the incompatible race 1. These results differ from those observed in common bean infected by *Colletotrichum lindemuthianum* (Nuss *et al.*, 1996; Devoto *et al.*, 1997). During this interaction an earlier and more intense accumulations of *pgip* mRNA in the incompatible interactions (host-resistant) compared to the compatible one (host-susceptible) has been always observed. However, our results are in agreement with a recent observation on soybean plants infected with the cyst nematode *Heterodera glycines* (Mahalingam *et al.*, 1999). Similarly, during this interaction an enhancement of *pgip* tran-

scripts has been observed only in compatible interactions.

On the basis of these results, it can be speculated that common bean and soybean *pgip* genes are regulated in a different manner, and/or a different array of signals are released during infection in the two species. This could also happen at subspecific level. As argued by Mahè *et al.* (1993) for the *C. lindemuthianum*-common bean interaction, kinetic of mRNA accumulation of a particular responsive gene may change with specific interacting genotypes (race-cultivar) and is not always consistent with plant resistance or susceptibility. This could also occur for *pgip* during interactions between soybean cvs and *P. sojae* races.

Moreover, the PGIPs are encoded by a multigene family (Frediani *et al.*, 1993) and members of the bean PGIP family with different inhibitory properties are also regulated in a diverse manner following environmental stimuli (Desiderio *et al.*, 1997; Devoto *et al.*, 1998). Determining the level of expression of single *pgip* members by hybridization analysis also complicated by their high degree of sequence similarity. Therefore, in different plant-pathogen interactions, similar *pgip* transcripts may subtend a different composition of specific *pgip* members with unpredictable inhibitory abilities. In our analyses two *pgip* transcripts of different size were detected, suggesting that a complex regulatory system may be in operation. The different signal intensity of the two *pgip* transcripts could also reflect a different degree of similarity with the PGIP probe used (*pGM7*). This possibility is supported by the existence of two soybean *pgip* genes with about 80% of nucleotide sequence similarity (D'Ovidio *et al.*, in preparation). Only complete characterization of the *pgip* gene family will allow a detailed analysis of gene expression and identification of the specific contribution of each PGIP in different plant-pathogen interactions.

Noteworthy is the observation that the induction of *pgip* transcripts during the interaction between soybean and *P. sojae* or *D. phaseolorum* var. *caulivora* is not mediated by PG activity. In fact, PG activity was not detected in seedlings infected by these pathogens, even in plants infected with the virulent *P. sojae* race 20. The absence of PG activity in infected tissue does not prove that these pathogens are unable to produce PG, because PGIP in plant tissue could have masked the presence of endo-PG, especially if produced at low level. However, Western blot analysis using a polyclonal antibody against endo-PG did not detect any PG in extracts of plants infected with *P. sojae* or *D. phaseolorum* var. *caulivora*. The wide range of cross-reactivity of the anti-PG antibody (De Lorenzo *et al.*, 1988; Peretto *et al.*, 1992, 1995) and the high sensitivity of the method suggest that endo-

PGs are not involved in the interaction between soybean and *P. sojae* or *D. phaseolorum* var. *caulivora*.

The induction of *pgip* transcript in spite of the apparent inability of *D. phaseolorum* var. *caulivora* and *P. sojae* to produce PG in soybean tissue (confirmed also in vitro for *P. sojae*; authors, unpublished), suggest that PG activity is not essential for induction. *Pgip* may belong to a group of responsive genes coordinately regulated by a number of aspecific elicitors independently of the presence of the target protein. Some types of oligogalacturonide, by-products of cell wall pectin depolymerization, are reported to act as elicitors of a number of defense genes including the bean *pgip* gene (Bergmann *et al.*, 1994). Although oligogalacturonides released by PG activity should be absent following *P. sojae* and *D. phaseolorum* var. *caulivora* infections, similar elicitors may be released by other pectolytic enzymes like pectate lyase or pectin lyase.

In conclusion, our data show that, in soybean, *pgip* transcripts are induced following attack by *S. sclerotiorum*, *D. phaseolorum* var. *caulivora* and *P. sojae*. In particular, we show that *pgip* transcripts accumulate with similar kinetics in both compatible and incompatible soybean-*P. sojae* interactions, with higher levels in the compatible one. The apparent inability of the virulent and avirulent races of *P. sojae* to produce PG suggests that PG-PGIP interaction is not required for the resistance response of soybean against this pathogen.

#### ACKNOWLEDGMENTS

We thank Prof. G. De Lorenzo (Università 'La Sapienza', Roma) for providing the anti-PG polyclonal antibody, Dr. B.M. Tyler (University of California, Davis, USA) and the Max-Planck-Institut für Züchtungsforschung, Köln (Germany) for providing races of *P. sojae*, Dr. J. Hill (USDA, Agricultural Research Service, Urbana, Illinois, USA) and Dr. P. Ciriani (Asgrow Italia, Lodi, Italy) for furnishing the soybean cvs. Research supported by the Italian 'Ministero delle Politiche Agricole', National Research Project 'Plant Biotechnology'.

#### REFERENCES

- Bergmann C.W., Ito Y., Singer D., Albersheim P., Darvill A.G., Benhamou N., Nuss L., Salvi G., Cervone F., De Lorenzo G., 1994. Polygalacturonase-inhibiting protein accumulates in *Phaseolus vulgaris* L. in response to wounding, elicitors and fungal infection. *The Plant Journal* 5: 625-634.

- Bradford M., 1976. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**: 248-254.
- Bradley D.J., Kjellbom P., Lamb C.J., 1992. Elicitor-induced and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein - a novel, rapid defence response. *Cell* **70**: 21-30.
- Collmer A., Keen N.T., 1986. The role of pectic enzymes in plant pathogenesis. *Annual Review of Phytopathology* **24**: 383-409.
- Cooper R.M., 1983. The mechanisms and significance of enzymatic degradation of host cell walls by parasites. In: Callow J.A. (ed.). *Biochemical plant pathology*, pp. 101-135. Wiley and Sons, New York.
- Cooper R.M., 1987. The use of mutants in exploring depolymerases as determinants of pathogenicity. In: Day P.R., Jellis G.J. (eds.). *Genetics and plant pathogenesis*, pp. 261-281. Blackwell, Oxford.
- De Lorenzo G., Castoria R., Bellincampi D., Cervone F., 1997. Fungal invasion enzymes and their inhibition. In: Carrol G., Tudzynski P. (eds.). *The mycota*, Vol. Va, Plant relationships, pp. 61-83, Springer Verlag, Berlin.
- De Lorenzo G., Scala F., Salvi G., Cervone F., 1988. Comparison of immunological reactivity of polygalacturonases from different fungi. *Giornale Botanico Italiano* **122**: 1-6.
- Desiderio A., Aracri B., Leckie F., Mattei B., Salvi G., Tigelaar H., Van Roekel J.S.C., Baulcombe D.C., Melchers L.S., De Lorenzo G., Cervone F., 1997. Polygalacturonase-inhibiting proteins (PGIPs) with different specificities are expressed in *Phaseolus vulgaris*. *Molecular Plant-Microbe Interactions* **10**: 852-860.
- Devoto A., Clark A.J., Nuss L., Cervone F., De Lorenzo G., 1997. Developmental and pathogen-induced accumulation of transcripts of polygalacturonase-inhibiting protein in *Phaseolus vulgaris* L. *Planta* **202**: 284-292.
- Devoto A., Leckie F., Lupotto E., Cervone F., De Lorenzo G., 1998. The promoter of a gene encoding a polygalacturonase-inhibiting protein of *Phaseolus vulgaris* L. is activated by wounding but not by elicitors or pathogen infection. *Planta* **205**: 165-174.
- D'Ovidio R., Anderson O.D., 1994. PCR analysis to distinguish between alleles of a member of a multigene family correlated with wheat bread-making quality. *Theoretical and Applied Genetics* **88**: 759-763.
- Eye L.L., Sneh B., Lockwood J.L., 1978. Factors affecting zoospore production by *Phytophthora megasperma* var. *sojae*. *Phytopathology* **68**: 1766-1768.
- Favaron F., Alghisi P., Marciano P., 1992. Characterization of two *Sclerotinia sclerotiorum* polygalacturonases with different abilities to elicit glyceollin in soybean. *Plant Science* **83**: 7-13.
- Favaron F., D'Ovidio R., Porceddu E., Alghisi P., 1994. Purification and molecular characterization of a soybean polygalacturonase-inhibiting protein. *Planta* **195**: 80-87.
- Favaron F., Peretto R., Bonfante P., Alghisi P., 1993. Differential absorption and localization of two *Sclerotinia sclerotiorum* endo-polygalacturonases in soybean hypocotyls. *Physiological and Molecular Plant Pathology* **43**: 353-364.
- Frediani M., Cremonini R., Salvi G., Caprari C., Desiderio A., D'Ovidio R., Cervone F., De Lorenzo G., 1993. Cytological localization of the *pgip* genes in the embryo suspensor cells of *Phaseolus vulgaris* L. *Theoretical and Applied Genetics* **87**: 369-373.
- Keeling B.L., 1976. A comparison of methods used to test soybean for resistance to *Phytophthora megasperma* var. *sojae*. *Plant Disease* **60**: 800-802.
- Kovats K., Binder A., Hohl H.R., 1991. Cytology of induced systemic resistance of cucumber to *Colletotrichum lagenarium*. *Planta* **183**: 484-490.
- Laemmli U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **222**: 680-685.
- Mahalingam R., Wang G., Knap H.T., 1999. Polygalacturonase and polygalacturonase inhibitor protein: gene isolation and transcription in *Glycine max-Heterodera glycines* interactions. *Molecular Plant-Microbe Interactions* **12**: 490-498.
- Mahé A., Grisvard J., Dron M., 1993. Two avirulent races of *Colletotrichum lindemuthianum* trigger different time courses of plant defence reactions in bean. *Molecular Plant-Microbe Interactions* **6**: 423-428.
- Milner Y., Avigad G., 1967. A copper reagent for the determination of hexuronic acids and certain ketohexoses. *Carbohydrate Research* **4**: 359-361.
- Nuss L., Mahé A., Clark A.J., Grisvard J., Dron M., Cervone F., De Lorenzo G., 1996. Differential accumulation of PGIP (polygalacturonase-inhibiting protein) mRNA in two near-isogenic lines of *Phaseolus vulgaris* L. upon infection with *Colletotrichum lindemuthianum*. *Physiological and Molecular Plant Pathology* **48**: 83-89.
- Peretto R., Bettini V., Favaron F., Alghisi P., Bonfante P., 1995. Polygalacturonase activity and location in arbuscular mycorrhizal roots of *Allium porrum* L. *Mycorrhiza* **5**: 157-163.
- Peretto R., Favaron F., Bettini V., De Lorenzo G., Marini S., Alghisi P., Cervone F., Bonfante P., 1992. Expression and localization of polygalacturonase during the outgrowth of lateral roots in *Allium porrum* L. *Planta* **188**: 164-172.
- Ride J.P., 1980. The effect of induced lignification on the resistance of wheat cell walls to fungal degradation. *Physiological Plant Pathology* **16**: 187-196.

- Ryder T.B., Cramer C.L., Bell J.N., Robbins M.P., Dixon R.A., Lamb C.J., 1984. Elicitor rapidly induces chalcone synthase mRNA in *Phaseolus vulgaris* cells at the onset of the phytoalexin defence response. *Proceedings of the National Academy of Sciences, USA* **81**: 5724-5728.
- Sambrook J., Fritsch E.F., 1989. Molecular cloning, a laboratory manual. Maniatis T. (ed.). Cold Spring Harbor Laboratory Press, New York.
- Walton J.D., 1994. Deconstructing the cell wall. *Plant Physiology* **104**: 1113-1118.
- Yao C.L., Conway W.S., Ren R.H., Smith D., Ross G.S., Sams C.E., 1999. Gene encoding polygalacturonase inhibitor in apple fruit is developmentally regulated and activated by wounding and fungal infection. *Plant Molecular Biology* **39**: 1231-1241.

Received 21 August 1999  
Accepted 1 February 2000