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 endopolygalacturonase (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 Athrow 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 fur Zuchtungsforschung, Köl. 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 10⁷ ml⁻¹ 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 RNAeasy 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 Na₂HPO₄, 0.019 M NaH₂PO₄, 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 MgCl₂, 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 pGMY 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⁻¹ fresh wt of 50 mM Tris-HCl buffer pH 7.8 containing 1 M NaCl and 2.5 mM dithiolreitol. 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⁻¹.

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-
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).

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).
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, 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 subsume 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 oligogalacturonides, 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.

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