Relationships Among Endo-Polygalacturonase, Oxalate, pH, and Plant Polygalacturonase-Inhibiting Protein (PGIP) in the Interaction Between *Sclerotinia sclerotiorum* and Soybean

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The necrotrophic fungal pathogen *Sclerotinia sclerotiorum* secretes oxalic acid and endo-polygalacturonase (endo-PG) in host plants. Oxalic acid acidifies the plant tissue to values more suitable to endo-PG activity. However, we observed that the infected soybean seedlings possessed a pH of 3.8, which is below that optimal for endo-PG activity (4.5 to 5.0). We investigated, therefore, the effects of pH (from 5.0 to 3.6) and oxalate (5 to 20 mM) on the activity of the major basic endo-PG (PGb) and towards an acidic endo-PG (PGa) secreted by *S. sclerotiorum* during soybean infection. We verified that only PGb activity is stimulated by oxalate, while at the lowest pH levels, PGa escapes the inhibition of a soybean polygalacturonase-inhibiting protein (PGIP). These results, performed on polygalacturonic acid, were apparently consistent with data obtained from studies with soybean hypocotyl segments, in which PGb activity was increased by oxalate and PGa maintained its activity also at pH 3.6, possibly because at this pH the PGIP contained in the plant tissue is inactive. Reverse transcription-polymerase chain reaction analysis showed that, during soybean infection, the expression of the putative *pga* gene is delayed in comparison to the basic one. The different temporal expressions of the two endo-PGs and their differing responses to pH, oxalate, and PGIP seem to be consistent with a possible maximization of the fungal PG activity in the host tissue.

Additional keywords: *Glycine max*, pathogenesis, *pg* genes.

Sclerotinia sclerotiorum is a necrotrophic fungal pathogen with a wide host range, including several important crops such as sunflower, soybean, and canola (Boland and Hall 1994). The fungus produces water-soaked lesions on infected organs or tissues in which large amounts of oxalic acid and cell-wall-degrading enzymes accumulate (Lumsden 1969, 1976; Maxwell and Lumsden 1970). Experimental work established that oxalic acid is a determinant of pathogenicity, since mutants of *S. sclerotiorum* that do not produce oxalic acid were unable to colonize infected tissues (Godoy et al. 1990) and transgenic soybean

plants able to scavenge oxalate via oxalate oxidase activity were resistant to the pathogen (Donaldson et al. 2001).

There are several mechanisms by which oxalic acid compromises plant cells. Oxalic acid is toxic to plant cells, chelates Ca^{2+} in the apoplast, thus weakening the cell wall, depresses defense responses such as phenol oxidase activity and the oxidative burst, and by lowering the pH of the apoplast, increases the activity of some fungal cell-wall-degrading enzymes (CWDE) (Cessna et al. 2000; Dutton and Evans 1996). Various CWDE are produced by *S. sclerotiorum* during plant infection (Hancock 1967; Marciano et al. 1983; Riou et al. 1991). Among these, pectinases and especially endo-polygalacturonases (endo-PG; EC 3.2.1.15) have received particular attention, since they appear likely to be the major determinants of water-soaked lesions produced by the pathogen when infecting host plants (Favaron et al. 1993; Hancock 1966; Lumsden 1976; Marciano et al. 1982). It is widely accepted that PG activity is greatly enhanced by acidification of plant tissues, which may in part be due to oxalic acid. However, the inconsistency between the optimum pH level for *S. sclerotiorum* endo-PG activity determined in enzymatic assays (between 4.5 and 5.0) (Favaron et al. 1992; Hancock 1966; Lumsden 1976) and the pH level measured in water-soaked lesions or in the fungal culture medium (between 3.0 and 4.0) (Cessna et al. 2000; Cotton et al. 2003; Godoy et al. 1990; Marciano et al. 1983) suggests a more complex role of oxalic acid in PG activity during pathogenesis. One possibility is that oxalate anion could affect PG activity directly, or alternatively, it could alter the recognition of PG by polygalacturonase-inhibiting protein (PGIP), a glycoprotein inhibitor localized in the plant cell wall (De Lorenzo et al. 2001).

Both these hypotheses were verified in the present work. First, we looked for a possible effect of oxalate on the total endo-PG activity and, particularly, on two prominent endo-PGs produced by *S. sclerotiorum* when infecting soybean plants. These PGs, formerly indicated as PG-II and PG-IV (Favaron et al. 1988, 1993), are here renamed PGa and PGb in consideration of their acidic or basic isoelectric point (pI), respectively. Secondly, we investigated the joint effect of oxalate, pH, and soybean PGIP on the activity of these two endo-PGs. The combined effect of oxalate and pH on the activity of PGa and PGb was also determined on soybean hypocotyls, which constitutively contain PGIP (Favaron et al. 1994).

Finally, taking into account that tissues infected by *S. sclerotiorum* undergo a progressive decrease of pH levels, determined by the accumulation of oxalic acid, and that pH may be

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a regulative factor of pectinase genes (Prusky and Yakoby 2003; Rollins and Dickman 2001), we also looked for possible differences in the temporal expression of polygalacturonase (pg) genes encoding basic or acidic PGs.

RESULTS

pH values and oxalic acid concentration in infected soybean hypocotyls

The pH and oxalic acid concentration in soybean hypocotyls were measured 24 and 48 h after inoculation with *S. sclerotiorum*. At 24 h, pH was 4.8 and oxalate measured about 7 mM. At 48 h, pH decreased to 3.8 and oxalate concentration increased to near 50 mM. However, only data at 48 h, when water-soaked lesions completely girdled the hypocotyl segments, appeared to represent the conditions of the infected tissue (Fig. 1), because the tissue collected at 24 h contained large amounts of healthy tissue that likely altered the correct determinations of pH and oxalic acid concentration.

Effect of pH and oxalate on *S. sclerotiorum* PG activity.

Since standard colorimetric copper-based methods for detecting reducing ends were unreliable due to the negative interference of oxalate, PG activity was determined viscosimetrically.

The effect of pH and oxalate (20 mM) was assessed on the PG activity secreted by *S. sclerotiorum* when grown in liquid culture (four days old) or produced in infected soybean plantlets (two days after inoculation). Both crude PG preparations showed their maximum activity at about pH 4.6, but oxalate modified their pH-dependent curves in opposite directions. At the higher pH values tested (4.6 and 5.0), oxalate decreased the activity of PG produced in liquid culture, while it did not modify the PG activity at pH 3.6 and 4.0 (Fig. 2). In contrast, oxalate did not effect the activity of PG produced in planta at the higher pH values but nearly doubled PG activity at pH 3.6 and 4.0 (Fig. 2).

In light of previous results (Favaron and Marciano 1992) that showed that a basic endo-PG isoform, named PGb, is abundantly produced only in infected plant tissue, we determined the effect of oxalate on this PG and against an acidic endo-PG, named PGa, that is instead produced both in culture and in planta. We found that oxalate enhanced the activity of PGb in a concentration-dependent manner and most noticeably at the lower pH values (Fig. 3). In contrast, oxalate was ineffective

Fig. 1. *Sclerotinia sclerotiorum*-infected soybean hypocotyls at different times after inoculation. A plug of mycelium-colonized agar was placed along the middle region of hypocotyls (as shown at 0 h) and was removed at the following times to take the picture showing the lesions. Hypocotyl segments (approximately 5 mm) were collected as indicated by thick lines. Lesion borders at 16 and 24 h are shown by dotted lines. At 12 h, the lesion was limited to the tissue under the plug of mycelium, whereas at 48 h, the lesion affected most parts of the hypocotyl.

on PGa at the lower pH values and decreased its activity at the higher pH values (Fig. 3). Therefore, the observed positive effect of oxalate on the overall PG activity extracted from the *S. sclerotiorum*-infected plants (Fig. 2) is likely due to PGb.

Effect of pH and oxalate on PGIP activity.

In these experiments the inhibitory activity of PGIP against PGa and PGb was analyzed. We confirmed previous observations that fivefold more soybean PGIP was necessary to inhibit PGa in comparison with PGb (Favaron et al. 1994). Therefore, 5 and 1 PGIP units were used in the inhibition assays against PGa and PGb, respectively.

PGa was inhibited by PGIP maximally at pH 4.6 to 5.0, lesser inhibited at pH 4.0, and not at all inhibited at pH 3.6 (Fig. 4). Consequently, the differences of PG activity at different pH levels appeared less pronounced than those observed in the absence of the inhibitor and optimum pH shifted from 4.6 to 4.0 (Fig. 4A). The addition of oxalate did not modify the PGIP inhibition pattern of PGa (Fig. 4B). On the contrary, in the presence of oxalate, PGb was inhibited by PGIP to a similar extent (about 50% of inhibition) at all pH values tested (Fig. 4B). In the absence of oxalate, PGb was apparently not inhibited by PGIP at pH 3.6 (Fig. 4). However, at this pH level, PGb activity was very low and largely lower than the activity obtained with oxalate (Fig. 4A). At pH 3.6, it was also observed that, without oxalate, the rate of substrate degradation dramatically decreased during the enzymatic assay. The addition of oxalate improved the rate of substrate degradation (data not shown).

Fig. 2. In vitro assay to assess the influence of the pH level and oxalate on total endo-polygalacturonase (endo-PG) activity produced and isolated from **A**, fungal culture or **B**, infection of soybean seedlings by *Sclerotinia sclerotiorum*. Total endo-PG activity was assayed at pH 3.6, 4.0, 4.6, and 5.0, with (broken line) and without (filled line) oxalate addition. Oxalic acid was assayed at 20 mM. In each assay, 25 enzyme units of PG, as determined at the optimum pH of 4.6, was used. Each datapoint is the mean of three replicates $\pm$ standard deviation. Arrow indicates pH optimum.

Effect of pH and oxalate on soybean hypocotyl slices treated with PGs.

Hypocotyl slices dipped in sodium acetate or sodium oxalate buffers at pH 4.6, 4.0, or 3.6 were treated with PGa and PGb, and galacturonides released in the buffer solution were measured after 4 h of incubation. In comparison with the control, the presence of oxalate, on average, doubled the uronides released by PGb, even if the lowering of pH levels decreased significantly the amount of uronides released by PGb ($P < 0.01$) (Fig. 5). A smaller (+20%) but significant increase of uronides ($P < 0.05$) was also induced by oxalate acting on PGa, while the effect of pH levels on PGa activity was not significant (Fig. 5).

N-terminal amino acid sequences, cloning, and *pg* expression during plant infection.

In the attempt to isolate the genes encoding PGa and PGb, reverse transcription-polymerase chain reaction (RT-PCR) experiments on total RNA extracted from soybean hypocotyls 24 h after inoculation with *S. sclerotiorum* were performed. Primers were designed on the basis of the N-terminal amino acid sequences of PGa and PGb and on the nucleotide sequences of *S. sclerotiorum* PGs reported in GenBank (Table 1). The first six amino acids of PGa were ATTXTE, which are identical to those of PG2 reported by Waksman and associates (1991). This sequence is also similar to that of

PG3 (Waxman et al. 1991) and to that deduced from the *pg5* gene (accession number Y13669) in which a glycine or a cysteine at position 4 replaces the undetermined X amino acid of PGa, respectively (Table 1). On the basis of the observed sequence similarity, we constructed two sense oligonucleotide primers differing only at their 3' ends for the triplet encoding glycine (PGaglyF) or cysteine (PGacyF) and an antisense primer corresponding to the terminal 19 bp of *pg5* (PGaR). RT-PCR experiments produced an amplicon only when the sense primer containing the cysteine triplet was used, whereas no amplification product was obtained with the primer containing the glycine triplet, even when the annealing temperature was decreased to 50°C. These results do not completely rule out the possibility that our *S. sclerotiorum* strain contains an additional acidic PG possessing a glycine at amino acid position 4 of the mature protein but demonstrates the presence of a gene having a cysteine at that position.

The RT-PCR product obtained with the cysteine triplet primer was cloned and sequenced. One of the resulting clones, named pSspga, contained an insert of 1,035 bp, and its deduced amino acid sequence showed 97% identity with the deduced protein encoded by the *pg5* gene (Fig. 6). The only difference between the two sequences was an indel of 7 aa in their N-terminal portions (Fig. 6). The actual presence of this indel was confirmed by performing RT-PCR experiments, using the same forward primer (PGacyF) and two different reverse primers selective for the presence (PGainsR) or absence (PGadelR) of the insertion. The expected amplicon was obtained only when the primer PGadelR was used (data not shown).

The N-terminal portion of PGb starts with AGSXTFSGS SGA, which is identical to that deduced from the *pg2*, *pg3* (Fraissinet-Tachet et al. 1995), and *Sspg1d* (accession number AF501307) genes of *S. sclerotiorum* (Table 1) and when a cysteine codon is present at the corresponding position of the undetermined X residue of PGb. Accordingly, primers for RT-PCR reactions were designed on the basis of the consensus sequence derived from the comparison between these sequences. The amplicon of the expected size, obtained with the primer pair PGBF1 and PGBR, was cloned, and one of the resulting clones, named pSspgb, was sequenced. The correctness of the pSspgb sequence was verified by sequence analysis directly on the RT-PCR product. pSspgb contained an insert of 1,017 bp, and its nucleotide sequence showed a high degree of similarity to members (*pg1*, *pg2*, and *pg3*) of the gene subfamily encoding neutral PGs (Fraissinet-Tachet et al. 1995; Raymond et al. 1994) and was nearly identical (only two amino acid substitutions) to the *Sspg1d* gene (accession number AF501307) (Fig. 6). Due to the very high sequence similarity between *pg* members encoding the basic or neutral PGs, the primers used are unable to discriminate between different members of this *pg* subfamily. However, the perfect correspondence between the pSspgb sequence and that obtained directly from the RT-PCR product indicates that the pSspgb clone derives very likely from the prevalently expressed member of this *pg* subfamily, which in our strain at protein level, is likely to correspond to PGb. The nucleotide and amino acid sequences of the two new reported *pg* genes, *Sspga* and *Sspgb*, share 64 and 68% identity, respectively.

In order to compare the temporal expression of *Sspga* and *Sspgb*, RT-PCR experiments were performed on total RNA extracted from soybean hypocotyls at different times after inoculation with *S. sclerotiorum*. The transcript putatively corresponding to the basic PGb appeared 8 h after *S. sclerotiorum* infection, whereas that corresponding to the acidic PGa was detected 4 h later, at 12 h after inoculation. Both types of transcripts were present at 48 h, the last point analyzed (Fig. 7).

Fig. 3. In vitro assay to assess the effect of the pH level and oxalate on the activity of acidic endo-polygalacturonase (PGa) (top panel) or basic endo-PG (PGb) (bottom panel) isoforms of *Sclerotinia sclerotiorum*. Endo-PG activity was assayed at pH 3.6, 4.0, 4.6, and 5.0 with (broken lines) and without (filled line) oxalate addition. Oxalic acid concentrations were 0, 5, 10, and 20 mM. In each assay, 25 enzyme units of PG, as determined at the optimum pH of 4.6, was used. Each datapoint is the mean of three replicates $\pm$ standard deviation. Arrow indicates pH optimum.

DISCUSSION

Most work on oxalic acid-producing fungi deals with the synergistic action of oxalic acid and PG during pathogenesis (Dutton and Evans 1996). Oxalic acid is a well-established pathogenicity factor of *S. sclerotiorum* (Cessna et al. 2000; Godoy et al. 1990). Endo-PG, however, is likely indispensable in producing the rotting symptoms observed with this pathogen. Oxalic acid would favor PG activity by lowering the pH level of plant tissues to more suitable values and by increasing accessibility of PG to the substrate, notably by sequestering calcium ions bound to the cell wall pectate. In addition to these established effects, we found a direct effect of oxalic acid on the activity of a specific endo-PG and on the interaction between PG and its inhibitor PGIP.

We demonstrate that oxalate increases the total activity of the endo-PG secreted by *S. sclerotiorum* during plant infection, and we observed that this effect is particularly evident when the pH is below the optimum for PG activity. In contrast, oxalate does not increase the total activity of the endo-PG produced by the fungus when grown in liquid culture. This finding can be explained by the observation that oxalate enhances the activity of the basic isoform PGb, which is produced only when the fungus infects the host plant (Favaron and Marciano 1992), but not the activity of the other PG isoforms, including PGa, which are prevalently acidic and secreted both in liquid culture and in infected plants.

Another difference between the purified PGa and PGb isoforms is their different sensitivity to soybean PGIP inhibition at pH levels below the optimum value of 4.6. In particular, at pH 4.0 and 3.6, PGa was at most only slightly inhibited by PGIP so that its activity was similar at the different pH values tested, and its optimum pH value shifted from 4.6 to 4.0. Also PGb escaped PGIP inhibition at pH 3.6 but only in the

absence of oxalate. The addition of oxalate not only enhanced PGb activity but also restored PGIP inhibition. A possible explanation for the ability of oxalate to both enhance PGb activity and influence its interaction with PGIP is that oxalate binds to PGb (especially at low pH) and stabilizes the enzyme-active site. Consequently, the enzyme affinity for intact and especially for partially digested polymeric substrate increased. This possibility is supported by our observation that, at pH 3.6, oxalate restored the rate of substrate degradation that dramatically decreased during the enzymatic assay, when the amount of partially digested substrate is progressively increasing. In this view, PGIP, which likely competes with the substrate for the enzyme-active site (Di Matteo 2003; Federici et al. 2001), also recovers its inhibitory activity. However, a direct effect of oxalate on PGIP activity can not be ruled out.

The above results, obtained on polygalacturonic acid as substrate, were partially mirrored by data obtained on soybean hypocotyl slices treated with the two purified endo-PGs. In these experiments, the effect of pH on PG activity is consistent with data obtained on polygalacturonic acid, if we consider that soybean tissue contains PGIP. In particular, PGa shows similar activities at the different pH values, as was observed on commercial substrate in the presence of the inhibitor. The slight increase in PGa activity in the presence of oxalate, not observed in the assays with polygalacturonic acid, could be due to its capability to chelate cell wall Ca^{2+} , which can favor substrate accessibility. According to what was observed on polygalacturonate, in the hypocotyl assay, the activity of PGb increased strongly with oxalate, even if the magnitude of the oxalate effect at pH 4.6 was greater than that obtained in the assay with the commercial substrate. These discrepancies between data attained on soybean hypocotyls and those obtained on polygalacturonic acid, however, are not surprising because additional

Fig. 4. In vitro assay to assess the effect of pH and oxalate on the inhibition activity of a purified soybean polygalacturonase-inhibiting protein (PGIP) against acidic endo-PG (PGa) or basic endo-PG (PGb) of *Sclerotinia sclerotiorum*. **A**, PG activities obtained with and without PGIP. **B**, Inhibition of PG activity expressed as a ratio (%) between the decrease of PG activity with PGIP and the PG activity without PGIP. Endo-PG activity was assayed at pH 3.6, 4.0, 4.6, and 5.0 with (broken line) and without (filled line) oxalate addition. Oxalic acid was assayed at 20 mM. In each assay, 25 enzyme units of PG, as determined at the optimum pH of 4.6, was used. A total of 5 and 1 PGIP units were used against PGa and PGb, respectively, to obtain the same inhibition (50%) at the optimum pH (4.6). Each datapoint is the mean of three replicates $\pm$ standard deviation. Arrow indicates pH optimum.

factors occurring in plant tissue may condition PG activity, PG-PGIP interaction, or the effect of oxalate.

In order to understand whether PGa and PGb can contribute to the infection process in a different manner, we first cloned two *pg* genes putatively coding for these PG isoforms, and then verified their temporal expression during plant infection. As demonstrated by N-terminal amino acid sequence and by deduced amino acid sequence of cDNA clones, PGa and PGb are members of the two PG subfamilies described in *S. sclerotiorum* and reported as coding for neutral and acidic PGs, respectively (Cotton et al. 2002; Fraissinet-Tachet et al. 1995). Our basic PGb appears to belong to the subfamily encoding neutral PGs and, similar to those members, shows a prominent induction in planta (Favaron and Marciano 1992; Fraissinet-Tachet et al. 1994). The different classification of these PGs as basic or neutral may depend on the methods used for estimating the pI: measured by isoelectric focusing (IEF) separation of the PG protein or inferred from the deduced amino acid sequence. Our basic PGb possesses a measured pI of 8.3 (Favaron and Marciano 1992); instead, the deduced proteins of the neutral PGs have a pI around 7.0.

PGa activity, on the other hand, is induced in culture by galacturonic acid and derived polymeric substrates (Favaron and Marciano 1992) in a manner similar to the acidic isoforms described by Fraissinet-Tachet and Fevre (1996). PGa is also probably induced by acidic pH values (below 5.0), as already observed for the expression of some *pg* genes in *S. sclerotiorum* (Cotton et al. 2003; Rollins and Dickman 2001) and in the related fungus *Botrytis cinerea* (Wubben et al. 2000).

RT-PCR analysis showed that the putative gene encoding the basic PGb is expressed earlier than that encoding the acidic PGa, confirming the occurrence of a different regulation mechanism of the two PGs. This result is in accordance with the recent observation, obtained from a study of carrot root infected with *S. sclerotiorum* that, in comparison with neutral PG-encoding genes, the expression of the acidic PG-encoding genes was delayed (Kasza et al., 2004).

On the whole, results of activities and expression analyses support a different role of the two PGs during pathogenesis and

Fig. 5. Effect of pH and oxalate on the release of uronides from soybean hypocotyl slices, 4 h after the treatment with acidic endo-polygalacturonase (PGa) or basic endo-PG (PGb) of *Sclerotinia sclerotiorum*. A total of 10 hypocotyl slices were dipped in 0.25 ml of acetate (40 mM) or oxalate (20 mM) solutions buffered at different pH levels. A total of 20 enzyme units of PG activity, as determined in the in vitro assay at the optimum pH of 4.6, was used in each treatment. Control values from treatments performed with boiled enzymes were subtracted to each activity value. Values are the means of four separate experiments, each performed with three replicates. * indicates significant at the 0.05 level of probability, ** significant at 0.01 level of probability, and N.S. not significant, respectively, at analysis of variance. Within each PG treatment, bars with the same letters represent values that are not significantly different at $P = 0.01$ according to Duncan's multiple range test.

Table 1. N-terminal amino acid sequences and isoelectric points (pI), as determined from purified proteins or deduced from gene sequences of polygalacturonases (PGs) from *Sclerotinia sclerotiorum*^a

Endo-PG designation	N-terminal aa sequence ^b	Measured or calculated pI ^c	Clone type	Accession no.	Reference
Acidic endo-PGs					
PG2	ATTXTFSG	4.8		S13661	Waksman et al. 1991
PG3	ATTGTFSG	4.9		B60155; S13945	Waksman et al. 1991
<i>Sspg3</i>		(6.35)	mRNA	AY312510	
<i>pg5</i>	<i>ATTCTFSG</i>	(4.33)	genomic	Y13669	Kasza et al. 2004
<i>sppg6</i>		(6.30)	mRNA	AF501308	
<i>pg6</i>		(6.30)	genomic	AJ539086	Kasza et al. 2004
<i>pg7</i>		(5.08)	genomic	AJ539087	Cotton et al. 2003
PGa	ATTXTF	4.85			This study, Favaron and Marciano 1992
<i>Sspga</i>	<i>ATTCTFSG</i>	(4.24)	mRNA	AJ620513	This study
Neutral or basic endo-PGs					
<i>pg1</i>	<i>AGSCTFSDPAVPL</i>	(6.55)	genomic	L12023	Reymond et al. 1994
<i>pg2</i>	<i>AGSCTFSGSSGA</i>	(7.05)	genomic	L29040	Fraissinet-Tachet and Fevre 1996
<i>pg3</i>	<i>AGSCTFSGSSGA</i>	(6.21)	genomic	L29041	Fraissinet-Tachet and Fevre 1996
<i>Sspg1d</i>	<i>AGSCTFSGSSGA</i>	(7.52)	mRNA	AF501307	
PGb	AGSXTFSGSSGA	8.3			This study, Favaron and Marciano 1992
<i>Sspgb</i>	<i>AGSCTFSGSSGA</i>	(7.35)	mRNA	AJ620514	This paper

^a Only deduced N-terminal amino acid sequences with similarity to known N-terminal protein sequences are indicated. PG designation (acidic or neutral) was based on the authors' classification. PGa = acidic endo-PG, PGb = basic endo-PG.

^b Sequences reported in italics are those deduced from gene sequences.

^c Calculated pI values are reported in parentheses. pI were deduced from mature amino acid sequences or from the entire coding region (italics) when no similarity with known N-terminal PG sequences were available.

provide us with elements to describe a possible sequence of events occurring during colonization of the soybean tissue by *S. sclerotiorum*. The early expression of PGb and its sensitivity to PGIP inhibition account for its early role in tissue penetration. This possibility is also supported by previous results demonstrating the ability of PGb to penetrate intact plant tissues better than PGa (Favaron et al. 1993) and to be already active at the pH level of healthy tissue (Favaron et al. 1992). Both these properties are very useful at the initial stage of infection, when the tissue is still intact and the secretion of oxalic acid is just beginning. Furthermore, at this stage, the PGIP content in plant tissue may not be sufficient to impair significantly the PG activity (Favaron et al. 1994). Subsequently, the increased secretion of oxalic acid would parallel the expression of acidic PGs and, by lowering the pH value toward the optimum, would better support the overall PG activity. During this infection stage, the pathogen also induces the expression of *pgip* genes and, likely, PGIP accumulates in the infected tissue (Favaron et al. 2000; D'Ovidio et al. 2002). Further accumulation of oxalic acid decreases the pH level below the optimum for PG activity but also can favor the escape of PGIP inhibition by the acidic PGs. The success or failure of tissue colonization could rely on several factors including the presence of a proper PGIP in the tissue undergoing attack as well as the ability of a PG to escape recognition.

<i>pg1</i>	MVHILSSALSLLRLGAAVSAAPA--PAPTAAPNVADALAAVEKRAKSGCTFSDPAVPLPAI
<i>pg2</i>	MVEILSSALSLLRLGAAVSAAPA--PAPTAAPNVADALAAVEKRAKSGCTFSGSSGAAAAI
<i>pg3</i>	MVHILSSALSLLRLGAAVSAAPA--PAPTAAPNVADALAAVEKRAKSGCTFSGSSGAAAAI
<i>Sspg1d</i>	MVEILSSALSLLALGAAVSAAPA--PAPTAAPNVADALAAVEKRAKSGCTFSGSSGAAAAI
<i>Sspgb</i>	-----AGSCTFSGSSGAAAAI
<i>Sspga</i>	-----ATTGTFSGSSGAASAS
<i>pg5</i>	MV-NLSAPLILGGLSALASALPAAPAPTAAP---D-LA---KRATTCTFSGSSGAASAS
<i>pg1</i>	KSKASCATIVISAVAVPSGTTLDLTGLKSGTHV-----VFEGTTTFGYEWSGGLVSV
<i>pg2</i>	KSKASCATIVISAVAVPSGTTLDLTGLKSGTHV-----VFEGTTTFGYEWSGGLVSV
<i>pg3</i>	KSKASCATIVISAVAVPSGTTLDLTGLKSGTHV-----VFEGTTTFGYEWSGGLVSV
<i>Sspg1d</i>	KSKASCATIVISAVAVPSGTTLDLTGLKSGTHV-----VFEGTTTFGYEWSGGLVSV
<i>Sspgb</i>	KSKASCATIVISAVAVPSGTTLDLTGLKSGTHV-----VFEGTTTFGYEWSGGLVSV
<i>Sspga</i>	KSKASCATIVLSALAVPSGTTLDLTGLTQGTQV-----IFEGITTFGYEWSGGLVSV
<i>pg5</i>	KSKASCATIVLSALAVPSGTTLDLTGLTQGTQVQVVFEGITTFGYEWSGGLVSV
<i>pg1</i>	SGTDITVTGASGSLDNGAKYWDGKTNGGKTKPKFFFYAHSKLGKSSINNKKILNSPVQ
<i>pg2</i>	SGTDITVTGASGSLDNGAKYWDGKTNGGKTKPKFFFYAHSKLGKSSINNKKILNSPVQ
<i>pg3</i>	SGTDITVTGASGSLDNGAKYWDGKTNGGKTKPKFFFYAHSKLGKSSINNKKILNSPVE
<i>Sspg1d</i>	SGTDITVTGASGSLDNGAKYWDGKTNGGKTKPKFFFYAHSKLGKSSINNKKILNSPVQ
<i>Sspgb</i>	SGTDITVTGASGSLDNGAKYWDGKTNGGKTKPKFFFYAHSKLGKSSINNKKILNSPVQ
<i>Sspga</i>	SGTDITVTQSGSAYLDGKASYWDGEGSNGGKTKPKFFFAHNLI-STIENIYLNPVQ
<i>pg5</i>	SGTDITVTQSGSAYLDGKASYWDGEGSNGGKTKPKFFFAHNLI-STIENIYLNPVQ
<i>pg1</i>	VFSINSASGLTSLGITIDNSAGN--SLGHNTDAFDVGSSTDIISGANVQNDCLAINS
<i>pg2</i>	GFINSASGLTSLGITIDNSAGN--SLGHNTDAFDVGSSTDIISGANVQNDCLAINS
<i>pg3</i>	GFINSASGLTSLGITIDNSAGN--SLGHNTDAFDVGSSTDIISGANVQNDCLAINS
<i>Sspg1d</i>	VFSINSASGLTSLGITIDNSAGN--SLGHNTDAFDVGSSTDIISGANVQNDCLAINS
<i>Sspgb</i>	VFSINSASGLTSLGITIDNSAGN--SLGHNTDAFDVGSSTDIISGANVQNDCLAINS
<i>Sspga</i>	VFSIDNANGLTINNVITINASAGDTSLGHNTDGFIDGDSSTVTITGANVQNDCLAINS
<i>pg5</i>	VFSIDNANGLTINNVITINASAGDTSLGHNTDGFIDGDSSTVTITGANVQNDCLAINS
<i>pg1</i>	GTGITFTGGTCSGGHLSIGSVGGRDNVSDVIESSTVKNANGVRIKTVLGATGSVS
<i>pg2</i>	GTGITFTGGTCSGGHLSIGSVGGRDNVSDVIESSTVKNANGVRIKTVLGATGSVS
<i>pg3</i>	GTGITFTGGTCSGGHLSIGSVGGRDNVSDVIESSTVKNANGVRIKTVLGATGSVS
<i>Sspg1d</i>	GTGITFTGGTCSGGHLSIGSVGGRDNVSDVIESSTVKNANGVRIKTVLGATGSVS
<i>Sspgb</i>	GTGITFTGGTCSGGHLSIGSVGGRDNVSDVIESSTVKNANGVRIKTVLGATGSVS
<i>Sspga</i>	GTGITFTGGTCSGGHLSIGSVGGRDNVSDVIESSTVKNANGVRIKTVLGATGSVS
<i>pg5</i>	GTGITFTGGTCSGGHLSIGSVGGRDNVSDVIESSTVKNANGVRIKTVLGATGSVS
<i>pg1</i>	GVTYKDITLSGITSYGVVIEQDYENGSPGKPTSGVPIITGVTLNHNHGTSSSATNVVYL
<i>pg2</i>	GVTYKDITLSGITSYGVVIEQDYENGSPGKPTSGVPIITGVTLNHNHGTSSSATNVVYL
<i>pg3</i>	GVTYKDITLSGITSYGVVIEQDYENGSPGKPTSGVPIITGVTLNHNHGTSSSATNVVYL
<i>Sspg1d</i>	GVTYKDITLSGITSYGVVIEQDYENGSPGKPTSGVPIITGVTLNHNHGTSSSATNVVYL
<i>Sspgb</i>	GVTYKDITLSGITSYGVVIEQDYENGSPGKPTSGVPIITGVTLNHNHGTSSSATNVVYL
<i>Sspga</i>	GVTSGITLSGITDGIIVNQAYNGN--AGSPTNGVAISKFILDNVGTVESGATNIYIE
<i>pg5</i>	GVTSGITLSGITDGIIVNQAYNGN--AGSPTNGVAISKFILDNVGTVESGATNIYIE
<i>pg1</i>	CAK--CSGWTD--VNVTTGGKSTKCAGLPSGVK---CAK--CSGWTD--VNVTTGGKSTKCAGLPSGVK---
<i>pg2</i>	CAK--CSGWTD--VNVTTGGKSTKCAGLPSGVK---CAK--CSGWTD--VNVTTGGKSTKCAGLPSGVK---
<i>pg3</i>	CAK--CSGWTD--VNVTTGGKSTKCAGLPSGVK---CAK--CSGWTD--VNVTTGGKSTKCAGLPSGVK---
<i>Sspg1d</i>	CAK--CSGWTD--VNVTTGGKSTKCAGLPSGVK---CAK--CSGWTD--VNVTTGGKSTKCAGLPSGVK---
<i>Sspgb</i>	CAK--CSGWTD--VNVTTGGKSTKCAGLPSGVK---CAK--CSGWTD--VNVTTGGKSTKCAGLPSGVK---
<i>Sspga</i>	CGSGSCTNNTWSGVSVTGGKSTSTCLNVTSGGVSCSL
<i>pg5</i>	CGSGSCTNNTWSGVSVTGGKSTSTCLNVTSGGVSCSL

Fig. 6. Amino acid sequence alignment of *pg* members of the neutral (*pg1*, *pg2*, *pg3*, *sspg1d*, and *Sspgb*) and acidic (*pg5* and *Sspga*) subfamilies. Only sequences sharing similarity with known N-terminal protein sequences have been reported. Asterisks and dots indicate identical or similar residues in all analyzed sequences, respectively.

Simultaneous analysis of PGs and PGIP expression and activities, oxalic acid accumulation, and pH changes during plant infection could better substantiate the role of these different components in the interaction. However, the lack of information on the complete gene families of both the PGs and PGIPs in addition to the very high sequence similarity between members of the acidic and neutral PG subfamilies could complicate the identification of the specific contribution of the different PGs during tissue colonization.

Overexpression of *pgip* genes has been considered as a possible strategy to increase plant resistance to diseases. Tomato and *Arabidopsis* plants overexpressing *pgip* have shown a decrease of disease symptoms produced by *B. cinerea* (Ferrari et al. 2003; Powell et al. 2000), another oxalic acid-producing pathogen. However, in light of the present results, the effectiveness of plant PGIPs may depend not only on the specific molecular characteristics of the fungal PGs (Cook et al. 1999; Desiderio et al. 1997; Favaron 2001) but also on the presence of additional factors such as oxalic acid, which can influence PG activity directly or indirectly by favoring its escape from PGIP inhibition. Therefore, the identification of more effective *pgip* genes for transfection into plants to combat a fungal pathogen producing oxalic acid should also include investigations on PGIP activity at specific pH levels and oxalic acid concentrations occurring during plant infection.

MATERIALS AND METHODS

Plant, fungus, and infections.

Soybean seeds (*Glycine max* [L.] Merr. cv. Harosoy) were surface-sterilized by immersion in sodium hypochlorite (0.5 % vol/vol) for 30 min and then were rinsed thoroughly in sterile water. Plants were grown for six days in the dark at 25°C in sterilized moist vermiculite.

The B-24 isolate of *Sclerotinia sclerotiorum* (Lib. De Bary) was grown for three days at 25°C on potato dextrose agar to obtain mycelium for the inoculation of soybean seedlings.

For the infection experiments, soybean seedlings were placed horizontally on plastic trays. Roots were covered with a layer of moist paper towel. Plants were inoculated by placing

Fig. 7. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of transcripts putatively corresponding to acidic endo-polygalacturonase (endo-PG) (*Sspga*) or basic endo-PG (*Sspgb*) of *Sclerotinia sclerotiorum*. The same amount of total RNA (0.5 µg) from soybean seedlings infected with *S. sclerotiorum* was used in the one-step RT-PCR assays. Time represents hours after fungal inoculation. Experiment was repeated four times with identical results. A representative experiment is reported.

small plugs (5 × 2 mm) of mycelium-colonized agar, cut from marginal zones of colonies, along the middle region of hypocotyls (Fig. 1). The trays containing inoculated seedlings were sealed with plastic film and were incubated at 25°C in the dark. At various times after inoculation, hypocotyl segments (approximately 5 mm) cut transversally with a razor blade exactly below the agar plugs were collected, were frozen in liquid nitrogen, and were stored at -80°C for subsequent analyses.

PG and PGIP production and purification.

PGs of *S. sclerotiorum* were obtained from infected soybean hypocotyl segments or from liquid culture containing 0.2% (wt/vol) polygalacturonic acid (Fluka AG, Buchs, Switzerland) as substrate (Favaron and Marciano 1992; Favaron et al. 1997). PGa and PGb were purified from infected soybean hypocotyls 48 h after inoculation, by a combination of IEF and chromatographic techniques (Favaron et al. 1997). Purified PGs were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli 1970), were electroblotted on an Immobilon-P membrane (Millipore, Billerica, MA, U.S.A.) and, after staining with Coomassie blue R-250, were sequenced by a 477A Applied Biosystems sequencer equipped with a 120 A on-line phenylthiohydantoin analyzer.

Soybean PGIP was extracted from 6-day-old soybean hypocotyls and was purified by preparative IEF, PG-sepharose, and cation-exchange (Mono-S column) chromatography, as previously reported (Favaron et al. 1994).

PG and PGIP assays.

PG activity was determined viscosimetrically, measuring the decrease in relative viscosity at 30°C of a 2-ml reaction mixture containing 0.5% (wt/vol) polygalacturonic acid substrate (from orange, grade III, purity 98%; Sigma, St. Louis) dissolved in 75 mM sodium acetate buffer, bovine serum albumin (0.1 mg ml⁻¹) and a varying amount of sodium oxalate (0 to 20 mM). The mixtures were adjusted to various pH levels (3.6, 4.0, 4.6, and 5.0) with NaOH. Micro-Ostwald capillary viscosimeters (i.d. = 0.70 mm) were used. Each viscosimeter was mounted on a measuring stand AVS/S provided with light barriers and was connected to an automatic viscosity measuring system AVS 310 unit (Schott Geräte, Mainz, Germany). One enzyme unit was defined as the amount of enzyme causing a 50% decrease of the initial viscosity of the reaction mixture at pH 4.6 in 1,000 min. PG assays were performed using 25 enzyme units. In the inhibition assays, one PGIP unit was defined as the amount of inhibitor able to reduce by 50% at pH 4.6 the activity of 25 units of PGb. Five PGIP units were necessary to inhibit to the same extent (by 50%) the activity of 25 PGa units.

Determinations of oxalic acid and pH.

A total of 10 infected hypocotyl segments, weighing approximately 150 mg fresh weight (FW), were collected as described above at 24 and 48 h after inoculation with *S. sclerotiorum* and were homogenized in 0.45 ml of distilled water at 4°C, using a potter. After a brief centrifugation (5 min) at 10,000 × g, the pH level of the supernatant was measured and oxalic acid concentration was determined by the oxalic acid enzymatic (UV test) kit (Boehringer Mannheim, Roche, Germany), following the manufacturer's instructions.

PG treatment of soybean hypocotyl slices.

The ability of PGa and PGb to release uronides from soybean hypocotyls as a function of pH level and oxalate concentration was assayed as follows. Slices (1 mm thick) were cut transversely from the middle region of noninoculated soybean hypocotyls. Slices were repeatedly washed with distilled water for 20 min and then were briefly dried on a filter paper and

were immersed in 2.0-ml Eppendorf vials (10 slices per vial, approximately 30 mg FW). The vials contained 0.25 ml of 40 mM sodium acetate or 20 mM sodium oxalate adjusted to different pH levels (3.6, 4.0, or 4.6) with NaOH and 20 enzyme units of PGa or PGb. After 4 h of incubation at 30°C on a platform shaker (70 strokes per min), each vial containing PG-treated slices was briefly decanted and the reaction mixture was collected and centrifuged briefly at 10,000 × g to remove debris. Two aliquots of 20 µl each were taken from each reaction mixture, and the uronides released were measured by the *m*-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen 1973) using D-galacturonic acid as a standard. Control treatments were performed with boiled enzyme. For each treatment, three replicates were performed. For each endo-PG, the full experiment was repeated four times in successive periods. Since the four experiments were performed with different plant samples, each one of them was considered as a block, and data were submitted to analysis of variance in a completely randomized block fashion.

Extraction of RNA, RT-PCR, and cloning of amplified products.

Total RNA was extracted from infected hypocotyl segments with the Nucleo spin RNA plant kit (Machery-Nagel, Düren, Germany). RT-PCR was performed using the Ready-to-go RT-PCR beads (Amersham Pharmacia Biotech, Uppsala, Sweden), following the manufacturer's instructions. Forward and reverse oligonucleotide primers were designed on the basis of the N-terminal amino acid sequences of the two purified PGs (PGa and PGb) and on the nucleotide sequences of *S. sclerotiorum* *pg* genes sharing a high degree of identity with the N-terminal amino acid sequences of PGa and PGb. Oligonucleotide primers specific for the PGa and PGb sequences were: PGaglyF, 5'-AAGCGAGCAACTACTGGC-3'; PGacysF, 5'-AAGCGAGCAACTACTTGT-3'; PGaR, 5'-CTACAAGGAGCAAGAGACG-3'; PGainsR, 5'-CCTTCGAAGATGACAACACG-3'; PGadelR, 5'-GTGATACCTTCGAAGATGACTTT-3'; PGbF1, 5'-GCCG GTTCCTGACCTTCTC-3'; PGbF2, CATCAAGTCCAAGGCTTCCT; and PGbR, 5'-TTAACACTTGACACCAGATGGG-3'. Each RT-PCR reaction was performed in a total volume of 50 µl, using 0.5 µg of total RNA obtained from 24-h-infected hypocotyls and 50 ng of each of the two primers. cDNA synthesis and amplification conditions were: 30 min at 42°C for first-strand cDNA synthesis; 5 min at 95°C to inactivate the reverse transcriptase; and then, 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for cDNA amplification.

Amplified products were analyzed on a 1.2% agarose gel, and amplicons of the expected size were excised from the gel, were purified with the GFX PCR DNA and gel band purification kit (Amersham Pharmacia Biotech), and then, were cloned into the pGEM-T Easy vector (Promega, Madison, WI, U.S.A.) according to the manufacturer's protocol. The ligation mixture (10 µl) was used to transform 200 µl of competent cells (*Escherichia coli* DH5α; Gibco BRL, Gaithersburg, MD, U.S.A.), using standard procedure (Sambrook et al. 1989). Identification of transformed colonies was performed by blue/white screening according to Sambrook and associates (1989). Plasmid DNA was purified from recombinant bacterial clones with the Nucleobond plasmid purification midi kit (Clontech, Palo Alto, CA, U.S.A.). Nucleotide sequences of the cDNA clones, pSpSga and pSpSgb, were determined using the ABI PRISM dye terminator cycle sequencing ready reaction kit on a semiautomatic ABI PRISM 310 sequencer (Applied Biosystems, Foster City, CA, U.S.A.). Sequence analysis and primers selection were performed using the DNAMAN sequence analysis package (Lynnon BioSoft, Vaudreuil-Dorion, Quebec, Canada).

Time course RT-PCR analysis on infected tissues was performed using the oligonucleotide primer pairs PGacysF/PGaR and PGBF2/PGBR targeting PGa and PGB, respectively. RT-PCR conditions were the same as described above, except that an annealing temperature of 55 or 60°C was chosen for cDNA amplification with PGacysF/PGaR and PGBF2/PGBR, respectively. Correctness of the amplicons was verified by determining their nucleotide sequence.

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