

EXPRESSION OF TWO *SCLEROTINIA SCLEROTIORUM* ENDO-PG GENES CORRELATES WITH ENDO-POLYGALACTURONASE ACTIVITY DURING *GLYCINE MAX* COLONIZATION

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SUMMARY

Quantitative expression of *Sclerotinia sclerotiorum* genes encoding two endo-polygalacturonase (endo-PG) isoforms (PGa and PGb), malate dehydrogenase (MDH), a key enzyme in fungal biosynthetic pathway of oxalic acid, and plant polygalacturonase-inhibiting protein (PGIP) were monitored by real-time reverse transcription-polymerase chain reaction (qRT-PCR) during the early stages (0-48 h) of soybean seedling infection. The activity of the two endo-PGs was also investigated during plant infection. PGa and PGb activity reflected very closely the pattern of their transcript accumulation as determined by qRT-PCR. In particular, the PGb encoding gene (*Sspgb*) was induced at 8 h after inoculation and reached a maximum at 16 h; expression of the PGa encoding gene (*Sspga*) was comparatively lower, reaching its maximum level later and its rate of increase paralleled that of the *S. sclerotiorum* β -tubulin gene; the expression of the MDH encoding gene (*Ssmdh*) was maximal 16 h after infection; soybean *pgip* transcript began to accumulate 8 h after inoculation reaching a maximum after 24 h. Expression patterns of reported genes are discussed in relation to the ability of *S. sclerotiorum* to induce disease by regulating endo-PGs and oxalate accumulation to elude the effect of plant PGIP.

Key words: endopolygalacturonase, PGIP, soybean, oxalic acid, real-time RT-PCR.

INTRODUCTION

Sclerotinia sclerotiorum is a necrotrophic pathogen able to infect a wide range of host plants (Boland and Hall, 1994). During pathogenesis, this fungus secretes a large number of cell-wall degrading enzymes (CWDEs) to penetrate, colonize and macerate host tissues (Marciano *et al.*, 1983; Riou *et al.*, 1991). Among CWDEs,

endo-polygalacturonase (endo-PG) produced by *S. sclerotiorum* is able to macerate intact tissue (Favaron *et al.*, 1993). The endo-PG of *S. sclerotiorum* is encoded by several *pg* genes which, based on sequence similarity, can be classified into four groups. The first group encodes neutral or basic isoforms and comprises three genes (*sspg1a*, *sspg1b*, *sspg1c*) isolated from strain S5 of *S. sclerotiorum* (Reymond *et al.*, 1994; Fraissinet-Tachet *et al.*, 1995) and two additional ones, *sspg1d* and *Sspgb*, isolated from strains 100 and B-24, respectively (Favaron *et al.*, 2004; Li *et al.*, 2004b). The second group encodes acidic PG isoforms and includes three genes (*pg5*, *sspg5* and *Sspga* isolated from S5, 100 and B-24 strains, respectively) showing a high degree of sequence similarity (Favaron *et al.*, 2004; Kasza *et al.*, 2004; Li *et al.*, 2004b). The third and fourth group code for acidic PGs with a peculiar N-terminal sequence and comprise the pair *pg6/sspg6* and the pair *pg7/sspg3*, respectively. The genes *pg6* and *pg7* were isolated from strain S5 while the genes *sspg3* and *sspg6* were isolated from strain 100 (Kasza *et al.*, 2004; Li *et al.*, 2004b). Only the *Sspga*, *Sspgb* and *sspg1b* gene products have been characterized (Cotton *et al.*, 2002; Favaron *et al.*, 2004) and analysis of gene expression performed by RT-PCR showed that the basic or neutral PGs are the first to be expressed by *S. sclerotiorum* when infecting host plants (Favaron *et al.*, 2004; Kasza *et al.*, 2004).

Previously, we have found that some biochemical properties of PGb, encoded by *Sspgb*, support its prominent role at an early stage of soybean seedling infection (Favaron and Marciano, 1992; Favaron *et al.*, 1992, 1993). PGb also induces programmed cell death on soybean cells, an important property for a necrotrophic fungus such as *S. sclerotiorum* (Zuppini *et al.*, 2005). In addition, we observed that oxalic acid secreted by *S. sclerotiorum* during plant infection has a dual effect on PGb activity. Initially, it changes the pH toward more suitable values for PG activity and then, at sub-optimal pH, has a direct enhancing effect on the activity of the enzyme (Favaron *et al.*, 2004). We observed also that PGa, the acidic isoform encoded by the *Sspga* gene, escapes inhibition by soybean polygalacturonase-inhibiting protein (PGIP) at acidic pH determined at later stages of soybean infection.

Based on these observations, we hypothesized that *S.*

sclerotiorum modulates the expression of PGa and PGb to efficiently degrade pectic polymers in a host cell wall environment where pH, oxalate and PGIP levels are modified during plant infection (Favaron *et al.*, 2004).

In this paper, we have first established the occurrence of PGa and PGb activity of *S. sclerotiorum* within 48 h after inoculation of soybean seedlings. Then, we monitored, by qRT-PCR, the quantitative expression of *Sspga* and *Sspgb* genes. Due to the effect of oxalate on endo-PG activity, we also analysed the quantitative expression of *Ssmdh*, the putative *S. sclerotiorum* gene encoding malate dehydrogenase (Li *et al.*, 2004a), a key enzyme in fungal oxalate biosynthesis (Dutton and Evans, 1996; Munir *et al.*, 2001) immediately upstream of oxaloacetase, the final enzyme of the biosynthetic pathway in *S. sclerotiorum* (Maxwell, 1973). Because endo-PG activity may also be affected by the plant PGIP, we monitored the quantitative expression of *Gmpgip* gene encoding the soybean PGIP (Favaron *et al.*, 1994; D'Ovidio *et al.*, unpublished).

MATERIALS AND METHODS

Disease assay. Soybean seeds [*Glycine max* (L.) Merr. cv Harosoy] were surface sterilized by immersion in sodium hypochlorite (0.5% v/v) for 30 min, and then rinsed thoroughly in sterile water. Plants were grown for 6 days in the dark at 25°C in sterilized moist vermiculite.

The B-24 isolate of *S. sclerotiorum* (Lib. De Bary) was grown for 3 days at 25°C on potato dextrose agar (PDA) to obtain mycelium for the inoculation of soybean seedlings.

Soybean seedlings were inoculated by mycelium-colonized agar plugs as previously reported (Favaron *et al.*, 2004). At various times after inoculation, hypocotyl segments (*ca.* 5 mm long), cut transversally with a razor blade exactly below the agar plugs, were collected, frozen in liquid nitrogen and stored at -80°C for subsequent analyses.

On gel detection of PG activity. To monitor the presence of PG isoforms in the infected soybean seedlings, infected hypocotyl segments, collected as above reported, were directly placed upon the surface of 0.8 mm thick polyacrylamide (PAA) gel containing 3.3% (v/v) carrier ampholytes (Amersham Biosciences, Uppsala, Sweden) covering the pH range 3-10 or 4.5-5.4. PG isoforms were detected by the agarose overlay technique described by Ried and Collmer (1985).

Isoelectrofocusing (IEF) standards (broad range pI, Bio-Rad Laboratories, Hercules, Ca, USA; and low range pI, Amersham Bioscience, Uppsala, Sweden) were used to estimate the pI in the pH range 3-10 and 4.5-5.4, respectively.

Separation of acidic PG isoforms and activity assays.

PG isoforms extracted from 48 h soybean infected hypocotyls were separated by preparative IEF in the pH range 3-10 and then pI fractions between 4.0 and 6.0 were refocused in the pH range 4.5-5.4, as previously reported (Favaron *et al.*, 1997). PG activity of the IEF fractions was determined by the reducing end-groups and viscosimetric assays, by using polygalacturonic acid as a substrate (PGA, from orange, grade III, Sigma, Saint Louis, Missouri, USA) as previously reported (Sella *et al.*, 2004). An aliquot of the most active fraction of each PG peak was analysed by PAA-IEF as above reported. One reducing unit was defined as the amount of enzyme required to release 1 $\mu\text{mol min}^{-1}$ of reducing groups. One viscosimetric unit was defined as the amount of enzyme causing a 50% decrease of the initial viscosity of the reaction mixture in 1,000 min.

Extraction of RNA and primers design. Total RNA was extracted from infected hypocotyl segments at various times after inoculation with the Nucleo Spin RNA Plant kit (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions. Five hypocotyl segments were used for each time point. Contaminating DNA was removed using DNA-freeTM (Ambion, Austin, TX, USA).

Forward and reverse oligonucleotide primers specific for the endo-polygalacturonase (*Sspga* and *Sspgb*; accession number AJ620513 and AJ620514), the putative malate dehydrogenase (*Ssmdh*; accession number CD645605) and β -tubulin (*Sstub*; accession number AF421379) genes from *S. sclerotiorum* and the soybean ubiquitin (*Gmubi*; accession numbers D28123) and *pgip* (*Gmpgip*) genes were designed using the Primer 3 software (Rozen and Skaletsky, 2000) and have the following sequence: PGA198F and PGA198R (5'-CAACGC-TAATGGGTTGACAAT-3'; 5'-CAGACACCCCCTGAGAAAGTA-3') (*Sspga*); PGbasicF and PGbasicR (5'-TCCTCCATCAACAACGTCAA-3'; 5'-GTGTTGTGTCCGAGGGAGTT-3') (*Sspgb*); SsMD FOR and SsMD REV (5'-CCAAACGCAAACATCCTCGT-3'; 5'-CCAAAGTGGTAACGCCGAAA-3') (*Ssmdh*); SstubF and SstubR (5'-TGAAGGAGGTTGAGGACCAA-3'; 5'-GGGGAGGAATGGAGCAAAG-3') (*Sstub*); GmUbiF1 and GmUbiR1 (5'-TGCGTCTTCGTGGTGGTATG-3'; 5'-TCAGCGAGGGTCCCTCCAT-3') (*Gmubi*); GmPGI PcosF and GmPGIPcosR (5'-CAACCACTCTCTCCATGG-3'; 5'-TAAGATCGGAGAGGTCGAGG-3') (*Gmpgip*).

Primers for *Gmpgip* amplified all the soybean *pgip* genes reported (D'Ovidio *et al.*, unpublished).

Primer specificity was confirmed by PCR on plasmid clones containing the corresponding genes or by RT-PCR on total RNA of uninfected and infected soybean tissue. PCR reactions were performed in a total volume of 25 μl , using as template 20-50 ng of plasmid DNA.

The reaction mixture also contained 12.5 µl of REDTaq™ Mix (Sigma, Saint Louis, Missouri, USA) and 50 ng (300 µM) each of forward and reverse primers. Amplification conditions were the following: 94°C for 2 min, then 30-35 cycles at 94°C for 1 min, 60°C for 1-2 min and 72°C for 1-2 min. RT-PCR reactions were performed using the Ready-To-Go RT-PCR beads (Amersham Biosciences, Uppsala, Sweden) following the manufacturer's instructions. Each reaction was performed in a total volume of 25 µl using 250 ng of total RNA obtained from 0 or 24 h infected hypocotyls and 50 ng (300 µM) 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; then, 35 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min for cDNA amplification. The absence of contaminating DNA was verified by including a negative control without reverse transcriptase.

Amplicons of the expected size were obtained only when specific primers were used with correspondent clones containing the specific gene as template. Primers specific for *Sstub* and *Ssmdb* genes were assayed by RT-PCR using the RNA of uninfected and infected soybean tissue as template, and amplified only in the presence of the fungus (data not shown).

Real-Time RT-PCR (qRT-PCR). qRT-PCR experiments were performed using the *iCycler iQ* (Bio-Rad Laboratories, Hercules, CA, USA) and an RT-PCR mix containing the fluorogenic dye SYBR® Green I. qRT-PCR analysis of infected tissues were performed in a total volume of 25 µl, using as template 12.5 ng of total RNA obtained from infected hypocotyls at different times after inoculation (0, 8, 16, 24 and 48 h). The reaction mixture contained also 12.5 µl of 2x QuantiTect SYBR Green RT-PCR Master Mix (Qiagen, Milano, Italy), 0.25 µl of QuantiTect RT Mix (Qiagen, Milano, Italy) and 50 ng (300 µM) of each forward and reverse primers. Each reaction was made in triplicate.

Only replicates showing threshold cycle values differing for less than 0.5 cycles were used. cDNA synthesis and amplification conditions were the following: 30 min at 50°C for reverse transcription; 15 min at 95°C to inactivate the Reverse Transcriptases and activate Hot-StarTaq DNA Polymerase; then, 40 cycles at 94°C for 15 s, 60°C for 30 s and 72°C for 30 s for cDNA amplification. To check the specificity and the size correctness of the RT-PCR products, a melting curve analyses at the end of each Real-Time amplification and amplicons fractionation on a 1.5% agarose gel were performed. The absence of contaminating DNA in each RNA samples was verified by performing a qRT-PCR without reverse transcriptase.

Comparison between samples was based on the threshold cycle (C_T), the cycle at which there is the first

detectable increase in fluorescence compared to baseline. Relative expression analysis was determined by using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001; Applied Biosystems User Bulletin No. 2-P/N 4303859). Since this method can be applied when the amplification efficiencies of target and endogenous reference genes are similar, we calculated them by performing qRT-PCR on serial dilutions of the RNA template over a 100-fold range (Livak and Schmittgen, 2001). Amplification efficiencies of target and endogenous reference genes were between 96% and 98%. Real time RT-PCR experiment was carried out on RNA extracted from two separate experiments of plant inoculation. In the different experiments the expression trend of each gene was the same.

Results of a single experiment are reported in the present paper.

RESULTS

On gel detection of PG activity. *S. sclerotiorum* infected hypocotyl segments were loaded directly on the PAA gel surface, and after the IEF run the PG isoforms were detected by the overlay technique. This procedure, exploiting the IEF electric field for the extraction of the proteins accumulating in the diseased tissue, provides a realistic representation of the relative amounts between PG isoforms produced during plant infection. Alternative extraction procedures of PGs based on tissue homogenisation with saline buffers and desalting of extracted samples can alter the relative amount between the PG isoforms.

The analysis of the overlay gel showed that PGb (estimated pI 8.3) activity was perceivable 8 h after inoculation, and its activity increased strongly with time (Fig. 1A). Instead, PGa (estimated pI 4.9) activity was barely visible from 12 to 24 h becoming strong only at 48 h after infection (Fig. 1B).

Additional basic/neutral and acidic PG bands were also present but, in order to establish whether these isoforms were of endo- or exo-PG type, we further characterized only the three acidic isoforms with estimated pI of 4.7, 4.8 and 5.0 (Fig. 1B). To this aim, PG activity extracted from 48 h infected hypocotyls was subjected to preparative IEF and the fractions obtained assessed viscosimetrically and by the reducing end-groups assay. Four peaks of PG activity were detected: the first two displayed very high reducing activity and a low viscosimetric activity, thus denoting mostly exo-PG activity; in contrast, the next two peaks showing a negligible reducing-end activity in comparison to a relevant viscosimetric activity were classified as endo-PG (Fig. 2A). Therefore, as inferred by analysis of each peak fraction on PAA-IEF gel, the isoform bands with pI at 4.7 and 4.8 were exo-PG, whilst that with pI about 5.0, like the PGa isoform (estimated pI 4.9), was an endo-PG (Fig. 2B).

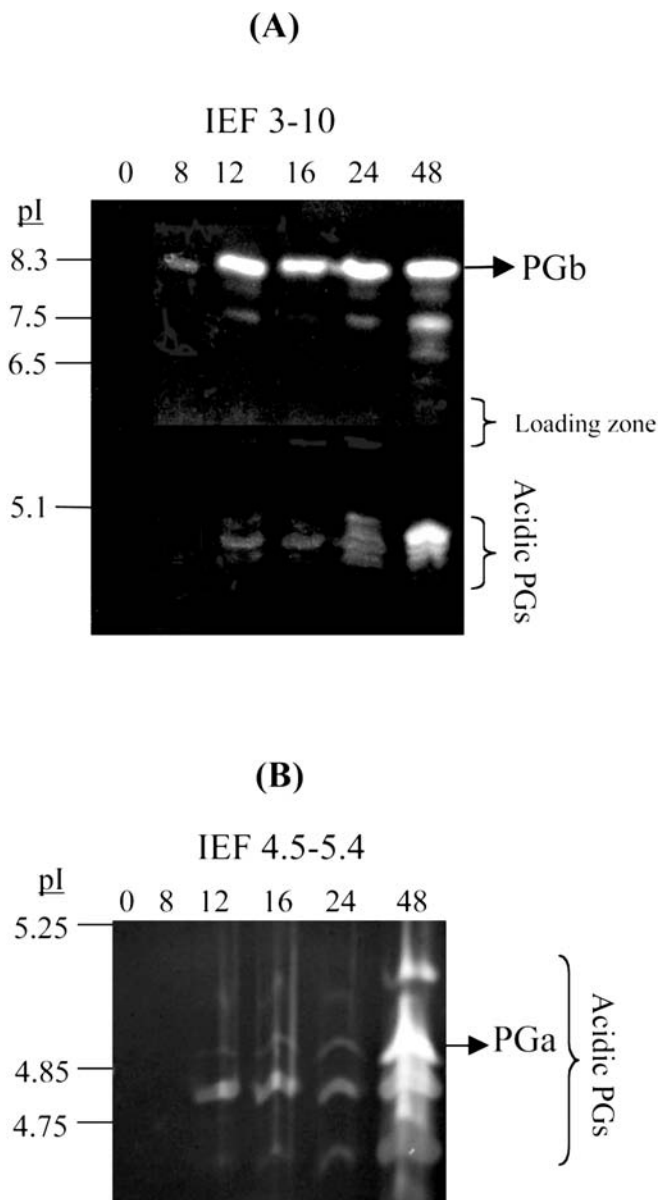


Fig. 1. Thin layer IEF in the pH range 3-10 (A) and 4.5-5.4 (B) of hypocotyl fragments harvested from soybean seedlings infected with isolate B-24 of *S. sclerotiorum* at 0, 8, 12, 16, 24 and 48 h after inoculation. Fragments (5 mm in length) were taken below the agar-colonized plugs used for seedling infection. Two hypocotyl fragments were loaded at 0, 8, 12, 16, 24 h. At 48 h one hypocotyl fragment was loaded in (A) and two in (B). pI values are indicated on the left. Bands corresponding to PGa and PGb are indicated on the right.

Quantification of gene expression during *S. sclerotiorum*-soybean infection. In order to compare the temporal expression of *Sspga*, *Sspgb*, *Ssmdb* and *Gmpgip*, we performed qRT-PCR experiments on total RNA extracted from soybean hypocotyls at different times after inoculation with *S. sclerotiorum* (0, 8, 16, 24 and 48 h). Melting curve analysis and agarose gel electrophoresis performed to check the specificity and the correct size of the PCR products confirmed the presence of a single

amplicon of the expected size (not shown). For the relative quantification, two housekeeping genes were used: the *S. sclerotiorum* β -tubulin gene, which allowed the expression analysis of the fungal genes (*Sspga*, *Sspgb*, *Ssmdb*) during tissue colonization and the soybean ubiquitin gene, which allowed the analysis of the relative accumulation of *Gmpgip* transcript in the tissue.

By using the fungal β -tubulin gene as a normalizer, we monitored *S. sclerotiorum* gene expression in comparison to the fungal growth in the plant tissue during infection. The results showed that *Sspgb* transcription was strongly induced (30 fold increase) in the early 16 h after infection and then the rate of expression progressively decreased (Fig. 3). In contrast, *Sspga* gene was not appreciably induced and therefore its expression, which maximally showed <0.5 fold increase at 48h, was proportional to fungal growth (Fig. 3). Besides, *Ssmdb* transcription was induced in the first stage of infection, reaching the maximum value of about 1 fold increase after 16 h (Fig. 3). By using the soybean ubiquitin gene as housekeeping, *Gmpgip* transcript noticeably accumulated until 24 h after *S. sclerotiorum* infection and maintained that level in the next 24 h (Fig. 3). However, at this stage of infection (48 h) the tissue appeared completely macerated and, therefore, plant defence responses, including PGIP accumulation, are likely impaired.

DISCUSSION

Endo-PG and oxalic acid are the most likely factors responsible for the well-known disease symptoms produced by *S. sclerotiorum*. The two molecules act synergistically, elicit cell death and depress defence responses of the host (Dutton and Evans, 1996; Cessna *et al.*, 2000; Favaron *et al.*, 2004; Zuppini *et al.*, 2005). Pg genes show differences in their temporal expression, with those predicting neutral or basic endo-PG isoforms being expressed earlier than those encoding the acidic endo-PGs (Favaron *et al.*, 2004; Kasza *et al.*, 2004; Li *et al.*, 2004b). Previously, it was shown that the basic PGb isoform produced by the B-24 isolate of *S. sclerotiorum* when infecting soybean seedlings possesses properties more suitable than the acidic PGa isoform to an early phase of host infection (Favaron and Marciano, 1992; Favaron *et al.*, 1992, 1993).

Plant PGIP may represent a barrier to endo-PG activity and a potential mechanism of defence against fungal pathogens. However, to be effective, PGIP should possess high affinity to fungal endo-PG and be active at pH conditions of plant tissue during fungal infection. We hypothesized that *S. sclerotiorum* modulates expression of PGa and PGb to efficiently degrade pectic polymers in a host cell wall environment where pH, oxalate level and PGIP expression and activity are modified during plant infection (Favaron *et al.*, 2004).

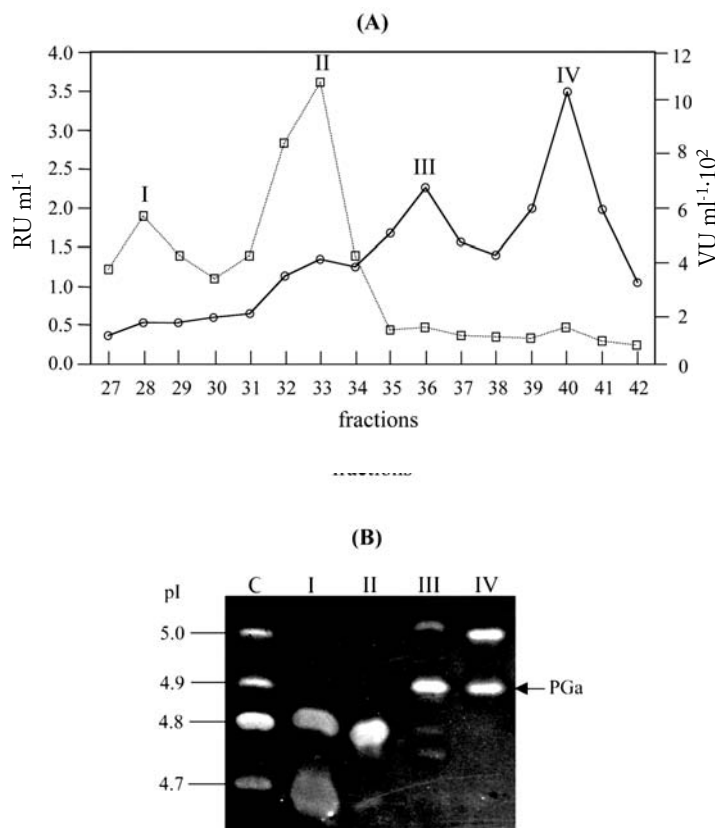


Fig. 2. Separation of acidic PG isoforms by preparative IEF (pH range 4.5-5.4) of crude PG extracted from soybean seedlings after 48 h from inoculation with *S. sclerotiorum*. **(A)** Profile of active fractions obtained by reducing-end groups (—□—) and viscosimetric (—○—) assays. **(B)** Thin layer IEF in the pH range 4.5-5.4 of the most active fraction of each PG peak (I-IV) shown in **(A)**. Estimated pI are reported on the left. Band corresponding to PGa is indicated on the right. A sample of the crude PG extract was loaded in the C lane. Ten VU of PG activity were loaded on lanes C, I and II; 20 VU were loaded on lanes III and IV, respectively. RU = reducing units; VU = viscosimetric units.

In order to obtain a more complete picture of crucial events concerning early stages of soybean infection by *S. sclerotiorum*, in the present paper we have first determined the presence of PGa and PGb activity in soybean tissue. Then, we have analysed the temporal expression of (i) *Sspga* and *Sspgb* genes, encoding PGa and PGb, (ii) *Ssmdh* gene, which encodes the malate dehydrogenase enzyme involved in the oxalic acid biosynthesis, and (iii) *Gmpgip* gene, encoding the soybean PGIP. The analysis was conducted up to 48 h from inoculation, when the tissue was completely macerated (Favaron *et al.*, 2004).

PGa and PGb band intensity on the gel after IEF separation provides a realistic representation of the contribution of the two isoforms on the total PG activity produced in soybean tissue during pathogen infection. PGb was clearly the earlier and predominant endo-PG at all time assayed while PGa activity appeared later and

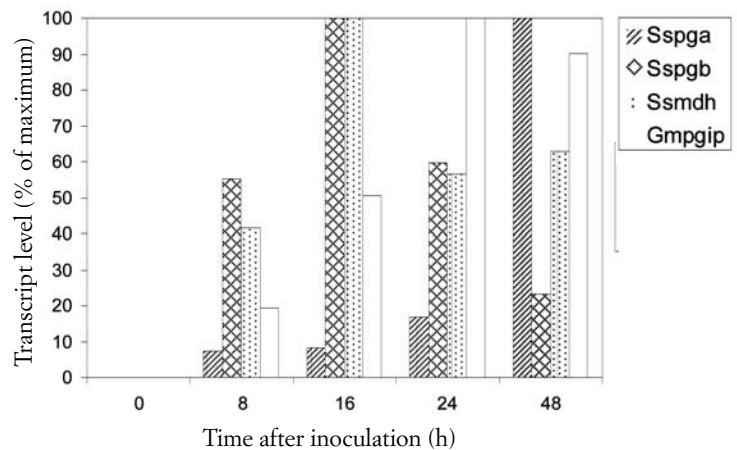


Fig. 3. Transcript quantification of *S. sclerotiorum* *Sspga*, *Sspgb*, *Ssmdh* and soybean *Gmpgip* genes accumulating in soybean tissue after *S. sclerotiorum* infection. Each value is reported as the percent of the maximum transcript value. Each transcript was normalized with the *S. sclerotiorum* β -tubulin gene as housekeeping of the fungal genes or with the endogenous soybean ubiquitin gene as housekeeping of the plant *Gmpgip* gene.

with a lesser band intensity. PGa focused on a narrow acidic region where two exo-PG isoenzymes and a further endo-PG were present. This second endo-PG occurred with a timing resembling that of PGa. Similarly, in a different strain of *S. sclerotiorum*, Waksman *et al.* (1991) reported the presence of two acidic endo-PGs possessing pIs comparable to our acidic isoforms and determined their N-terminal amino acidic sequence. One is identical to PGa while the other shows a single amino acidic substitution at the N-terminal. This second sequence, however, has not been detected in any of the sequences deduced from the *pg* genes cloned so far (Favaron *et al.*, 2004; Kasza *et al.*, 2004; Li *et al.*, 2004b). Therefore, the molecular characteristics and properties of this additional endo-PG secreted during *S. sclerotiorum* infection remain to be elucidated together with minor basic/neutral PGs.

The differential transcript accumulation and enzyme activity of PGa and PGb in infected soybean hypocotyls mirror the expression pattern of *Sspga* and *Sspgb* genes as determined by qRT-PCR analysis. In fact, a quick and relevant induction of *Sspgb* gene was observed in comparison to the *Sspga* gene. These results confirm previous data implicating a different mechanism of regulation of basic/neutral and acidic endo-PGs. It was reported that acidic pH conditions of the culture medium induce the expression of neutral PGs, even if the pH values indicated as inducers are not coincident (Rollins and Dickman, 2001, Cotton *et al.*, 2003). In infected plants, as well as in culture media, oxalic acid is the factor affecting pH values responsible for the induction of neutral PG. But oxalic acid concentration is only at about 1 mM until 16 h after soybean seedlings inocula-

tion (not shown) and pH changes in infected tissue become appreciable only after 24 h (Favaron *et al.*, 2004).

Besides, *Ssmdh* gene, encoding malate dehydrogenase, a key enzyme of the oxalate biosynthetic pathway, was expressed coincidentally with the *Sspgb* gene. These observations would not support a clear role of pH in regulating the *Sspgb* gene expression. However, it should be noticed that, at the early stages of fungal infection, oxalate and pH determinations in plant extract may be altered by the presence of significant amount of healthy tissue, and pH values could be lower in localized portion of infected tissue. Moreover, high amount of malate dehydrogenase may not be necessary to support a relevant oxalate biosynthesis if this enzyme possesses the extraordinary high specific activity as shown in the fungus *Fomitopsis palustris* (Munir *et al.*, 2001). Other factors in addition to pH are likely involved in the *Sspgb* gene induction. We reported that PGB is almost undetectable in culture medium where the pH conditions are very acidic, and that addition of plant extracts different from pectic polymers stimulates the production of PGB (Favaron and Marciano, 1992).

Recently, also Li *et al.* (2004b) have suggested that physical and nutritional factors are positive regulators of *ssp1d*, a gene from strain 100 of *S. sclerotiorum* homologue to *Sspgb*. In contrast to *Sspgb*, *Sspga* transcript accumulates progressively in the infected tissue correlating with fungal growth. Thus, *Sspga* behaves as a constitutively expressed gene and its encoded product PGa likely works in a very acidic pH environment.

Soybean PGIP could provide an obstacle to *S. sclerotiorum* endo-PG activity, and as previously observed, the PGIP transcript accumulates in the infected tissue in response to pathogen infection (Favaron *et al.*, 2000; D'Ovidio *et al.*, 2002). Now, by quantitative expression analysis we show that accumulation of the soybean *Gmpgip* transcript is delayed in comparison to induction of the pathogen *Sspgb* transcript. Though soybean PGIP is effective against PGB, its amount in the early stage of hypocotyl tissue colonisation could not be enough to counteract the fungal PGB. Later, a likely reduction of PGB activity due to the accumulation of PGIP may be compensated by the increase of PGa activity, which, at acidic pH conditions of the infected tissue, escapes PGIP inhibition (Favaron *et al.*, 2004).

Results obtained give a more precise representation of the initial events occurring during tissue colonization involving plant cell wall degradation by *S. sclerotiorum* endo-PGs. The major features are the high correlation between expression of *S. sclerotiorum* endo-*pg* genes and endo-PG activity during colonisation of soybean plants, the strong expression of PGB in the initial stage of infection and the delayed accumulation of PGIP transcript in the infected tissue. Therefore, to efficiently counteract PGB activity, soybean PGIP should be strongly expressed at early stages of infection.

Knock-out experiments of the *Sspgb* gene should definitively clarify the role played by this PG in *S. sclerotiorum* pathogenesis. Similarly, the constitutive over-expression of PGIP should clarify the role of this protein in counteracting PGB during *S. sclerotiorum* infection.

ACKNOWLEDGMENTS

We acknowledge Carla Castiglioni for valuable technical assistance in PG production and purification. This work was supported by MIUR-FIRB 2002-2005.

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Received 5 May 2005

Accepted 1 September 2005

