Spatial and temporal expression patterns of *Avr1b*-1 and defense-related genes in soybean plants upon infection with *Phytophthora sojae*

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Received 28 August 2006; accepted 4 September 2006.
First published online 29 September 2006.

DOI:10.1111/j.1574-6968.2006.00467.x

Editor: Holger Deising

Keywords
Avr1b; gene-for-gene interaction; *Glycine max*; *Phytophthora sojae*; RT-PCR.

Abstract

The *Avr1b* locus is required for avirulence of the oomycete pathogen *Phytophthora sojae* on soybeans carrying resistance gene *Rps1b*. One of the *Avr* genes of the locus (*Avr1b*-1) was shown to encode an elicitor. We have analyzed the spatial and temporal expression patterns of *Avr1b*-1 in comparison to defense-related genes induced in soybean. *Avr1b*-1 expression was detectable mainly in close proximity to the site of infection, in wound-inoculated hypocotyls as well as in roots infected with zoospores. Usually, in compatible interactions, higher expression levels of *Avr1b*-1 were observed in roots when compared with incompatible *P. sojae*–soybean interactions, whereas neither the timing nor the amount of transcript accumulation of defense-related genes showed cultivar-specific differences. In contrast, the *PsojNIP* gene encoding a proposed virulence factor was expressed only during the necrotrophic phase in the compatible interaction.

Introduction

Phytophthora sojae is an aggressive hemibiotrophic oomycete that causes root and stem rot on soybean (*Glycine max* L.) plants. Resistance or susceptibility of soybean to *P. sojae* is attributed to a complex exchange of signaling cues between the pathogen and the host that results either in colonization and disease, or in recognition of the invading pathogen by the host, leading to a successful defense response. In this system, the primary determinants of compatibility among different *P. sojae* races and soybean cultivars are pathogen avirulence (*Avr*) genes and host resistance (*Rps*) genes (Shan *et al.*, 2004). Recently, two genes within the *Avr1b* locus from *P. sojae* were identified that are required for the Avr1b phenotype (Shan *et al.*, 2004). It was proposed that the Avr1b-1 protein might act as a specific elicitor on soybean plants containing the *Rps*1b resistance gene. In support of this hypothesis, recombinant Avr1b-1 protein was infiltrated into leaves of soybean seedlings and shown to trigger a severe necrosis in the leaves of *Rps*1b cultivars 2–3 days after infiltration (Shan *et al.*, 2004). Other recently isolated *Avr*-genes from oomycete pathogens were suggested to encode secreted avirulence proteins as well (Allen *et al.*, 2004; Armstrong *et al.*, 2005; Rehmany *et al.*, 2005). Other *P. sojae*-derived molecular patterns or motifs have been characterized that elicit host and nonhost responses. Although these do not seem to be determinants of *Avr* gene-mediated race specificity, they are thought to be involved in plant disease resistance at the species level (nonhost resistance). Among the latter group of factors are a distinct oligo-β-glucoside motif of the β-glucan structural cell wall polysaccharide (Sharp *et al.*, 1984a, b), the Pep-13 fragment of an extracellular transglutaminase (Brunner *et al.*, 2002), and cysteine-rich proteins called elicitins that are inactive in soybean but stimulate an HR-like response when infiltrated in tobacco leaves (Kamoun

et al., 1994; Becker *et al*., 2000). In addition, a necrosis-inducing protein (PsojNIP) was found that is a powerful inducer of necrosis and cell death in bioassays and was suggested to facilitate colonization of host tissues during the necrotrophic phase of growth (Qutob *et al*., 2002).

Phytophthora sojae is a soil-borne pathogen and produces free-swimming zoospores at temperatures between 25 and 30 °C, particularly under wet conditions. Upon reaching the root surface, the zoospores attach, encyst, and develop a germ tube that penetrates into host tissues. Soybean cultivars that carry an effective *Rps* gene against an attacking *P. sojae* strain rapidly develop a hypersensitive response (HR) within hours after zoospore attachment. As a consequence of the resistance response, the oomycete growth is limited to the cortex and stele of lateral roots and remains confined to the vicinity of the infection site (Beagle-Ristaino & Rissler, 1983; Enkerli *et al*., 1997a). In susceptible cultivars, *P. sojae* infects lateral roots and progresses into tap roots and hypocotyls, without occurrence of an early HR. An initial biotrophic phase of about 12 h (Ward, 1990; Enkerli *et al*., 1997a) is followed by a necrotrophic phase, spreading growth mode and causing severe, large necrotic lesions at about 24 h.

Numerous reactions have been described that form a multi-component and rapid differential defense response in resistant vs. susceptible soybean cultivars. These reactions range from callose deposition at infection sites (Bonhoff *et al*., 1987; Enkerli *et al*., 1997b), cell wall cross-linking (Brisson *et al*., 1994), induction of genes encoding pathogenesis- and defense-related proteins (Moy *et al*., 2004; Vega-Sánchez *et al*., 2005) to a complement of enzymes involved in the accumulation of pterocarpanoid-type phytoalexins, the glyceollins (Ebel & Grisebach, 1988; Ebel, 1998; Subramanian *et al*., 2005). It has been concluded that, in the resistance response, invading *P. sojae* hyphae will come into contact with an antimicrobial environment very soon after infection (Hahn *et al*., 1985; Ebel & Grisebach, 1988; Enkerli *et al*., 1997a).

In the present studies, we describe, at early stages after infection of soybean, the changes of mRNA levels for Avr1b-1, for two enzymes of glyceollin biosynthesis, isoenzyme 3 of 4-coumarate:CoA ligase (4CL3) (Lindermayr *et al*., 2002) and dihydroxypterocarpan 6a-hydroxylase (D6aH) (Schopfer *et al*., 1998), and for two pathogenesis-related proteins, PR1a (Graham *et al*., 2003) and the putative basic peroxidase IPER (Yi & Hwang, 1998). Our results show that *Avr1b*-1 mRNA is hardly detectable at early hours postinoculation when *P. sojae* biomass is low and when mRNA levels for the biosynthetic enzymes are significantly increased. High *Avr1b*-1 mRNA levels are maintained transiently at later stages when massive defenses are known to occur. *Avr1b-1* is expressed during infection only, as was reported for other oomycetal avirulence genes (Shan *et al*., 2004; Rehmany *et al*., 2005). This is also the case for *PsojNIP* encoding a necrosis-inducing protein. However, further investigations are required to identify the mode of production and action of Avr1b-1 at the infection interface, in conjunction with the proposed corresponding Rps1b protein.

Materials and methods

Plant material and *P. sojae* cultures

Soybean [*Glycine max* (L.) Merr.] seeds of cultivars Harosoy (*rps*1b) and Williams L77-1863 (*Rps*1b) were obtained from T. Anderson, Agriculture and Agri-Food, Canada. Seedlings were grown under aseptic conditions in water-soaked vermiculite for 3 days (root infection) or 9–12 days (hypocotyl infection), as described previously (Hahn *et al*., 1985), in a growth chamber using a 15 h (26 °C) day and a 9 h (22 °C) night period at 60% relative humidity. *Phytophthora sojae* races 1 (laboratory culture collection) and 2 (strain P6497) were routinely grown on Lima bean agar plates at 25 °C in the dark (Ayers *et al*., 1976). Zoospores were obtained from 12-day-old cultures as reported previously (Eye *et al*., 1978). Large cultures were grown in 1.8-L Fernbach flasks containing 400 mL of modified Erwin medium (Keen, 1975) in the dark for 3–4 weeks (hypocotyl infection) or for 2–6 weeks (RNA extraction).

Plant inoculation procedures

Assays with hypocotyls of 9–12-day-old soybean seedlings were performed according to Albersheim & Valent (1978). The seedlings were mounted in sets of four plants, each on a horizontal glass rod, by piercing the hypocotyls 5 mm below the cotyledons. A small piece of *P. sojae* mycelium was placed in the vertical slit wound of about 1 cm length. Control plants were wounded but not inoculated with mycelium. After incubation in a closed chamber at 26 °C for 5 days at 100% humidity, each set of plants was removed from the rod and a 2-cm hypocotyl segment, centered on the wound, was cut from each seedling, frozen in liquid nitrogen, and stored at − 80 °C.

Groups of 15 unwounded 3-day-old soybean seedlings were dip-inoculated with suspensions containing *c*. 10 000 *P. sojae* zoospores per seedling as described (Hahn *et al*., 1985). Seedlings placed in distilled water were used as control. Following incubation for varying lengths of time at 26 °C in the darkness at 100% humidity, root segments were excised 1 cm above and 1 cm around the infection site, and from the root tip. Root segments were frozen in liquid nitrogen and stored at − 80 °C. Inoculations were performed at least three times and data from representative analyses are shown.

RNA isolation

Total RNA from *P. sojae* mycelium was isolated using published methods (Chang *et al.*, 1993). Total RNA from soybean seedlings was prepared using the RNeasy Plant Mini Kit (Qiagen).

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was used to monitor gene expression in *P. sojae*, in soybean, and during plant–pathogen interaction. Two micrograms of DNase-treated RNA were transcribed in a 20-μL reaction volume containing 200 U of M-MLV reverse transcriptase (Invitrogen), 80 U of RNasin RNase inhibitor (Promega), 80 ng random primers (Invitrogen), 1 mM dNTPs, and 10 mM dithiothreitol, in first-strand buffer (Invitrogen). The reaction mixture was incubated under the following conditions: 25 °C, 15 min; 37 °C, 90 min; 90 °C, 5 min. Two microliters of the RT reaction mixture were used for PCR in a 10-μL volume containing 0.25 U of *Taq*-DNA-polymerase (Qbiogene) and 10 pmol of each specific forward and reverse primers in Taq incubation mix (Qbiogene) containing 2.5 mM $MgCl_2$. In the case of *4CL3*, 2 pmol of primers and 1.5 mM $MgCl_2$ was used.

The sequences of *P. sojae* oligonucleotides were: *Avr1b*-1 (GenBank accession no. AY426744), forward 5′-CCAAGTA TCACGAACCATGCG-3′, reverse 5′-TGCGATTTCGGCG AGACC-3′; *PsojNIP* (GenBank accession no. AF320326), forward 5′-GCACAGCGGATACAACAAGTA-3′, reverse 5′-ACAAAGTCGTCACATTCAAATGCTG-3′; *Act*A (GenBank accession no. X15900), forward 5′-TCATGGTCGGCATG GACCA-3′, reverse 5′-GGCCGTGGTCGTGAACGAG-3′. *Glycine max* gene-specific primers used were: *4CL3* (GenBank accession no. AF002258), forward 5′-ACGGTAGCT GCTTCTCTTGATGC-3′, reverse 5′-GTCGTCGACGGTCA CAACCTT-3′; *D6aH (Cyp93a1*, GenBank accession no. D83968), forward 5′-GCAAGAAAAACCTTCCACCAAGT-3′, reverse 5′-AAACGCGAAAAGGAAGTCTTGGG-3′; *PR1a* (GenBank accession no. AF136636), forward 5′-TGAT GTTGCCTACGCTCAAG-3′, reverse 5′-ATCCAAGACGCA CCGAGTTA-3′; *IPER* (GenBank accession no. AF007211), forward 5′-CTCAGGTGCTCATACATTCGG-3′, reverse 5′-ACAACAAGTAGTGGCGGGC-3′; *tub*B2 (GenBank accession no. M21297), forward 5′-GTGACTTGAACCATCTG ATCTCAGC-3′, reverse 5′-GTTGAAGCCATCCTCAAGC CAG-3′.

PCR conditions were: Step 1: 94 °C, 3 min (1 cycle); step 2: 94 °C, 30 s; 57–61 °C, 30–60 s; 72 °C, 30–45 s (21–38 cycles); step 3 (for experiments shown in Figs 2 and 3): 72 °C, 10 min. PCR products were analyzed on 1.2% or 1.4% agarose gels and visualized after ethidium bromide staining. To verify the specificity of the *Avr1b*-primers, products of the amplification reactions were excised from the gels, purified, cloned, and sequenced (data not shown).

Results

Infection of soybean tissues with mycelium or zoospores of *P. sojae*

Two previously employed bioassays, which reproducibly show the expected race specificity of the host–pathogen interaction, were used in the present studies. Hypocotyls of 9–12-day-old soybean seedlings with a vertical slit wound of about 1 cm length were mounted on horizontal glass rods according to Albersheim & Valent (1978) and inoculated by placing a small piece of *P. sojae* mycelium into the wound. After 5 days of infection (Fig. 1), a 2-cm hypocotyl segment, centered on the wound, was cut from each seedling and used for RNA extraction. At this stage of infection, massive maceration and tissue collapse could be observed in susceptible soybean hypocotyls (Fig. 1). In a second assay, unwounded roots of 3-day-old soybean seedlings were dip-inoculated with zoospore suspensions of *P. sojae* and incubated for varying lengths of time (Hahn *et al.*, 1985). The incompatible interaction was characterized by a darkening of the infection site on the roots as early as 5 h after inoculation, later identifiable as a hypersensitive ring necrosis, whereas the compatible interaction was characterized by a slight browning of the root 8–12 h after inoculation. The water-treated control seedlings rarely showed any discoloration or tissue reaction at the inoculation sites.

Analysis of transcript levels for *P. sojae Avr1b*-1, *P. sojae* actin (*ActA*), and soybean tubulin (*tubB2*) in inoculated hypocotyls

After 5 days of infection, the pathogen enters the necrotrophic growth mode in compatible interactions, as characterized by abundant hyphae proliferation, in contrast to incompatible interactions. At this stage, high transcript levels for *P. sojae* actin were detected in susceptible soybean hypocotyls of cv. Harosoy, infected with race 1 (*Avr1b*) or 2 (*avr1b*), and Williams L77-1863, infected with race 2 (Fig. 2). In the incompatible interaction between soybean cv. Williams L77-1863 and race 1 of *P. sojae*, the actin transcript level was low. *Avr1b*-1-specific, infection-induced transcripts were found with race 1, but not with race 2. *Avr1b*-1 transcript level was higher in tissues from the susceptible than from the resistant soybean cultivar, reflecting probably the restriction of pathogen growth in the incompatible interaction as suggested by low actin transcript levels (Fig. 2). As observed earlier (Shan *et al.*, 2004), *Avr1b*-1 expression was detectable in infected plants only but neither in *P. sojae* zoospores nor in mycelium of race 1 at various growth stages (data not shown).

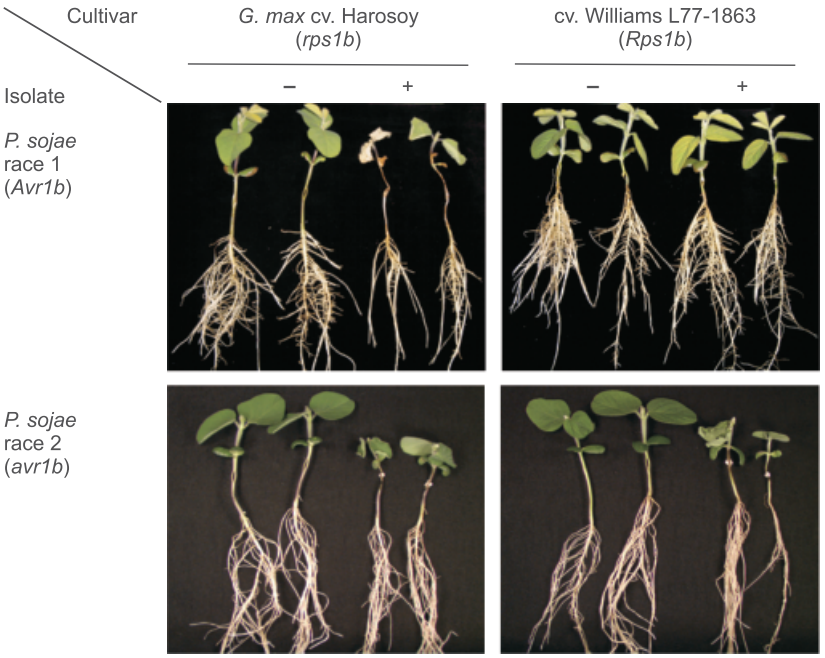

Fig. 1. Bioassay using soybean hypocotyls. Hypocotyls of 9–12-day-old seedlings of cultivars Harosoy and Williams L77-1863 were wounded and inoculated with small pieces of *Phytophthora sojae* mycelium of either race 1 or race 2 (+) or wounded and not inoculated (–) (Albersheim & Valent, 1978). After 5 days of treatment, pictures were taken and hypocotyl segments around the infection sites were then excised for isolation of total RNA.

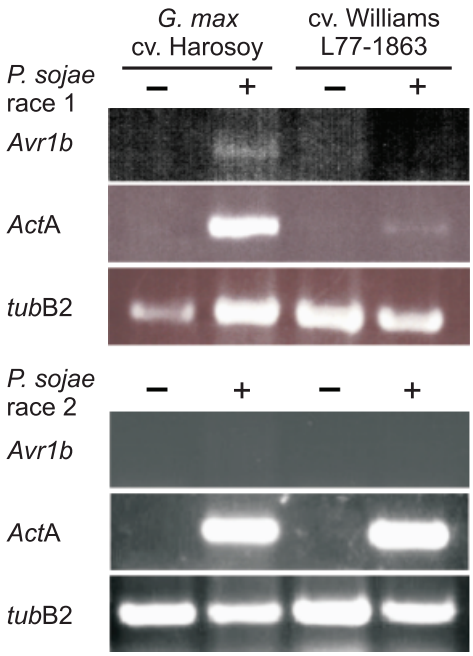

Fig. 2. Gene expression patterns in soybean hypocotyls. Transcript levels of *Phytophthora sojae Avr1b*-1 (*Avr1b*), *P. sojae* actin (*Act*A) and soybean tubulin (*tub*B2) genes were monitored in the compatible cv. Harosoy – *P. sojae* race 1 and race 2 interaction, the compatible cv. Williams L77-1863 – *P. sojae* race 2 interaction, and the incompatible cv. Williams L77-1863 – *P. sojae* race 1 interaction (see Fig. 1). Material derived from the hypocotyl bioassay was analyzed by semi quantitative RT-PCR using gene-specific primers and 35 amplification cycles. Symbols denote: –, untreated hypocotyls; +, soybean infected with either race 1 or race 2, as indicated.

Expression levels of *P. sojae Avr1b*-1 and actin genes in different parts of infected soybean roots

In earlier work on the spatial course of hyphae growth and glyceollin I accumulation in infected soybean seedling roots (Hahn *et al.*, 1985), large differences between resistant and susceptible plants were detected when the interaction between one soybean cultivar and two *P. sojae* races that differed in virulence were analyzed. The incompatible interaction was characterized by extensive colonization of the root cortex by the oomycete at the infection site concomitantly with high glyceollin I levels. No hyphae were observed in advance of detectable phytoalexin levels. In contrast, the compatible interaction was characterized by extensive unchecked oomycete colonization of the root stele, with lesser growth in the rest of the root. Only small amounts of glyceollin I were detected in whole root extracts during the first 14 h after infection. In the present study, expression of *P. sojae Avr1b*-1 and actin genes after 24 h of infection was detected mainly in root parts placed in the zoospore suspensions (Fig. 3, tip and ring), no expression was recorded in root sections above the basal parts (Fig. 3, up). In addition, *Avr1b*-1 appeared to be more abundant in the compatible interaction with cv. Harosoy as opposed to the incompatible interaction with cv. Williams L77-1863.

Time course of gene expression in incompatible and compatible *P. sojae*–soybean interactions

Genes encoding two enzymes of phytoalexin biosynthesis in soybean were chosen to monitor time courses of infection-

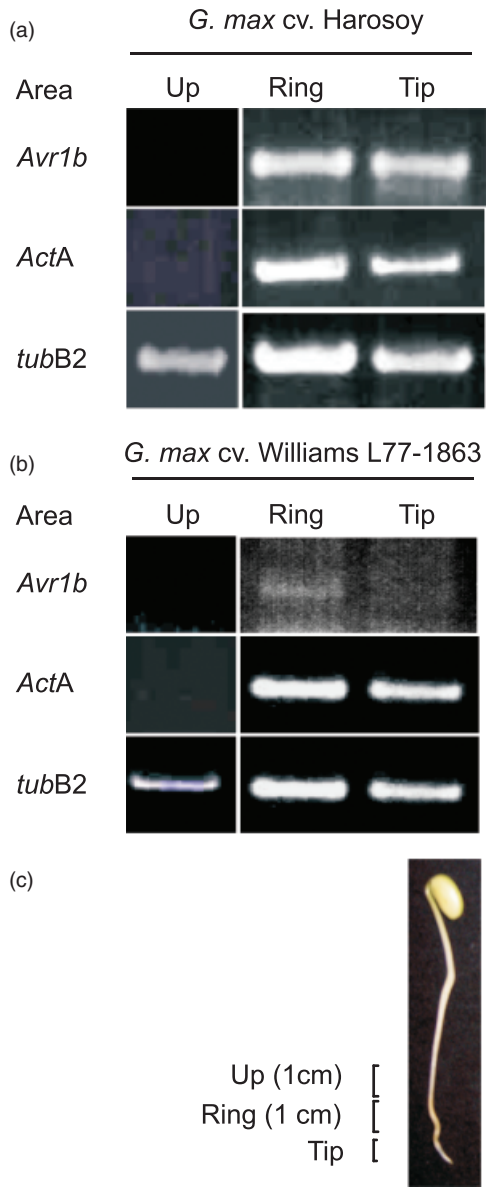

Fig. 3. Spatial course of transcript accumulation in infected soybean roots. Roots of unwounded 3-day-old soybean seedlings of cv. Harosoy (a) and cv. Williams L77-1863 (b) were dip-inoculated with zoospores of *Phytophthora sojae* race 1. After 24 h of inoculation, root segments were excised from the ring close to the water-air interface, above the ring (up) and below the ring (tip) according to the schematic representation (c). Transcript abundance was analyzed by RT-PCR using gene-specific primers and 35 cycles in the compatible cv. Harosoy (a) – and in the incompatible cv. Williams L77-1863 (b) – *P. sojae* race 1 interaction.

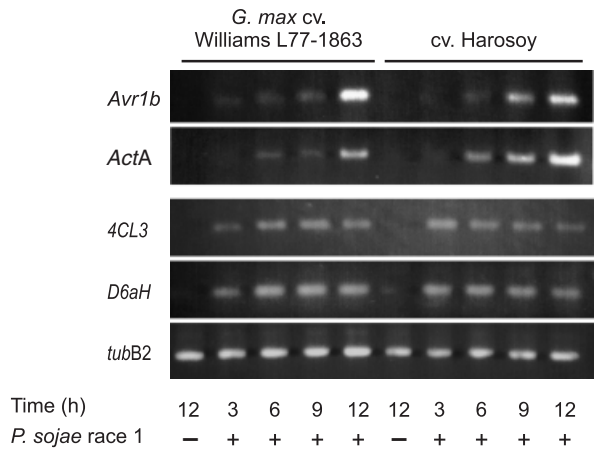

Fig. 4. Time course of phytoalexin biosynthesis gene expression in compatible and incompatible soybean–*Phytophthora sojae* interactions in comparison with expression of *Avr1b*-1 and actin (*Act*A) from *P. sojae*. Roots of unwounded 3-day-old soybean seedlings of cv. Harosoy (susceptible) or cv. Williams L77-1863 (resistant) were dip-inoculated with zoospores of *P. sojae* race 1 and transcript levels were analyzed by RT-PCR using gene-specific primers. Root segments close to the infection site (ring) were excised for the isolation of total RNA after the indicated time (h). Transcript levels of the following genes were monitored: *Avr1b*-1 (*Avr1b*, 38 cycles) and actin A (*Act*A, 38 cycles) of *P. sojae;* isoenzyme 3 of 4-coumarate:CoA ligase (*4CL3*, 21 cycles), dihydroxypterocarpan 6a-hydroxylase (*D6aH*, 24 cycles) and tubulin B2 (*tub*B2, 30 cycles) of soybean. Symbols denote: +, roots inoculated with *P. sojae*; –, roots of untreated control plants.

induced changes of mRNA levels of defense-related metabolic processes in the host plant in conjunction with *Avr1b*-1 expression. Of these, one codes for 4CL3 (Lindermayr *et al.*, 2002), a central enzyme of general phenylpropanoid metabolism involved in the synthesis of one of the phytoalexin precursors, and the second codes for D6aH (Schopfer *et al.*, 1998), a hydroxylase specifically involved in a late step of glyceollin biosynthesis (Ebel, 1998). In both the compatible and incompatible interaction of *P. sojae* race 1 with either cv. Harosoy or Williams L77-1863 roots, *4CL3* and *D6aH* mRNA levels were detectable at 3 h after the onset of infection. High mRNA levels were maintained in both the compatible and the incompatible interaction (Fig. 4), and could be detected even after 24 h (*4CL3*) or 48 h (*D6aH*) in both interactions (data not shown). Low mRNA levels for the two enzymes were observed at the time of inoculation and in uninfected control roots, which did not change during the period of the experiments (data not shown). In contrast, expression of genes encoding the two pathogenesis-related proteins PR1a and basic peroxidase (IPER) was not detectable in roots of untreated seedlings, but was induced over time in water-treated plants and to a higher extent in seedlings infected with *P. sojae* zoospores (Fig. 5). The fold-induction over the respective gene expression levels for water-treated seedlings during the time course was seemingly higher for the incompatible interaction (Fig. 5b) when compared with the compatible interaction (Fig. 5a).

Avr1b-1 expression appeared to be constitutive throughout infection, transcripts were detectable concomitantly (Fig. 4) or even before transcripts encoding the constitutively expressed actin gene (Fig. 6). At 3 h after the onset of

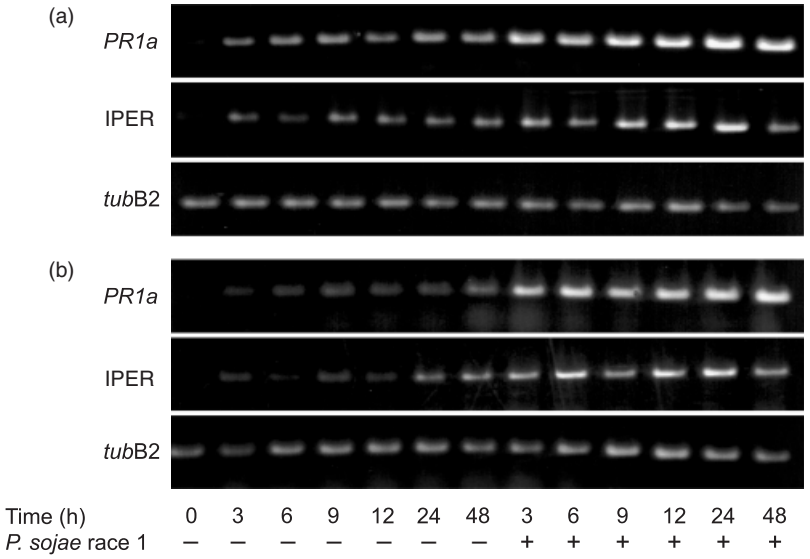

Fig. 5. Time course of pathogenesis-related gene expression in compatible (a) and incompatible (b) soybean–*Phytophthora sojae* interactions. Transcript levels of the following soybean genes were monitored after inoculation of roots of soybean cv. Harosoy (a, susceptible) and cv. Williams L77-1863 (b, resistant) with *P. sojae* race 1: pathogenesis-related gene *PR1a* (30 cycles) and basic peroxidase (IPER, 32 cycles), and tubulin B2 (*tub*B2, 30 cycles) as control. For further details see legend to Fig. 4.

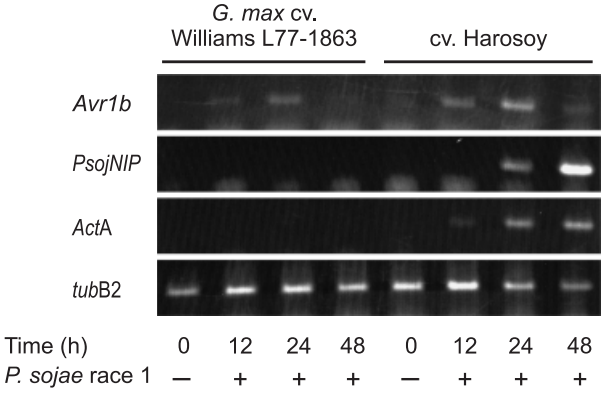

Fig. 6. Time course of expression of avirulence and virulence factors after infection of roots of susceptible (cv. Harosoy) and resistant (cv. Williams L77-1863) cultivars of soybean with zoospores of *Phytophthora sojae* race 1. Transcript levels of the following genes were monitored: *Avr1b* (38 cycles), necrosis-inducing protein 1 *(PsojNIP,* 38 cycles) and actin (*Act*A, 38 cycles) from *P. sojae*, and tubulin B2 (*tub*B2, 30 cycles) from soybean as control. For further details see legend to Fig. 4.

infection, *Avr1b*-1 *and Act*A mRNAs were barely detectable in either of the interactions. At 6 h, low levels were found, which subsequently increased in both types of interaction (Fig. 4) and remained high up to 24 h after the onset of infection (Fig. 3 and data not shown). With the exception of the 12 h timepoint shown in Fig. 4, a higher level of *Avr1b*-1 transcript accumulation was always detectable in hypocotyls or roots of the susceptible cultivar infected with *P. sojae* race 1 when compared with the resistant cultivar (Figs 2–4 and 6). The increase of transcript levels for *Avr1b*-1 roughly correlated with the increase of mycelium biomass, as monitored by actin expression. In contrast, the necrosis-inducing protein NIP was clearly induced above the actin level at a late time point, and was detectable in the compatible interaction only (Fig. 6).

Discussion

In an attempt to describe the 'interaction transcriptome' in plant–pathogen interactions (Birch & Kamoun, 2000), patterns of overall transcriptional profiles of soybean and *P. sojae* at the infection interface of the compatible interaction were analyzed (Moy *et al.*, 2004). In these studies, it was confirmed that soybean genes encoding enzymes of phytoalexin biosynthesis, among others, were upregulated with highest expression levels occurring at 24 h after infection. The number of pathogen genes expressed during infection reached a maximum at 24 h as well. It was concluded that in the soybean–*P. sojae* interaction, the pathogen transits from biotrophy to necrotrophy between 12 and 24 h after infection (Moy *et al.*, 2004). These metabolic shifts appear to be associated with an amazingly large number of gene functions, especially at the side of the pathogen.

A considerable number of upregulated pathogen genes represented those whose functions are possibly involved in facilitating infection or countering host defenses, such as proteinases, glucanases, elicitins, and others (Moy *et al.*, 2004). A gene that has been proposed to control race-specificity between soybean and *P. sojae*, and that was not included in the analyses mentioned above, is *Avr1b*-1. *Avr1b*-1 is expressed in a race- and infection-specific manner (Shan *et al.*, 2004). For the comparison of the expression of this presumptive avirulence gene with early induced plant defenses, genes encoding phytoalexin biosynthesis enzymes and pathogenesis-related proteins were selected in the present work, which are differentially regulated in infected vs. noninfected soybean roots (Ebel & Grisebach, 1988;

Schopfer *et al.*, 1998; Lindermayr *et al.*, 2002; Vega-Sánchez *et al.*, 2005). The results disclosed that the expression of defense-related genes, *4CL3* and *D6aH*, was enhanced at the earliest time point of 3 h. Expression of the pathogenesis-related genes *PR1a* and *IPER* was sensitive towards the experimental procedures but responded to infection with an induction above the levels observed for mock-infected seedlings. Differential expression of these genes was reported to possibly contribute to resistance in race-specific resistance as well as in partial resistance of soybean to *P. sojae* (Yi & Hwang, 1998; Vega-Sánchez *et al.*, 2005). Interestingly, expression of *Avr1b*-1 appeared to be constitutive *in planta*, correlating largely with oomycete growth in dip-inoculated roots. Conversely, the putative virulence factor PsojNIP was expressed only in the compatible interaction, and only at late timepoints, where the gene was clearly induced above the levels of actin (Fig. 6), suggesting a role during necrotrophy of the pathogen (Qutob *et al.*, 2002).

It was reported earlier that the extent of root tissue colonization in the incompatible and compatible interactions of two different races on one soybean cultivar could be clearly differentiated starting 8 h after dip-inoculation. In the same study it was shown that the phytoalexin, glyceollin I, was present 5 h after inoculation and rapidly accumulated thereafter (Hahn *et al.*, 1985). The rapid increase of phytoalexin accumulation correlated well with the changes at the enzyme activity (Ebel & Grisebach, 1988) and mRNA levels of the biosynthetic enzymes, as reported here and earlier.

An open question is the contribution of *Avr1b*-1 to the above scenario. It was confirmed that *Avr1b*-1 was not expressed in mycelium or zoospores of *P. sojae* race 1 (data not shown; Shan *et al.*, 2004) but was expressed *in planta* starting 3–6 h postinoculation (data not shown; Fig. 4). It also appeared that the time course of *Avr1b*-1 expression was rather similar in the compatible and incompatible interaction and the amount of mRNA was generally higher in roots during the compatible interaction (Figs 3 and 6). A reason for the apparent difference in the levels of *Avr1b*-1 and defense-related gene expression could be that very small amounts of Avr1b-1 protein are sufficient to trigger plant defense responses, or that the Avr1b-1 protein is not required during the initial but during the intermediate biotrophic phase until host cell death occurs, at which stage the expression of Avr1b-1 is repressed in contrast to the putative virulence factor PsojNIP. A later role for the avirulence protein would require another factor(s) mediating initial recognition of the pathogen by the host plant and induction of genes involved in early defense responses like those encoding enzymes of phytoalexin production. Because defense responses occur in both compatible and incompatible interactions, factors for initial pathogen recognition might be race nonspecific and might be present at the onset of infection, such as some of the general elicitors localized in the cell wall of the pathogen including the oligo-β-glucoside motif of the β-glucan structural polysaccharide (Albersheim & Valent, 1978; Sharp *et al.*, 1984a, b; Ebel, 1998). Recently, it was demonstrated using ion-selective microelectrodes that β-glucan elicitors induce rapid K^+ and Ca^{2+} fluxes as well as a depolarization of the plasma membrane potential of soybean root cells within a few minutes after application of the stimulus (Mithöfer *et al.*, 2005) and preceding defense gene activation. These changes may represent early signaling events that are required for initiating at least some of the inducible defense responses (Ebel & Grisebach, 1988; Mohr & Cahill, 2001; Lindermayr *et al.*, 2002; Subramanian *et al.*, 2005). Therefore, the final outcome of resistance or susceptibility of soybean to *P. sojae* may depend on more than one signaling event between the pathogen and its host. Further analysis of the host responses triggered by both the race-specific avirulence protein as well as the broad-host elicitor in soybean might allow the discrimination of specific vs. common signaling pathways.

Recent advances in cloning avirulence genes from the rust fungus *Melampsora lini* and three oomycete species (*Hyaloperonospora parasitica*, *P. sojae* and *Phytophthora infestans*) have provided the novel insight that these eukaryotic plant pathogens may deliver small proteins, such as Avr proteins, into the host cell cytoplasm where they are recognized by resistance proteins (Ellis *et al.*, 2006, for review). In the case of the flax rust fungus, it has been reported that *Avr* gene expression occurs in haustoria and the products are taken up by and are recognized inside the host plant cells (Dodds *et al.*, 2004). Rehmany *et al.* (2005) have recently identified a potential host-targeting signal in Avr proteins from the oomycete pathogens, including Avr1b-1 from *P. sojae*, which could be important for transporting proteins into the host cell. Future research towards the function of these Avr proteins and the cellular site of interaction with host components has to take these advances into account.

Acknowledgements

The excellent technical assistance by K. Schmieja is gratefully acknowledged. We thank J. Leclercq, A. Mithöfer, and M.V. Silber for valuable discussions. This work was supported by the Deutsche Forschungsgemeinschaft (EB 62/14-1, 2) to J.E. and by Grants to B.M.T. from the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, numbers 2001-02748 and 2003-05434.

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