Elicitin Genes Expressed In Vitro by Certain Tobacco Isolates of *Phytophthora parasitica* Are Down Regulated During Compatible Interactions

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Phytophthora spp. secrete proteins called elicitors in vitro that can specifically induce hypersensitive response and systemic acquired resistance in tobacco. In *Phytophthora parasitica*, the causal agent of black shank, most isolates virulent on tobacco are unable to produce elicitors in vitro. Recently, however, a few elicitor-producing *P. parasitica* strains virulent on tobacco have been isolated. We investigated the potential diversity of elicitor genes in *P. parasitica* isolates belonging to different genotypes and with various virulence levels toward tobacco as well as elicitor expression pattern in vitro and in planta. Although elicitors are encoded by a multigene family, *parA1* is the main elicitor gene expressed. This gene is highly conserved among isolates, regardless of the elicitor production and virulence levels toward tobacco. Moreover, we show that elicitor-producing *P. parasitica* isolates virulent on tobacco down regulate *parA1* expression during compatible interactions, whichever host plant is tested. Conversely, one elicitor-producing *P. parasitica* isolate that is pathogenic on tomato and avirulent on tobacco still expresses *parA1* in the compatible interaction. Therefore, some *P. parasitica* isolates may evade tobacco recognition by down regulating *parA1* in planta. The in planta down regulation of *parA1* may constitute a suitable mechanism for *P. parasitica* to infect tobacco without deleterious consequences for the pathogen.

Wild and cultivated plants are in constant contact with many microorganisms yet have developed a number of strategies in order to defend themselves from disease. Indeed, among the range of interactions between plants and pathogens, only a small minority lead to disease development. Plant defense strategies have been divided into two distinct classes (Heath 1981). The most common one, nonhost resistance, implies the existence of preformed physical and chemical barriers and/or the activation of nonspecific plant defense

responses triggered upon contact with microorganisms (Heath 1981). Nonhost resistance, which is assumed to be under polygenic control, is therefore not specific because it is effective against all individuals of a given pathogen species. During their evolutionary history, some microorganisms have acquired the pathogenicity factors necessary to infect one or a number of plant species, which then become host plants. These host plants can acquire the ability to protect themselves by specifically recognizing a given pathogen. This second type of resistance, the host-specific resistance, follows the “gene-for-gene concept” (Flor 1971), where the product of a pathogen avirulence gene (*Avr*) directly or indirectly interacts with a plant resistance gene (*R*) product in a very specific manner. After recognition, the subsequent steps of the interaction often lead to the hypersensitive reaction (HR) (Goodman and Novacky 1996) and to the activation of plant-defense responses (Hammond-Kosack and Jones 1996). Host resistance, therefore, represents a highly specialized form of interaction because a limited number or only two matching genes are necessary for the recognition event that leads to the subsequent resistance reaction (De Wit 1992).

Host resistance was first demonstrated in pathogen race–plant cultivar interactions. Proteins produced by the phytopathogenic fungus *Magnaporthe grisea* and the oomycete *Phytophthora infestans*, however, were shown to act as avirulence factors toward hosts and nonhosts, respectively, at the plant-species level (Kamoun et al. 1998b; Kamoun et al. 1999; Sweigard et al. 1995). Two kinds of avirulence genes can therefore be defined: cultivar- and species-specific *Avr* genes (Laugé and De Wit 1998). The fact that pathogen–nonhost interactions may involve species-specific *avr* genes has led some authors to hypothesize that host and nonhost resistance mechanisms may proceed in the same manner, that is by a recognition between an *Avr* and a *R* gene product (Kamoun et al. 1999).

Because there is a growing interest in engineering disease-resistant plants through the introduction of *R* and complementary *Avr* genes in crop plant genomes (De Wit 1992), it remains crucial to investigate how pathogens can overcome the action of naturally occurring resistance mechanisms in plants. These types of studies will undoubtedly help to evaluate the durability potential of artificial resistance.

Resistance and avirulence genes are generally dominant, and disease occurs when one of the genes is absent or defec-

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Nucleotide sequence of *parA1.1* and *parA1.2* are in the GenBank database, accession nos. AF316428 and AF316429, respectively.

tive. This has been shown for cultivar- and species-specific *Avr* genes in plant–fungi interactions. Two single-copy, cultivar-specific *Avr* genes, for example, have been cloned from *Cladosporium fulvum*. Named *Avr9* and *Avr4* (Joosten et al. 1994; Van den Ackerveken et al. 1992), these genes correspond to the *Cf-9* and *Cf-4* tomato resistance genes, respectively (Jones et al. 1994; Thomas et al. 1997). The deletion of *Avr9* and single-base-pair changes in the open reading frame (ORF) of *Avr4*, allow virulent strains to circumvent *Cf-9*- and *Cf-4*-mediated resistance of tomato (Joosten et al. 1994; Joosten et al. 1997). The *PWL* gene family, a group of species-specific *Avr* genes, has been identified in the blast fungus *M. grisea*. Mutations in *PWL3* or incorrect expression of *PWL4* appear to confer virulence to *M. grisea* isolates toward weeping lovegrass (Kang et al. 1995).

Tobacco (*Nicotiana tabacum*) is a nonhost plant species for the devastating plant pathogen genus *Phytophthora*, except for some *Phytophthora parasitica* isolates. *Phytophthora* spp. secrete small proteins called elicitors (Ricci 1997), which are encoded by a small multigene family (Kamoun et al. 1993a; Panabières et al. 1995). Elicitors induce HR and systemic acquired resistance (SAR) in tobacco (Bonnet et al. 1996; Kamoun et al. 1993b; Keller et al. 1996a). Some *P. parasitica* isolates do not secrete elicitors in vitro in detectable amounts, although elicitor genes are present in their genomes (Kamoun et al. 1993a; Ricci et al. 1993). These elicitor-nonproducing *P. parasitica* isolates also are virulent on tobacco. Taken together, the results suggest that elicitors are species-specific avirulence factors toward tobacco (Kamoun et al. 1994; Ricci et al. 1992). *P. parasitica*, however, is not easily amenable to DNA-mediated transformation and, therefore, the role of elicitors in the interaction between this pathogen and its host plant tobacco has not been established clearly. Nevertheless, recent results suggested that INF1, an elicitor produced by *P. infestans*, may govern nonhost resistance of *Nicotiana benthamiana* to this pathogen (Kamoun et al. 1998b).

The situation became more complex following characterization of *P. parasitica* elicitor-producing isolates that were moderately (Bonnet et al. 1994) or highly virulent on tobacco (Colas et al. 1998). An analysis of the genetic diversity of *P. parasitica*, including elicitor-producing and -nonproducing isolates, provided new insights into the understanding of the molecular events leading to the emergence of *P. parasitica* isolates virulent on tobacco (Colas et al. 1998). Some *P. parasitica* isolates may be able to circumvent tobacco resistance by losing the ability to secrete elicitors, a type of a loss that may have arisen from rare, independent mutational events because elicitor-nonproducing isolates belong to distinct genotypes. Conversely, the virulence on tobacco of some elicitor-0itin genes leading to proteins that no longer have elicitor activity or from the down regulation of elicitor gene expression in planta.

To test these hypotheses, we focused on the sequences and in vitro and in planta expression of elicitor genes from several *P. parasitica* isolates. In this report, we show that *parA1* is the main, if not the only, elicitor gene expressed in mycelium grown in vitro and in planta by *P. parasitica*. Furthermore, *parA1* is highly conserved in *P. parasitica* field isolates collected on various plant species, regardless of their in vitro elicitor production and virulence levels toward tobacco. We

present evidence that elicitor-producing isolates pathogenic on tobacco down regulate *parA1* during compatible interactions, although this down-regulation event does not occur in a *P. parasitica* isolate collected on tomato and avirulent on tobacco.

RESULTS

Selection of a subset of *P. parasitica* isolates.

We selected six *P. parasitica* isolates that belong to different genetic backgrounds and are representative of the pathological behavior of this pathogen toward tobacco, in relation to its ability to produce elicitors in vitro. Previously, we have shown that *P. parasitica* isolates fall into three classes (Colas et al. 1998): i) isolates collected on various host plants, except tobacco, all produce elicitors (NTE+) and are avirulent on tobacco; ii) elicitor-nonproducing tobacco isolates (TE–) are highly virulent and specialized to tobacco; iii) elicitor-producing tobacco isolates (TE+) are pathogenic and display a large range of virulence levels on tobacco. Furthermore, TE+ isolates do not appear to be specialized on this host plant. In the present study, carnation 26 and tomato 179 isolates are representative of the NTE+ class. Tobacco isolates 397 and 388, and 310 and 408 are representative of the TE– and TE+ classes, respectively.

Quantification of elicitor production in vitro.

Qualitative analysis of elicitor production in vitro has been performed for *P. parasitica* isolates differing in pathogenicity toward tobacco by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or Western blotting methods (Colas et al. 1998; Kamoun et al. 1994; Ricci et al. 1992). These techniques, however, may not be sufficiently sensitive to assess a possible link between quantitative differences in elicitor production and the variation in virulence levels on tobacco.

Therefore, isolates 26, 179, 310, 408, and 397 were grown in liquid medium for 5 days and the culture filtrates were analyzed for their elicitor content with a double antibody sandwich form of enzyme-linked immunosorbent assay (DAS-ELISA). Elicitor production levels did not differ significantly among elicitor-producing isolates, despite the large variation in their pathogenicity and virulence level on tobacco (Table 1). In addition, a signal was observed in the culture filtrate of the TE– isolate 397, although at a 1,000-fold-lower amount than in the culture filtrates of elicitor-producing isolates. Because the cross reactivity of the sera with heterologous elicitors from various species is known (Devergne et al. 1994), the signals observed in this experiment could have resulted from the expression of variant gene(s) distinct from *parA1*, encoding protein(s) devoid of elicitor activity on tobacco.

In addition, the very low level of elicitor production observed in the TE– 397 isolate could be the outcome of point mutations, leading to a dramatic decrease of mRNA or protein stability. In view of these possibilities, we analyzed the sequences of elicitor genes actually expressed in several *P. parasitica* isolates.

Sequences of elicitor genes expressed during in vitro growth.

Total RNA was extracted from the mycelium of several *P. parasitica* isolates grown in vitro and converted into cDNA

before being used as a template for reverse-transcription polymerase chain reaction (RT-PCR). Oligo(dT) and the degenerate primer uni1 (see below) were used. uni1 defines a region located in the signal peptide of elicitin genes. This region is highly conserved among all genes identified that encode “canonical” elicitins, or ORFs of 98 amino acids primarily identified as biologically active proteins (Kamoun et al. 1993b; Pernollet et al. 1993; Ricci et al. 1989). uni1 has been used previously for the efficient amplification of a large set of elicitin-encoding cDNAs from numerous *Phytophthora* and *Pythium* species (Panabières, unpublished results; Panabières et al. 1997; Ponchet et al. 1999). We therefore assumed this would amplify every potentially expressed elicitin gene.

A single fragment of approximately 550 bp was generated in all cases (Fig. 1A). The amplification signal was lower in TE– isolates than in TE+ and NTE+ isolates, which may reflect the relative abundance of mRNA encoding elicitin in the various isolates. The amplification products were purified, cloned, and sequenced.

Five independent clones were sequenced for each of five isolates (26, 310, 408, 397, and 388) and compared with *parA1* genomic sequence (Kamoun et al. 1993a). Among the 25 sequences, two different transcripts were identified that perfectly match the whole coding region of *parA1*. These were subsequently designated *parA1.1* and *parA1.2*. The *parA1.1* and *parA1.2* cDNAs differ from *parA1* only by two mismatches at nucleotides 366 and 385 (Fig. 2). These differences occur within the 3′ untranslated region (UTR), and both consist of G–A transitions. Because all clones analyzed exhibited adenine residues at these positions, the *parA1* allele described by Kamoun et al. (1993a) probably represents a variant of the canonical sequence of the parasiticein gene. *parA1.1* and *parA1.2* differ from each other by a G–A transition at position 461 in the 3′ UTR. One to four out of the five clones analyzed corresponded to one of the two cDNAs for each isolate, except for isolate 26, where *parA1.1* only was detected. Because *Phytophthora* spp. are diploid organisms, *parA1.1* and *parA1.2* may correspond either to two parasiticein genes or to two distinct alleles of a single gene.

Sequencing experiments allowed characterization of the first entire 3′ UTR for a parasiticein gene to date. The canonical sequence of *parA1*, extended with the newly determined 3′ UTR, was therefore compared with similar regions of elicitin-encoding sequences isolated from *Phytophthora* or *Pythium* species (Kamoun et al. 1997; Mao and Tyler 1996; Panabières et al. 1995; Panabières et al. 1997). No significant similarity

was found outside the coding region (data not shown), but the overall size and the base composition of the 3′ UTR was close to the UTRs from other elicitin genes. Moreover, a sequence identified as a polyadenylation signal (ATGAA), located 19 bp upstream from the poly(A) tail, was identical to that observed in several elicitin sequences (Panabières et al. 1995; Panabières et al. 1997).

Taken together, these results suggest that *parA1* is clearly the most highly expressed gene encoding canonical elicitin in *P. parasitica* in mycelium during in vitro growth. Furthermore, although elicitin gene expression could not be detected by Northern blot analyses in TE– isolates (Kamoun et al. 1993a; this study and data not shown), *parA1* is expressed in these isolates on the basis of RT-PCR. No sequence divergence among the analyzed clones could be observed no matter which pathogenicity toward tobacco and the elicitin gene expression level in vitro of the isolates were selected for this study. Hence, we reasoned that down regulation of *parA1* mRNA expression in planta could allow certain E+ isolates such as 408 to evade recognition when interacting with tobacco. We therefore studied the expression of *parA1* during the interaction between *P. parasitica* and tobacco.

***parA1* expression during *P. parasitica*–tobacco interaction.**

We originally hypothesized that differences in *parA1* expression in planta could contribute toward differences in the pathogenicity on tobacco among elicitin-producing *P. parasitica* isolates. To test this hypothesis, a positive control to determine the levels of elicitin gene expression for an isolate with low or null virulence on tobacco was necessary. This type of a control, however, could not be obtained a priori because a nonpathogenic strain would not develop in planta. Consequently, the pathogen biomass would not be sufficient to detect elicitin gene transcription even if it occurs. This biological limitation could, however, be circumvented by using tobacco plants, which would be more susceptible to *P. parasitica* than *N. tabacum* cv. Xanthi. In order to carry out this experiment, we used transgenic Xanthi tobacco plants (NahG8) that were transformed with the *nahG* gene from *Pseudomonas putida*, which encodes a salicylate hydroxylase. These transgenic plants no longer accumulate salicylic acid (SA) (Gaffney et al. 1993) and, because SA mediates the induction of SAR in tobacco upon elicitin treatment, NahG8 tobacco plants are more susceptible to elicitin-producing *Phytophthora* spp. than wild-type plants. There are, however, no differences in susceptibility after infection by TE– *P. parasitica* isolates (Keller et al. 1996b).

Table 1. Biological characteristics of the isolates in this study

Isolate	Host plant origin	Virulence on tobacco ^a	Elicitin production in vitro ^a	Elicitin produced in vitro (ng/ml/mg of dry weight) ^b
26	Carnation	Null	+	596.87 ± 36.60 ^d
179	Tomato	Null ^c	+	1137.51 ± 139.06 ^d
310	Tobacco	Low	+	899.81 ± 155.03 ^d
408	Tobacco	High	+	697.16 ± 43.21 ^d
397	Tobacco	High	–	0.66 ± 0.06 ^d
388	Tobacco	High	–	Not tested

^a Decapitated stem inoculation was used to measure virulence levels on tobacco. Elicitin production in vitro noted in the fourth column was qualitatively scored by Western blotting. These results have been published previously (Colas et al. 1998).

^b In vitro elicitin production was quantitatively measured by double antibody sandwich form of enzyme-linked immunosorbent assay. Values are means ± standard deviation of three independent replicates.

^c I. Lacourt and F. Panabières, unpublished results.

^d Values with the same symbol do not significantly differ according to student's *t* at *p* = 0.05.

Zoospores from isolates 26, 310, 408, and 397 were infiltrated into tobacco leaves of Xanthi wild-type NahG8 and NahG9 lines. Disease or resistance symptoms (HR-like necrosis or disease lesion) appeared 3 days after inoculation (Fig. 3). Isolate 26, collected on carnation, induced a HR-like necrosis on all tobacco lines. This result was expected because elicitor-induced necrosis is not SA dependent (Keller et al. 1996b). The susceptibility of NahG8, however, was comparable to that displayed by wild-type or NahG9 plants because no disease developed in the conditions used. Isolates 408 (TE+) and 397 (TE-) both induced disease symptoms on all tobacco lines. No necrosis was observed, even when plants were inoculated with the elicitor-producing isolate 408. Finally, the TE+ isolate 310 did not induce any symptoms of necrosis or

disease on wild-type and NahG9 plants, whereas an intermediate reaction between necrosis and disease was observed in NahG8 leaves.

Total RNA was extracted from inoculated leaves and hybridized with a *P. parasitica*-specific rDNA probe. Hybridization signals were observed only in the samples from leaves that displayed disease symptoms (Fig. 4A and C). Hence the lack of disease development always correlated with the absence of pathogen growth in planta. On the basis of symptom observations and Northern blot analysis, isolate 310 appears to colonize the leaves of NahG8 lines but not those of NahG9 or wild-type lines.

When *parA1*-encoding cDNA was used as a probe, in planta *parA1* expression could not be detected in Northern blot experiments (data not shown). Therefore, the RNA samples were used as templates for RT-PCR experiments in order to investigate in planta expression of the elicitor gene. Amplification products were resolved in agarose gels, blotted, and identified by hybridization with homologous, cloned probes. We used a cDNA encoding cytosolic cyclophilin from *P. parasitica* (*cyp1*) (F. Panabières, unpublished) as a positive control.

The expression time course of *cyp1* broadly followed the results obtained in Northern blots with the *P. parasitica* rDNA probe. A strong signal was obtained from the early stages of disease development, appearing as soon as 1 day after inoculation (Fig. 5B and D). In contrast, *cyp1* appeared on the first day after inoculation of wild-type plants with the incompatible isolates 26 and 310 and was no longer detected at later stages (Fig. 5B). This could reflect the efficient restriction of the pathogen growth once tobacco resistance mechanisms are induced. Surprisingly, the expression of *cyp1* was not detected in the incompatible interaction between isolate 26 and NahG8

Fig. 1. In vitro expression of elicitor-encoding sequences in various *Phytophthora parasitica* isolates. Reverse transcription-polymerase chain reaction amplifications were performed on **A**, cDNA or **B**, total RNA as a control for testing potential amplification of contaminating genomic DNA. The degenerate primer uni1, located in the highly conserved region encoding the signal peptide, was used in combination with oligo(dT). Lane 1, Control (no RNA); lane 2, isolate 26 (NTE+); lane 3, isolate 310 (TE+); lane 4, isolate 408 (TE+); lane 5, isolate 397 (TE-); lane 6, isolate 388 (TE-).

Fig. 2. Sequence alignment of the genomic sequence of *parA1* (Kamoun et al. 1993) and reverse transcription-polymerase chain reaction (RT-PCR)-based elicitor cDNAs (*parA1.1* and *parA1.2*). Coding regions are in capital letters and the putative polyadenylation site is boxed. Underlined sequence corresponds to uni1, the 5' primer used for RT-PCR amplifications.

line (Fig. 5D). This can be explained by a poor reproducibility as a result of a critical threshold for the detection of a pathogen transcript during incompatible interactions. Alternatively, because cyclophilin gene expression is induced under various stress conditions and external stimuli in plants such as SA (Marivet et al. 1994), it may be that plant resistance mechanisms in wild-type tobacco lines (which are abolished in transgenic plants) can, to some extent, induce the expression of the *P. parasitica cyp1* gene.

Although NTE+ isolate 26 induced a strong necrosis reaction on all tobacco lines, no expression of *parA1* could be detected at the mRNA level. This is likely to be a result of the low amount of pathogen biomass that hampered efficient detection of pathogen transcripts. The *parA1* probe did not reveal any signal in samples inoculated with the TE– isolate 397, as expected. More surprising was the absence of any signal when inoculating plant with TE+ isolate 408, which produces parasiticein in vitro. Conversely, a low level of *parA1* expression was detected in NahG8 plants inoculated with the weakly virulent TE+ isolate 310 3 days after inoculation. This signal was correlated with a faint necrotic symptom. Because both strains developed equally in NahG8 leaves (Fig. 4), we assumed that the absence of detection of *parA1* transcript in 408-inoculated plants reflects very low levels of elicitor expression. Moreover, this result is consistent with the observed symptoms of faint necrosis in 310–NahG8 interactions and no necrosis in 408–tobacco interactions (Fig. 3). Similar patterns were obtained when hybridizing with either the coding region (data not shown) or the highly specific 3' UTR probe (Fig. 5), demonstrating that *parA1* actually is the elicitor gene expressed during *P. parasitica*–tobacco interactions.

Isolates 310 and 26 were shown to produce similar amounts of parasiticein in vitro (Table 1). The differences of necroses

induced during interaction (Fig. 3) compared to their in planta development, reflected by rDNA and *cyp1* accumulation (Figs. 4 and 5), strongly suggests that the isolate 26 produces more elicitor in planta than does the isolate 310. Because the very low biomass of isolate 26 does not hinder the observation of visible symptoms of necrosis, we might expect the in planta expression of *parA1* to be exceptionally low in the interaction between isolate 310 and tobacco. In order to address whether the low *parA1* expression level observed during isolate 310–tobacco interaction is specific for this isolate or if it depends on the interacting host, we studied the expression of *parA1* in an alternative compatible interaction between *P. parasitica* and tomato.

***parA1* expression during *P. parasitica*–tomato interaction.**

The TE+ isolate 310 was collected on tobacco, but it can infect tomato (Colas et al. 1998) as well. Tomato leaves (*L. esculentum* hybrid 63.5 F₁) were therefore infiltrated with zoospores of 310. The *P. parasitica* isolate 179, which is pathogenic on tomato and produces equivalent amounts of parasiticein in vitro (Table 1), was included in the assay as a control. Similar disease symptoms were observed 3 days after inoculation with both strains (not shown). The extent of pathogen growth was evaluated by Northern blots with a *P. parasitica*-specific rDNA probe (not shown) and by RT-PCR amplification of the *P. parasitica cyp1* gene (Fig. 6B). The results indicate that 310 and 179 established a compatible interaction with tomato and that the pathogen biomass is equivalent 3 days after inoculation with both isolates.

The expression of elicitor genes was assessed by RT-PCR amplification of *parA1*, as previously performed in the tobacco–*P. parasitica* interactions. A strong signal was observed 3 days after inoculation with isolate 179 (Fig. 6A). This signal was observed when either the coding sequence or the 3' UTR

Fig. 3. Tobacco resistance reactions and disease symptoms observed 3 days after leaf infiltration of 200 zoospores of *Phytophthora parasitica* isolates 26, 310, 408, and 397 in Xanthi wild-type (left) and transgenic tobacco plants expressing (NahG8, center) or not expressing (NahG9, right) the *nahG* gene.

of *parA1* was used as probes. In contrast, no elicitin transcript was detected in tomato leaves inoculated with isolate 310, although the pathogen clearly demonstrated the ability to develop. We therefore conclude that the down regulation of parasiticein gene expression in planta is a mechanism that is dependent on the *P. parasitica* genotype rather than the host plant.

DISCUSSION

The absence of parasiticein production by some *P. parasitica* isolates has been shown to be associated with the ability to infect tobacco, whereas production of elicitin has been correlated with low or no virulence on this host plant (Bonnet et al. 1994; Kamoun et al. 1994; Ricci et al. 1992). Recent analysis of a large collection of *P. parasitica* isolates confirmed this correlation in most cases but also revealed that some isolates highly virulent on tobacco still produced large amounts of elicitin in vitro (Colas et al. 1998). Because elicitors specifically induce defense mechanisms on tobacco, we hypothesized that these isolates might be able to evade tobacco recognition by mutation in elicitin genes or through the regulation of elicitin gene expression. In this paper, we have shown that elicitin-producing isolates virulent on tobacco undergo down regulation of their elicitin genes in planta. This down regulation was observed in tobacco isolates with distinct genetic backgrounds. In contrast, one *P. parasitica* isolate collected on tomato and highly virulent to this host but avirulent on tobacco continues to express elicitin genes during the compatible interaction. Therefore, the down regulation of elicitin gene expression appears to be restricted to isolates that are pathogenic on tobacco.

Sequence analysis and subsequent hybridization with specific probes demonstrated that *parA1*, as described previously

(Kamoun et al. 1993a), is the main elicitin gene expressed in vitro and in planta by *P. parasitica*.

The mechanisms leading to *parA1* down regulation may be diverse such as *cis*-events (point mutations, deletions or insertions in the promoter, and mRNA stability) or on the basis of the action of *trans*-acting factors (repression or lack of induction). We have shown that *parA1* is functional in the "nonproducing" isolates of *P. parasitica*, even if expressed at a low rate. Two nearly identical transcripts encoding *parA1* were identified clearly in all isolates, regardless of elicitin production in vitro. On the basis of this analysis, it would appear that the low levels of parasiticein production do not originate from mutations in the coding and the 3' UTRs that might affect the stability of the protein or the mRNA. The two *parA1* transcripts could, perhaps, correspond to either two distinct genes or to two allelic forms of a single *parA1* gene. Although additional sequencing of promoter regions are required in producing and nonproducing isolates, *trans*-acting factors are more likely to be at the origin of this low expression than defective promoters for both copies or alleles. In two analyses of independent genetic crosses between one producing and one nonproducing isolate of *P. parasitica* (Colas 1997; Kamoun et al. 1994), the 17–21 and 13–14 progeny, respectively, failed to secrete elicitin in vitro. Despite the small number of progeny in these crosses, the results also favor the hypothesis of *trans*-acting repressor elements that control elicitin expression. This type of regulatory pathway would be well adapted for this multigene family.

The repression hypothesis could be evaluated with a transformation-based strategy. We recently performed efficient heterologous expression of cryptogein, an elicitin gene from *Phytophthora cryptogea*, under the control of its own promoter in *P. infestans* (Panabières et al. 1998). The same ap-

Fig. 4. Development of *Phytophthora parasitica* isolates 26, 310, 408, and 397 in **A and B**, wild-type and **C and D**, NahG8 tobacco plants, as reflected by Northern blot hybridization. Total RNA (15 µg) was extracted from infected tobacco leaves 1, 2, and 3 days after inoculation and hybridized with a *P. parasitica*-specific probe derived from 28S rDNA (**A and C**). To allow normalization for differences in loading samples among lanes, the same blots were stripped and reprobed with a nonspecific probe derived from the 18S rDNA from the root-knot nematode *Meloidogyne incognita* (**B and D**). NI = RNA isolated from noninfected leaves.

proach with a *parA1* gene isolated from a nonproducing strain of *P. parasitica*, would either lead to a failure of gene expression, if its promoter is defective, or to production of parasiticein, if *parA1* is submitted to a *trans*-repression in its native genetic background. Alternatively, the transformation of a tobacco isolate with the cryptogein gene would permit us to determine whether the *trans*-repressors, if they exist, act specifically on *parA1* sequences or other elicitor genes. The recent report of the transformation of a tobacco strain of *P. parasitica* (Bottin et al. 1999) offers the opportunity to test this hypothesis.

Recently, the existence of field isolates of *P. infestans*, which naturally fail to produce elicitor, has been described (Kamoun et al. 1998a). It would be interesting to develop transformation and genetic cross approaches in order to check whether the lack of elicitor production is mediated by similar mechanisms in *P. infestans* and *P. parasitica*.

For the analysis of elicitor gene expression during in planta growth, we used NahG8 transgenic tobacco plants (Gaffney et al. 1993) because we expected that, in this genetic background, the pathogen biomass would increase as a result of greater susceptibility to *P. parasitica* and, therefore, improve the detection of *P. parasitica* mRNA, even for isolates that are weakly virulent on tobacco. This attempt was successful for the tobacco isolate 310. The symptoms observed in the present study in wild-type and NahG8 tobacco plants following inoculation by several *P. parasitica* isolates are broadly consistent with those observed previously (Keller et al. 1996b), although slight differences were noted. This is mainly a result of differences in the inoculation methods. We performed leaf infiltrations with zoospores, whereas Keller et al. (1996b) inoculated decapitated stems with mycelial plugs. Stems are more susceptible organs to soilborne *Phytophthora* spp. than are leaves, whereas tobacco leaves are more responsive to HR-like necrosis induced by elicitors than are stems. In the present study, we selected the zoospore-leaf infiltration assay

because it is the most convenient one to study elicitor gene expression during infection, for several reasons. First, plant infections by *Phytophthora* spp. occur through zoospores in natural conditions (Erwin and Ribeiro 1996). Second, elicitors applied to decapitated tobacco stems translocate rapidly throughout the plant, whereas they remain restricted to the infiltration area when infiltrated into tobacco leaves (Keller et al. 1996a). Consequently, the leaf infiltration method allowed the comparison of disease symptoms and necrosis formation at the same time as elicitor gene expression, precisely at the inoculation site.

The tobacco pathogenic *P. parasitica* isolates 408 and 310 undergo down regulation of *parA1* mRNA in planta, but are indiscernible from other *P. parasitica* isolates that are non-pathogenic to tobacco with regard to their elicitor gene expression and production levels in vitro. Down regulation (either constitutive or induced during the interaction) might represent an advantage for some *P. parasitica* in order to evade the elicitation of tobacco resistance. This hypothesis is supported by a recent study where *P. infestans* strains deficient for the production of an INF1 elicitor were obtained through an antisense gene-silencing strategy (Kamoun et al. 1998b). Whereas wild-type strains are unable to infect *N. benthamiana*, the pathogenicity of INF1-deficient strains to this non-host plant is enhanced because 20 to 30% of inoculations lead to disease development.

The down regulation of elicitor gene expression has been observed in strains isolated from tobacco and virulent to this plant. The in planta down regulation of *parA1* mRNA for tobacco isolates occurs in compatible interactions with two host plants tested, tobacco and tomato. As tomato does not appear to react to elicitors, the down regulation of parasiticein gene expression by isolate 310 is not required for successful invasion. The parasiticein gene is expressed in the tomato isolate 179 during its interaction with tomato. Therefore, the mechanism of down regulation observed for E+ tobacco isolate 310

Fig. 5. Time-course expression of parasiticein and cyclophilin genes during the interaction between *Phytophthora parasitica* isolates 26, 310, 408, and 397 and **A and B**, wild-type and **C and D**, NahG8 tobacco plants. Reverse transcription-polymerase chain reaction (RT-PCR)-derived amplification products were separated on Tris-borate-EDTA agarose gels, Southern blotted, and hybridized with the *parA1* 3' untranslated region (**A and C**) or *cyp1* cDNA (**B and D**) as probes. RNA was isolated after 1, 2, and 3 days after inoculation. T = negative control for RT-PCR amplification (no RNA); NI = RNA isolated from noninfected leaves; M = template RNA isolated from isolate 26 mycelium.

appears to occur in compatible interactions, regardless of the reactivity of the given host to elicitors. Further experiments are needed to identify the origin of the signal that triggers this down-regulation event.

It should be noted that even when *parA1* is expressed in planta, its expression is never detected earlier than 3 days after inoculation. It has been reported that *infl* is not expressed in zoospores nor in germinating cysts of *P. infestans*, whereas it is expressed in the mycelium (Kamoun et al. 1997). Because *parA1* expression is detected in planta at a late stage, a down regulation of *parA1* in zoospores and germinating cysts of *P. parasitica* also is likely to occur.

Although avirulence genes restrict the host range of pathogens, they are maintained in natural populations. Therefore, avirulence genes may have pleiotropic intrinsic functions for plant pathogens (Knogge 1996; Laugé and De Wit 1998). The loss of avirulence genes leads to reduced virulence or fitness for several fungal and bacterial pathogens (Kearney and Staskawicz 1990; Laugé et al. 1997; Rohe et al. 1996), which supports this hypothesis. In the case of *P. parasitica*, *parA1* is highly conserved in field isolates, which suggests that a particularly strong selective pressure acts on this gene. Thus it is tempting to consider that parasiticein possesses an essential, intrinsic function for *P. parasitica*. Recently, elicitors have been shown to be sterol carrier proteins (Mikes et al. 1998). As *Phytophthora* spp. are auxotrophic for sterols (Hendrix 1970), elicitors probably possess a crucial biological function for these organisms. Down regulation of *parA1* mRNA in planta may therefore constitute a suitable mechanism to infect tobacco, without deleterious consequences for the pathogen. *parA1* is, strikingly, highly conserved among *P. parasitica*, even in TE- isolates, which are still able to express it, although at low levels. This indicates that for these isolates, the faint level of elicitor production is sufficient to fulfill its function or that *parA1* is expressed at higher rates at a different developmental stage that has not yet been characterized.

MATERIALS AND METHODS

P. parasitica growth conditions.

P. parasitica isolates were obtained from the INRA Antibes collection and were maintained on malt-agar (1%, 1%, wt/vol) at 24°C. For DAS-ELISA experiments and RNA extractions, isolates were grown on a defined liquid medium (Hall et al. 1969).

Quantification of elicitor production in vitro by DAS-ELISA.

Amounts of elicitor secretion in 5-day-old culture filtrates was quantified by DAS-ELISA, according to Devergne et al. (1994). A monoclonal antibody raised against cryptogein (an elicitor produced by *P. cryptogea*) that cross reacts with parasiticein and a polyclonal antibody raised against parasiticein were used as trapping antibodies. For each ELISA test, a range of dilutions of purified parasiticein were used for construction of a reference dose-response curve.

Infection assays.

For experiments on elicitor gene expression in planta, 100 µl of sterile water solution containing 200 zoospores was

infiltrated, as described (Galiana et al. 1997), into leaves of 2-month-old untransformed and transgenic tobacco plants (Xanthi) expressing (NahG8) or not expressing (NahG9) the *nahG* gene (Gaffney et al. 1993; this paper). Tomato plants (*Lycopersicon esculentum* hybrid 63.5 F₁) were inoculated, following the same procedure. Plants were grown in a growth chamber at 24°C, with 16 h of illumination at 40 m m⁻² per s.

Total RNA extraction and Northern blot experiments.

Total RNA was extracted from 3-day-old mycelium grown in vitro and from the infiltrated zone of *P. parasitica*-inoculated leaves 1 to 3 days after infection, according to Logemann et al. (1987). A subsequent purification step was added in order to remove any contaminating genomic DNA: sodium acetate (pH 5.2) was added at a 3 M final concentration. Samples were kept on ice for 3 h, centrifuged at 4°C for 20 min, and washed twice with 70% ethanol. Migration of total RNA (15 µg), capillary transfer, probe labeling, and hybridization at 42°C were performed according to standard procedures (Sambrook et al. 1989). Filters were washed at high stringency with a final wash at 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS at 65°C.

RT-PCR amplification.

First-strand cDNA was synthesized from 0.5 and 3 µg of total RNA extracted from mycelium or infected leaves in 25 µl of final volume with 250 µM deoxynucleoside triphosphate (dNTP) (each), 1 µM oligo(dT)₁₅, and 1 unit of avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, U.S.A.). For amplification of elicitor cDNAs, the degenerate primer uni1 (5'-ATGAACCTCCGCGCTCTSYTYGC), previously designed for the cloning of several elicitor-encoding sequences from various *Phytophthora* and *Pythium* species (Panabières et al. 1997), was used in combination with oligo(dT). A probe corresponding to the cytosolic cyclophilin *cyp1* from *P. parasitica* was amplified with a combination of oligo(dT)₁₅ and the primer *cyp1* (5'-ATGGACGTGGTCAAGGCCATC), designed after characterization of a cyclophilin-encoding cDNA from *P. cryptogea* (F. Panabières, unpublished results). Experimental data concerning the design and cloning of *cyp1* are available upon request. PCR amplification of 5 µl of first-strand cDNA were carried out in 50 µl of final volume containing 250 µM dNTP (each), uni1 or *cyp1*, and oligo(dT)₁₅ primers at 0.5 and 1 µM, respectively, and 1 unit of Taq DNA polymerase (Appligene, Illkirch, France). Reac-

Fig. 6. Time-course expression of **A**, *parA1* and **B**, *cyp1* genes during the compatible interaction between *Phytophthora parasitica* isolates 310 and 179 and tomato. Analysis of gene expression was performed as described in Figure 5.

tions were performed with a Robocycler (Stratagene, La Jolla, CA, U.S.A.) for 5 min at 95°C and for 30 cycles of 95°C for 1 min, 57°C for 1.5 min, and 71°C for 1.5 min. A final extension was performed at 71°C for 3 min. All RT-PCR experiments were repeated independently at least twice. Purification, cloning, Southern blotting, and hybridization of the RT-PCR amplified products were carried out as described (Sambrook et al. 1989). Southern blots were probed with PCR products encoding the coding sequence and the 3' UTR of *parA1* gene, respectively. Hence primers uni1 (see above) and uni2 (5'-CG AGAAGCCGTTTCGCGTA) allowed the amplification of the *parA1* coding sequence, whereas par1 (forward: 5'-GCGGGT TTAATCTCTGCGTCCAGAA) and par2 (reverse: 5'-ATACA AAAACCTTCATTACAGG) were designed specifically to amplify the 3' UTR of *parA1*. Sequencing was performed by Genome Express SA (Grenoble, France).

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