Two Avirulent Races of *Colletotrichum lindemuthianum* Trigger Different Time Courses of Plant Defense Reactions in Bean

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Received 1 December 1992. Accepted 15 March 1993.

A bean resistant line, carrying the ARE resistance gene, was challenged with two avirulent races of *Colletotrichum lindemuthianum* (races 1 and 9), the causal agent of bean anthracnose. The time course of the plant defense reaction was monitored by the observation of symptom development, fungal infection process, and accumulation of bean phenylalanine ammonia-lyase, chitinase, and hydroxyproline-rich glycoprotein mRNAs in infected leaves. Race 9-infected beans displayed a faster induction and a different pattern of all these events. When compared to previous results with an isogenic susceptible line, both race 1 and race 9 provoked an earlier induction of PAL and CHT mRNAs accumulation in incompatible interactions than in compatible ones. However, with race 1, the maximum of accumulation occurred almost at the same time after infection in both interactions. Instead, with race 9, it was observed 40 hr earlier in the incompatible interaction than in the compatible one. After infection by race 9, Hyp 4.1 transcripts were only slightly accumulated in the resistant line, whereas they were not accumulated at all in the susceptible line. In contrast, the pattern of induction of Hyp 4.1 mRNA was similar in these race 1-infected lines.

Additional keywords: compatible interaction, defense mRNAs, fungal infection process, incompatible interaction, near-isogenic lines, symptoms.

A successful infection of a plant by a pathogen requires a range of functions known as basic compatibility (Gabriel and Rolfe 1990). Race-specific resistance (incompatibility) may be considered to be superimposed on a basic host-parasite compatibility (Ellingboe 1976) and is generally controlled by a gene-for-gene interaction according to the Flor hypothesis (1942). A dominant resistance gene in the host would only confer resistance against races harboring the complementary dominant avirulence gene. In French bean, race-specific resistance against *C. lindemuthianum* (Sacc. & Magnus) Lams.-Scrib., the causal agent of anthracnose disease, is controlled by single dominant genes (Bannerot *et al.* 1971). A resistance gene in a particular host cultivar confers resistance against physiological races that are postulated to express the matching avirulence gene.

Plants respond to pathogen infections by mobilizing a complex network of active defense mechanisms (Bowles 1990). These include strengthening of plant cell walls, synthesis of antimicrobial compounds (phytoalexins), and accumulation of lytic enzymes such as chitinase. Many of these responses involve the transcriptional activation of the corresponding defense genes. Marked differences in the timing of defense gene activation depending on the compatibility/incompatibility of the interaction have often been reported (Bell *et al.* 1986; Dixon and Harrison 1990; Habereder *et al.* 1989; Hedrick et al. 1988; Mehdy and Lamb 1987). Bean-*C. lindemuthianum* interactions are well documented at the cytological level (O'Connell *et al.* 1985; O'Connell and Bailey 1988); molecular aspects of the bean defense response to this fungus have also been extensively studied (Corbin *et al.* 1987; Hedrick *et al.* 1988; Liang *et al.* 1989; Mehdy and Lamb 1987; Ryder *et al.* 1987; Showalter *et al.* 1985).

In a previous study (Mahé *et al.* 1992b), we have shown that even in a compatible context, different races of *C. lindemuthianum* were able to induce bean defense mechanisms with different kinetics. Thus, to avoid confusion between a specific resistance response (determined by the gene-for-gene interaction) and an undefined host resistance or pathogen fitness, plant and pathogen genetic backgrounds have to be controlled. In this report, incompatible interactions between a bean resistant line (P12 R) isogenic to the previously studied susceptible line (P12 S) (Mahé *et al.* 1992b) and two races of *C. lindemuthianum* were investigated.

The different interactions studied are summarized in Table 1.

In the present paper, only data concerning the incompatible interactions between P12 R and races 1 and 9 are presented. The other data were reported previously (Mahé *et al.* 1992b). Macroscopic symptoms, fungal development,

Table 1. The pattern of interactions between near-isogenic bean lines (P12 S and P12 R) and 3 races of *Colletotrichum lindemuthianum*

	P12 S	P12 R
Race 1	Compatible	Incompatible
Race 2	Compatible	Compatible
Race 9	Compatible	Incompatible

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MPMI Vol. 6, No. 4, 1993, pp. 423-428
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and defense gene mRNA levels were studied to monitor the defense responses. A chitinase (CHT) mRNA coding for a lytic enzyme, a hydroxyproline-rich glycoprotein mRNA (Hyp 4.1 mRNA) coding for a cell-wall protein, and a phenylalanine ammonia-lyase (PAL) mRNA playing a pivotal role in the lignin-isoflavonoid phytoalexin biosynthesis were chosen because they belong to different plant defense response pathways.

The comparison of these different interactions shows that an mRNA corresponding to a given defense gene does not always accumulate earlier in incompatible interactions than in compatible interactions. Moreover, the necessity of using isogenic material (plant or fungus) to compare defense responses in susceptible and resistant cultivars is demonstrated.

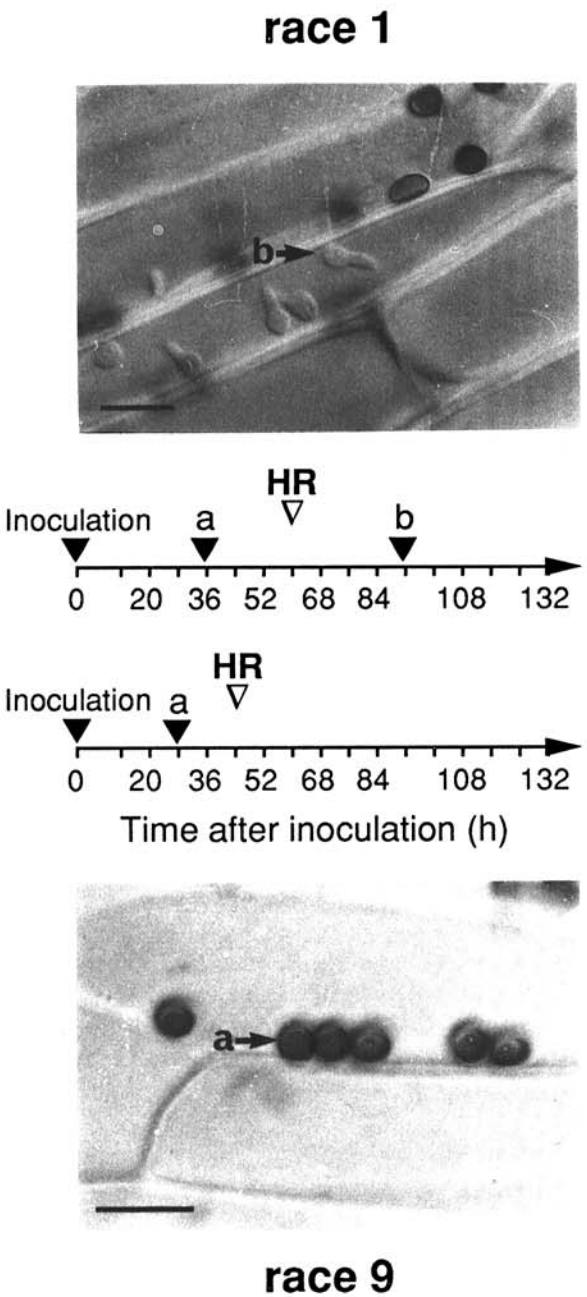

Fig. 1. Time course of symptom development and infection process during incompatible interactions of bean (P12 R) with races 1 and 9 of *C. lindemuthianum*. (▽) HR: hypersensitive reaction. Cytological observations (▼), a: appressorium, b: primary hyphae emerging from infection vesicles. The stage at which the fungus is restricted in P12 R/race 1 and P12 R/race 9 interactions is illustrated on the photographs. Bars = 10 μm.

RESULTS AND DISCUSSION

Symptom and fungal developments during incompatible interactions.

The initial infection process of *C. lindemuthianum* is similar in both compatible and incompatible interactions. The conidia germinate and appressoria differentiate on the surface of the plant tissue. An infection peg emerges from the appressorium and penetrates the epidermal plant cell as described in previous studies (O'Connell *et al.* 1985). The existence of this infection peg can be visualized from the outside of the cell by the presence of a dark point on the appressorium (O'Connell *et al.* 1985).

In incompatible interactions, the fungal development is seriously affected after penetration. In race 1-infected P12 R line, the fungus development was restricted at the stage of minute hyphae emerging from the vesicles observed at 92 hr after infection within the infected cell (Fig. 1). In this case, the symptoms were very small limited lesions appearing at 60 hr after inoculation, known as the hypersensitive reaction (HR). In P12 R infected by race 9, intracellular fungal structures were never observed (Fig. 1), thus, the fungus seems to be arrested soon after penetration. The macroscopic symptoms (HR) were visible at 44 hr after infection, 16 hr earlier than after infection by race 1. These necrotic lesions were darker and their area slightly larger than those observed after infection by race 1. Since race 9 is arrested earlier than race 1, these results are in good agreement with the data of O'Connell and Bailey (1988) who reported that the more rapidly the fungus is arrested, the earlier the HR symptoms appeared. However, in contrast with the present results, these authors reported on smaller lesion when the fungus is stopped earlier in its progression.

In the compatible interactions, the development of anthracnose symptoms and the fungal progression occurred faster for race 9- than for race 1-infected leaves of bean P12 S line (Mahé *et al.* 1992b).

Changes in plant defense mRNA amounts during incompatible interactions.

Changes in PAL, CHT, and Hyp 4.1 mRNA levels were examined at various times after infection of P12 R with races 1 and 9 of *C. lindemuthianum*. Dot blot hybridizations of total RNA were carried out for quantitative assessment of these changes.

In mock-infected leaves, no significant change in the level of PAL, CHT, and Hyp 4.1 mRNA transcripts were detected throughout the period investigated.

P12 R/race 1 interaction :

After infection of the resistant line with race 1, accumulation of PAL mRNA first became significantly detectable by 52 hr after infection and increased gradually until the end of the experiment (Fig. 2). The CHT tran-

scripts were first detected at 44 hr after spore inoculation. The CHT mRNA amount then continued to increase until the experiment was completed, in a manner similar to that observed for PAL mRNA (Fig. 2). The first induction of PAL and CHT mRNA thus occurred 8–10 hr before HR symptoms became visible. In contrast, the Hyp 4.1 transcripts started to accumulate later, by 76 hr postinoculation (Fig. 2). Similar results were obtained when studying the interaction with race 1 of another resistant line, Ey R (carrying the same resistance gene, the ARE gene, as P12 R), (data not shown). No effect of the plant genetic background has been observed yet when compatible interactions involving Ey S (a susceptible line isogenic to Ey R) and P12 S (isogenic to P12 R) were compared (Mahé *et al.* 1992b).

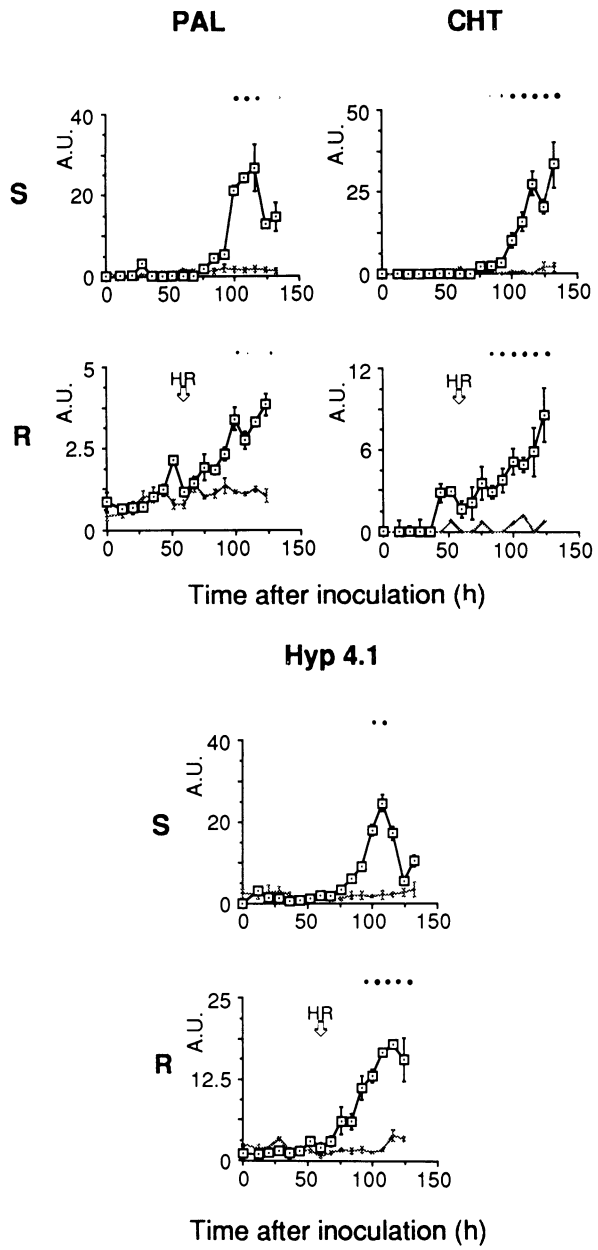

Fig. 2. Time course of induction of PAL, CHT, and Hyp 4.1 mRNAs during compatible (S) and incompatible (R) interactions of bean with race 1 of *Colletotrichum lindemuthianum*. The data for P12 S/race 1 interaction were from Mahé *et al.* (1992b). The level of mRNA was measured at the indicated times as described in methods. A representative autoradiograph of dot blot hybridization is included on the top of each corresponding graph. 0.5 and 1 μg of total RNA were spotted for infected P12 S and P12 R, respectively. For graph tracing, the data shown in the dots were quantitated by normalizing the densitometer scanning of each dot with the constitutive EF-1α gene control. Bars represent 2× SD from four replicates. A.U.: arbitrary units.

When resistant (present results) and susceptible lines (Mahé *et al.* 1992b) were compared (Fig. 2), the kinetics of PAL and CHT mRNAs accumulation differed significantly after infection by race 1 of *C. lindemuthianum*. In the compatible interaction, PAL and CHT mRNAs accumulated transiently during the latest stages of the interaction. In marked contrast, in the incompatible interactions, not only the induction period started earlier but in addition, it lasted longer. Nevertheless, the maximum accumulation of PAL and CHT mRNAs occurred at the same time in incompatible and compatible interactions. The kinetics of appearance of Hyp 4.1 transcripts followed nearly the same pattern in the susceptible and the resistant lines infected by race 1. The level of the induction was about sevenfold (for PAL mRNA), fourfold (for CHT mRNA), and 1.4-fold (for Hyp 4.1 mRNA) lower in the incompatible interaction than in the susceptible line infected with race 1. This may reflect the fact that only a small proportion of the cells became infected in the incompatible interaction (Bell *et al.* 1986).

PAL and CHT mRNA accumulation was already reported to start earlier in an incompatible interaction, for a bean-*C. lindemuthianum* interaction (Bell *et al.* 1986; Hedrick *et al.* 1988). But, in these cases, the maximal induction of PAL and CHT mRNAs also occurred earlier in the incompatible interaction than in the compatible one. These studies were performed using one cultivar and two different races of *C. lindemuthianum,* one avirulent and the other virulent. To verify whether the discrepancies observed between our results and the previous data (Bell *et al.* 1986; Hedrick *et al.* 1988) can be ascribed to a fungal genetic background effect, we have studied another incompatible interaction involving a different race of *C. lindemuthianum* (P12 R/race 9).

P12 R-race 9 interaction:

After infection of P12 R with race 9, a first rapid and transient increase in PAL mRNA starting 28 hr after infection was observed. Then, the PAL mRNA steady-state level remained nearly constant at about 40% of the maximal level until 84 hr postinfection before falling to almost the basal level (Fig. 3). The level of CHT mRNA also started to increase by about 28 hr after inoculation by race 9, it reached a maximum at 44 hr postinfection and remained constant until the end of the period examined, although some fluctuations were observed.

In the corresponding compatible interaction (Mahé *et al.* 1992b), PAL and CHT mRNAs were shown to accumulate transiently and their accumulation occurred later (at 84 hr postinfection) (Fig. 3). The extent of PAL and CHT mRNAs induction was 3- and 6.5-fold lower respectively in the infected resistant line than in the susceptible one.

After infection with race 9, while Hyp 4.1 mRNA was not detected in the susceptible line throughout the period

of interaction (Mahé *et al.* 1992b), it was slightly induced in the resistant line from 28 to 116 hr after infection with a maximum occurring about 76 hr postinfection. This induction appears real because its level was significantly above the level observed in mock-infected controls.

When the resistant bean line was challenged with race 9, the early onset of mRNA accumulation (PAL, CHT, and Hyp 4.1) appears correlated with the fungal penetration that occurred within an average period of 28–36 hr (O'Connell *et al.* 1985). In contrast, during compatible interactions with both race 1 and race 9, the PAL and CHT genes were activated only after a substantial fungal growth.

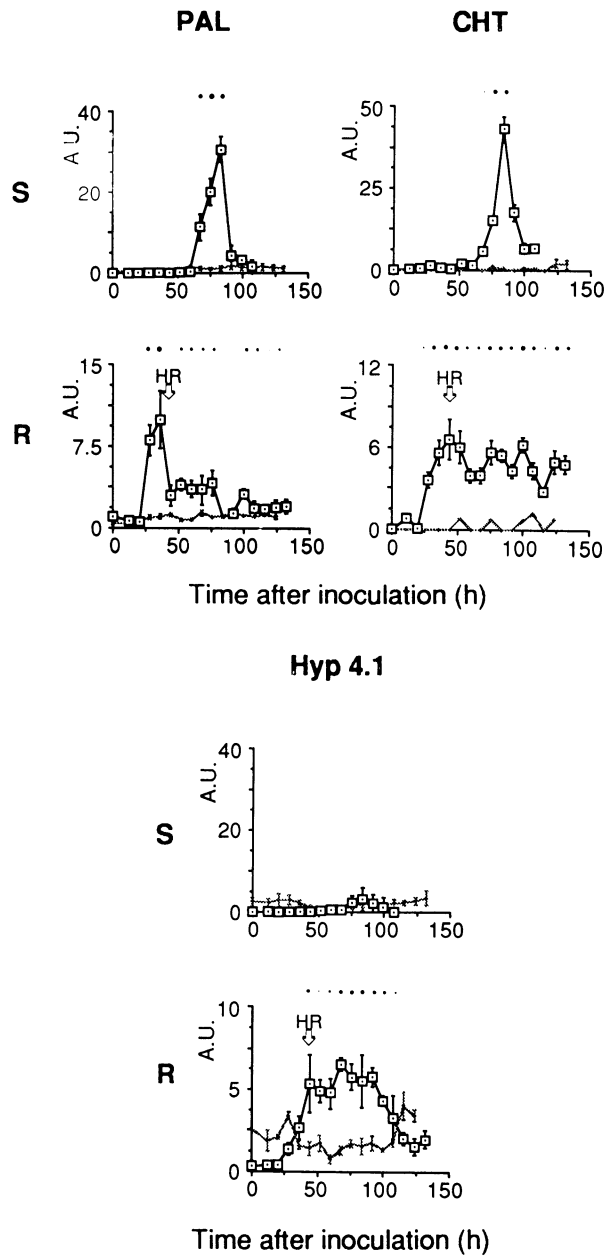

Fig. 3. Time course of induction of PAL, CHT, and Hyp 4.1 mRNAs during compatible (S) and incompatible (R) interactions of bean with race 9 of *Colletotrichum lindemuthianum*. The legends are the same as in Figure 2.

Races 1 and 9 are expected to carry the same avirulence gene because both are avirulent on bean genotypes carrying the ARE resistance gene. However, their genomes may be quite distinct. The observation of the compatible interactions (Mahé *et al.* 1992b) has already suggested differences in the genes controlling basic compatibility in these two races. Nevertheless, it cannot be excluded that a cluster of R genes exist at the ARE locus. In such a case, P12 R/race 9 and P12 R/race 1 would represent different gene for gene or gene for genes interactions.

The comparison of compatible (Mahé *et al.* 1992b) and incompatible interactions (present results) involving the near-isogenic lines P12 S and P12 R and the races 1 and 9 show that: 1) with race 1, the maximum levels of PAL and CHT mRNAs were observed at nearly the same time in compatible and incompatible interactions; and, 2) with race 9, the maximum levels of these mRNAs were observed 40 hr earlier in the resistant line than in the susceptible one. This result is in agreement with the observations of Bell *et al.* (1986) and Hedrick *et al.* (1988), although these authors reported a timing difference of about 70 hr.

In soybean roots infected by *Phytophtora megasperma* Drech. f. sp. *glycinea* T. Kuan & D.C. Erwin, Habereder *et al.* (1989) stated that PAL and CHS mRNAs accumulate earlier in incompatible than in compatible interaction, whereas Dhawale *et al.* (1989) did not observe a temporal difference in the CHS mRNA induction. These two groups used the same two races of the fungus, but the soybean cultivar they infected was different.

Within all these published experiments, the use of separate races to infect one cultivar makes a direct comparison of compatible and incompatible interactions difficult in terms of race-cultivar specificity, even if the two races used differ in the avirulence. Thus, the difference of 70 hr observed for defense mRNAs accumulation between compatible and incompatible interactions by Bell *et al.* (1986) and Hedrick *et al.* (1988), might be the result of a race-specific resistance superimposed on the differential aggressiveness of the two races. As a comparison, a difference of 32 hr in the timing of PAL and CHT mRNAs accumulation was observed when P12 S was challenged with race 9 or with race 1 (Mahé *et al.* 1992b). When P12 S/race 9 and P12 R/race 9 interactions were compared, a difference of 40 hr was observed in the timing of accumulation of these mRNAs. This difference can be ascribed to race-specific resistance because the difference between the two interactions compared, is most probably related to the presence of the ARE gene in the P12 R line. Bean P12 S and P12 R lines are very closely related genetically since a RFLP (restriction fragment length polymorphism) analysis (using 234 probe × enzyme combinations) and a RAPD (random amplified DNA) analysis (using 31 decanucleotides as primers) failed to reveal any polymorphism.

The study of Hyp 4.1 mRNA accumulation showed that the increases in the amount of this transcript, were almost identical in compatible and incompatible interactions involving race 1 and thus did not correlate with race-specific resistance. Similar results were obtained by Davidson *et al.* (1987) in barley. In contrast, Showalter *et al.* (1985) reported on an early marked increase in HRGP

mRNA in an incompatible interaction using bean hypocotyls and *C. lindemuthianum*; this accumulation was delayed in a compatible interaction. After infection with race 9, HRGP mRNA was not detected during compatible interaction (Mahé *et al.* 1992b), and it was only very slightly induced during incompatible interaction. There is a family of HRGP genes in the bean genome, and we do not know if the results we observed are specific to the Hyp 4.1 gene. Corbin *et al.* (1987) related earlier accumulation of Hyp 4.1 mRNA in the incompatible interaction, while Sauer *et al.* (1990) did not find any difference for Hyp 2.13 mRNA accumulation between compatible and incompatible interactions.

The accumulation kinetics of defense gene transcripts could be different in the presence or the absence of the ARE resistance gene. This strongly suggests the involvement of different signal transduction pathways in bean, in response to infection by *C. lindemuthianum*. The early PAL and CHT mRNAs accumulations observed in incompatible interactions with both races 1 and 9 support previous data obtained by Bell *et al.* (1986). This is not the case for the later accumulation of these mRNAs observed in the incompatible interaction with race 1 and for the absence of Hyp 4.1 transcript accumulation observed in the compatible interaction with race 9. Altogether, these data demonstrate that multiple specific and unspecific signals differentially regulate defense responses during bean-*C. lindemuthianum* interactions. These signals may be of fungal or plant origin or a combination of both. A survey of published data suggests that, depending on the biological system investigated (pathogen race, cultivar, tissue), the induction of a given defense gene might respond to specific or to unspecific signals (Dhawale *et al.* 1989; Corbin *et al.* 1987; Bell *et al.* 1986; Habereder *et al.* 1989; Dong *et al.* 1991; Fritzemeier *et al.* 1987).

In conclusion, the response of plant cells to pathogen is likely to be a highly coordinated network of events involving exchange and recognition of signals that lead to local and long-range activation of genes. The most specific responses leading to fungal arrest remain to be identified.

MATERIALS AND METHODS

Plant and fungal material.

Near-isogenic lines of *Phaseolus vulgaris* L., P12 R and P12 S, differing for the ARE resistance gene, have been developed by H. Bannerot and G. Fouilloux (INRA, Versailles, France). These lines result from 12 backcrosses and 14 subsequent selfings. The plants were grown under controlled conditions and leaf inoculations were as described previously (Mahé *et al.* 1992a). Control plants were mock-inoculated with water. Races 1 and 9 of *C. lindemuthianum* were provided by F. Legrand and J. Tailler (Université Paris XI, Orsay, France). They are both avirulent on bean cultivars harboring the ARE gene and correspond, respectively, to the α and γ races described by A. Charrier and H. Bannerot (1970).

Cytological procedures.

Leaves from P12 R plants were harvested every 8 hr after inoculation with races 1 or 9 of *C. lindemuthianum*. Immediately, four fragments (1 cm^2) were cut off from each leaf and treated for cytological observations as described (Mahé *et al* 1992a). Leaf pieces from four individual plants were surveyed for each sampling time.

RNA isolation and dot blot analysis.

Four leaves, each from a different plant, were collected, pooled, and frozen every 8 hr after inoculation, until 124 and 132 hr for race 1- and race 9-infected leaves, respectively. Total RNA was isolated from control and inoculated leaves by phenol-sodium dodecyl sulfate-chloroform extraction (Mahé *et al.* 1992a). RNA dot blot (with 0.5 or 1 μg of RNA) hybridizations and quantification of the transcript levels were achieved as described in Mahé *et al.* (1992b). The RNA hybridization experiments were performed independently for race 1- and race 9-infected leaves. This means that the autoradiograms signal strengths are not directly comparable because of a difference in the specific activity of the probe and in the exposure time lengths. The bean pPAL5, pCHT, and pHyp4.1 cDNA clones have been previously described by Edwards *et al.* (1985), Hedrick *et al.* (1988) and Corbin *et al.* (1987) respectively, and were kindly provided by C. J. Lamb (Salk Institute, La Jolla, CA). The EF-1α probe (pCHA0041), used as a constitutive marker of plant RNA, has been described by Axelos *et al.* (1989) and was obtained from B. Lescure (Université Paul Sabatier, Toulouse, France).

ACKNOWLEDGMENTS

We thank J. Tailler (Cryptogamie, Orsay, France) for fungal infection facilities; R. J. O'Connell and J. A. Bailey (Long Ashton Research Station, Bristol, UK) for training in cytological techniques; S. Brown (ISV, Gif-sur-Yvette, France) for the use of the Reichart Polyvar microscope; C. Thompson (Institut Pasteur, Paris) and the Kontron company for the use of the image analyzer.

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