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Fungal- and Plant-Specific Gene Markers to Follow the Bean Anthracnose Infection Process and Normalize a Bean Chitinase mRNA Induction

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A bean-specific translation elongation factor (EF-1α) and the *Aspergillus nidulans* actin gene were used to discriminate between bean (*Phaseolus vulgaris*) and *Colletotrichum lindemuthianum* RNA contents during a compatible anthracnose interaction. EF-1α is constitutively expressed in cotyledonary bean leaves during mock infection. EF-1α expression remains stable during the biotrophic phase of the infection process, but it drops rapidly during the necrotrophic phase at the time when the fungus actin transcript appears and accumulates. The EF-1α gene expression was also used to normalize the induction kinetics of a bean basic chitinase. The results show that the adjustment modified the induction quantitatively by 2.5-fold and shifted the maximal expression by 8 hr when compared to the uncorrected curve. The consequences of these results are discussed in terms of infection processes.

Additional keyword: expression normalization.

Plants elaborate a number of inducible defense responses after microbial attack, including the synthesis of phytoalexins, the reinforcement of cell walls by deposition of lignin, callose, hydroxyproline-rich glycoproteins, and the production of lytic enzymes such as chitinase and β-1,3-glucanase. Many of these responses involve the transcriptional activation of the corresponding defense genes; however, the kinetics are different when compatible and incompatible interactions are compared (Dixon *et al.* 1986; Collinge and Slusarenko 1987; Lamb *et al.* 1989; Dixon and Harrison 1990; Dixon and Lamb 1990). To study such host defense gene mRNA induction in a compatible interaction, it is necessary to consider the increasing amount of the infectious fungal material within the infected plant tissues. Consequently, for a given amount of total RNA isolated from infected plant tissues, the host/pathogen RNA ratio is expected to decrease during the process of infection. Thus, genes with a constitutive expression would be very useful to assess induction of plant defense or parasite pathogenicity genes.

To address this question, we investigated induction of plant gene expression in a compatible interaction between the bean *Phaseolus vulgaris* L. and the hemibiotrophic fungus *Colletotrichum lindemuthianum* (Sacc. & Magnus) Lams.-Scrib. In this report, bean translation elongation factor (EF-1α) and *Aspergillus nidulans* (Eidam) G. Wint. actin probes were used as specific markers for the plant and the fungus, respectively.

EF-1α is an essential element of protein synthesis. It catalyzes the binding of aminoacyl-tRNA to the A site of the ribosome in a reaction that requires guanosine triphosphate. It has been reported that cell viability requires the presence of at least one active EF-1α gene (Cottrelle *et al.* 1985a). An EF-1α gene has been cloned and sequenced from several species, including the yeast *Saccharomyces cerevisae* Hansen (Cottrelle *et al.* 1985b), the fungus *Mucor racemosus* Fresen. (Linz *et al.* 1986), tomato (Pokalsky *et al.* 1989), *Arabidopsis thaliana* (L.) Heynh. (Axelos *et al.* 1989), *Drosophila melanogaster* Meig. (Hovemann *et al.* 1988), and mice and humans (Brands *et al.* 1986; Rao and Slobin 1986). A high homology has been found among all the EF-1α genes (Pokalsky *et al.* 1989), but this homology is greater between plants than between plants and fungi. Comparisons of bean with *Arabidopsis* and of tomato with *Saccharomyces* deduced protein sequences revealed a similarity of 94% and 82%, respectively. The expression of plant EF-1α genes has been shown to be constitutive in a mature organ, whereas it increases in young tissues and decreases in senescent tissues (Pokalsky *et al.* 1989; Ursin *et al.* 1991).

Actin is an element of the cytoskeletal structure; it is involved in cellular motility, intracellular transport, and chromosome movement during mitosis and meiosis. Actin is an ubiquitous protein in eukaryotes and is highly conserved during evolution. Comparison of plant and fungal actin genes revealed nucleotide replacement substitution values of 17–18% (Hightower and Meagher 1986). So far, in all organisms examined, with the exception of two fungi, *S. cerevisae* (Ng and Abelson 1980) and *A. nidulans* (Fidel *et al.* 1988), actin has been found to be encoded by a multigene family. The actin genes are differentially expressed in organs and regulated during development (Hightower and Meagher 1986). For example, during the first few hours of the life cycle of *D. discoideum*, actin gene expression is induced threefold over the basal level found in vegetative cells, and the expression decays during fruiting body formation. The actin gene is constitutively expressed in vegetative cells and accounts for 1% of total poly(A)$^+$ transcripts (McKeown and Firtel 1981).

When investigating the induction of plant defense genes during infection, it is important to take into account the changes in the plant and fungal RNA ratios. As a model, we followed the induction of chitinase mRNA in the

compatible interaction between bean and the fungus *C. lindemuthianum*. Chitinase mRNA accumulation and enzymatic activity are induced in response to microbial infection (Hedrick *et al.* 1988; Voisey and Slusarenko 1989), hormonal treatment, and development (Shinshi *et al.* 1987; Broglie *et al.* 1986; Mauch *et al.* 1988a). Chitinase has been reported to degrade fungal walls leading to fungal growth inhibition *in vitro* (Schlumbaum *et al.* 1986; Mauch *et al.* 1988b). Chitinases may also release fungal wall fragments that are thought to be elicitors of plant stress secondary metabolites (Keen and Yoshikawa 1983). Chitinases exist as acidic and basic isoforms. Two acidic and two basic chitinases have been identified in tobacco (Legrand *et al.* 1987), whereas one basic and two acidic chitinases have been reported in bean (Awade *et al.* 1989). The acidic forms are secreted into the apoplast, whereas the basic forms accumulate intracellularly at a site supposed to be the vacuole (Bowles 1990).

MATERIALS AND METHODS

Plant and fungal material. Seeds of *P. vulgaris* '$P_{12}S$' were provided by H. Bannerot and G. Fouilloux (INRA, Versailles, France). Seedlings were grown in a greenhouse for 12 days in trays of vermiculite at 23° C with a relative humidity of 70% and a 12-hr photoperiod (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

C. lindemuthianum race 1 was supplied by F. Legrand and J. Tailler (laboratoire Cryptogamie, Orsay, France). *C. lindemuthianum* race 1 corresponds to an α race previously described by Charrier and Bannerot (1970). Mycelium was grown on malt (20 g/L) agar (20 g/L) plates at 19° C in darkness. Conidia were obtained from 12-day-old cultures by gentle scraping with a spatula, suspended in sterile water, and filtered through a sintered glass filter (20–40 μm). This suspension was used for plant inoculation. Liquid cultures were carried out in "Roux's flasks." Fifty milliliters of culture medium (potato-dextrose agar medium, 1 g/L of yeast extract, 1 g/L of casein hydrolysate) was inoculated with 2–5 $\times$ 10^5 conidia and incubated 3 days at 19° C in darkness. The mycelium was harvested by filtration, frozen in liquid nitrogen, and conserved at −80° C.

DNA probes. The bean cDNA clones coding for chitinase (pCHT; Hedrick *et al.* 1988) and EF-1α (pCHA0041; Axelos *et al.* 1989) and the *A. nidulans* genomic DNA clone coding for actin (pSF5; Fidel *et al.* 1988) were, respectively, gifts from C. J. Lamb (Salk Institute, San Diego, USA), B. Lescure (Université Paul Sabatier, Toulouse, France), and N. R. Morris (University of Medicine and Dentistry of New Jersey, Piscataway, USA). The 0.65-kb *Eco*RI-*Hin*dIII insert fragment of the pCHT clone, the 1.1-kb *Eco*RI-*Bam*HI insert fragment of the EF-1α clone, and the 0.5-kb *Sal*I insert fragment of the actin clone were used as probes and labeled by primer extension (Maniatis *et al.* 1989).

Leaf inoculation. Twelve-day-old plants were inoculated by spraying a suspension of conidia (10^7 spores per milliliter) on the lower surface of the cotyledonary leaves. The mock-infected plants were sprayed with sterile water. The infected and mock-infected plants were placed in an infection chamber at 20° C. The plants were grown in a confined saturated humidity atmosphere, with 10-hr dark and 14-hr light photoperiods, under fluorescent bulbs (Philips PL 24W183, 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Eight leaves were collected every 8 hr from the inoculation time (0 hr) up to 132 hr after inoculation. Four leaves were immediately frozen in liquid nitrogen and conserved at −80° C until they were used for RNA extraction; the four other leaves were immediately processed for cytological observations.

Cytology. Pieces of fresh leaves were cut into 1-cm^2 fragments, placed in specimen tubes, and immersed in 0.15% (w/v) trichloroacetic acid in a 3:1 mixture of ethanol and chloroform, according to a procedure used by R. J. O'Connell (personal communication). The tubes were placed in an oven at 70° C until the tissues became colorless. The ethanol/chloroform solution was then removed and replaced with lactophenol (British Drug House, Dorset, England). The samples were observed with Nomarski interference contrast optics (Reichart Polyvar microscope; Liecap, Austria).

RNA blot hybridization. Total cellular RNA was isolated as previously described by Haffner *et al.* (1978), except that 0.1% sodium dodecyl sulfate (SDS) was added in the extraction buffer, and 0.1% hydroxyquinoline was added to the phenol. The RNA concentration was measured spectrophotometrically.

RNA samples (10 μg) were fractionated by electrophoresis on a denaturing formaldehyde 1.5% agarose gel, according to the method described by Maniatis *et al.* (1989). RNA gels were then vacuum-blotted onto a nylon membrane (Hybond N^+, Amersham Corp., Arlington Heights, IL) in 2$\times$ SSC (1$\times$ SSC = 150 mM NaCl, 15 mM sodium citrate) (Vacugene Pharmacia LKB, Broma, Sweden) at 55 cm of H_2O pressure for 1 hr and 30 min and fixed for 5 min under UV light (Germicide tubes TUV 6, Philips). The prehybridization was performed in 2$\times$ SSC and 5$\times$ Denhardt's solution at 42° C for 5 hr. Hybridization was carried out at 42° C for 16–18 hr in 0.5% SDS, 5$\times$ Denhardt's solution, 50% formamide, and 3$\times$ SSC for the actin probe and in 4$\times$ SSC and 100 μg/ml of salmon sperm DNA for the EF-1α and pCHT probes. After hybridization, the filters were washed twice with 0.1$\times$ SSC and 0.5% SDS at room temperature for 30 min and twice with 0.1$\times$ SSC and 0.1% SDS at 46° C for 30 min. The filters were exposed to X-ray Curix Agfa Gevaert film (Agfa Gevaert, Belgium) or X OMAT-AR Kodak film (Eastman Kodak, Rochester, NY) depending on the intensity of the expected signal. The quantification of the transcript levels was achieved by densitometric scanning of the autoradiograms (Densitometer CD 60; DESAGA, Heidelberg, Germany). To remove the probe and reuse the RNA blots, the filters were washed two times by immersing in boiling 0.01$\times$ SSC and 0.5% SDS for 5 min and by shaking at room temperature for 30 min.

RESULTS

EF-1α mRNA level in bean leaves infected by *C. lindemuthianum*. To use EF-1α mRNA as a constitutive plant RNA marker in infected bean leaves, two conditions have to be fulfilled. First, EF-1α needs to be constitutively expressed in cotyledonary leaves under the infection growth

conditions. Second, the EF-1α probe must hybridize specifically to plant RNA.

The constitutive expression of EF-1α was investigated by northern analysis of total cellular RNA isolated from leaves of the mock-infected bean plant. Hybridization with the EF-1α probe (Fig. 1A) detected a transcript of 1.8 kb at each timepoint of the mock-infection kinetics. The length of the EF-1α transcript corresponds to the estimated size from sequence data of a complete bean cDNA clone (M. Axelos and B. Lescure, personal communication). Despite small variations between timepoints, the intensity of the hybridization signals appeared quite constant during the mock-infection kinetics (Fig 1A). Thus, the EF-1α expression can be considered constitutive in cotyledonary leaves.

The specificity of the EF-1α probe towards bean RNA was established by hybridization of the probe to total cellular RNA extracted from healthy bean leaves or from mycelium of *C. lindemuthianum* race 1 grown in liquid medium. Under high stringency conditions, the EF-1α transcripts were detectable only in plant RNAs and not in the fungal RNA (Fig. 1B). The absence of signal with the EF-1α DNA probe on fungal RNA is probably attributable to a low degree of homology between bean and fungal EF-1α sequences; the bean EF-1α probe does not hybridize with fungal DNA under the conditions used (data not shown).

The expression of EF-1α was examined in a compatible interaction between the susceptible bean cultivar $P_{12}S$ and *C. lindemuthianum* race 1 at various times during the course of infection. As shown in Figure 2, the level of the EF-1α mRNA appeared to be quite stable until 108 hr after infection, after which the amount of this RNA decreased significantly (116–132 hr). The lower amount of EF-1α transcript observed at 0 hr and at 76 hr appeared to be fortuitous, because this was not reproducible in different experiments. Such was not the case for the three last samples of the infection kinetics, which always presented a signal three times lower than the average of earlier points. For a constant load of total RNA to be maintained for each timepoint, the decrease of the EF-1α mRNA may be attributed to a decrease of the overall bean RNA transcripts; this decrease was correlated to an increase of fungal RNA or to a down regulation of the EF-1α gene expression during the infection process.

Fungal actin mRNA levels in bean leaves infected by *C. lindemuthianum*. To check whether the threefold decrease of EF-1α mRNA level during the late stages of the infection was due to the appearance of fungal RNA, we used an *A. nidulans* actin probe. First, the specificity of the fungal probe towards *C. lindemuthianum* RNA was assessed. As shown in Figure 3A, a 1.65-kb transcript was detected in *C. lindemuthianum* RNA but not in bean RNA. Although the size of the *C. lindemuthianum* actin mRNA appears to be smaller than that of *A. nidulans* (2.4–2.6 kb; Fidel *et al.* 1988), it is sufficient to code for the 377 amino acids of the actin protein. In fact, the actin mRNA size varies in different organisms and ranges from 1.5 to 1.7 kb in petunia (Baird and Meagher 1987), soybean (Shah *et al.* 1982), and rice (McElroy *et al.* 1990), and is even

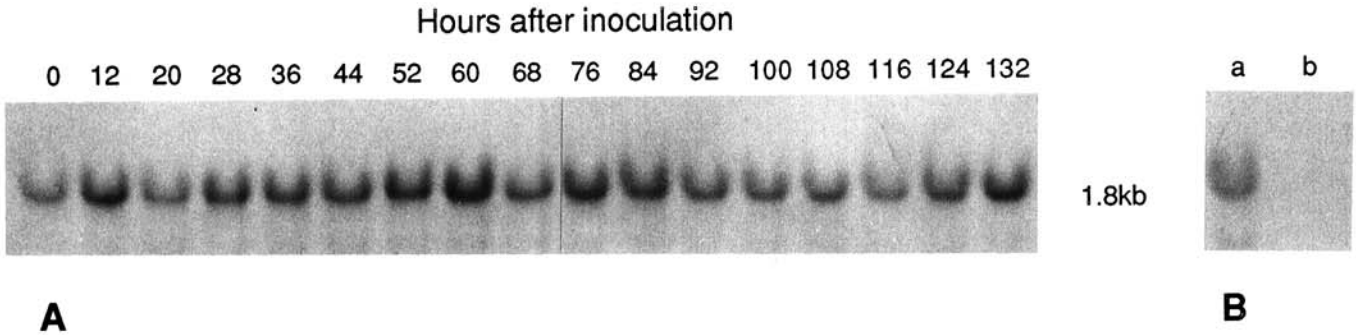

Fig. 1. Northern blot analysis of translation elongation factor (EF-1α) mRNA. Total cellular RNA (10 μg per lane) was fractionated by electrophoresis in denaturing agarose gels, blotted onto nylon filters, and hybridized to a ^{32}P-labeled bean EF-1α DNA probe. **A,** RNA isolated at the indicated times after mock infection of *Phaseolus vulgaris* '$P_{12}S$' cotyledonary leaves. **B,** RNA from healthy bean leaves (lane a) and *Colletotrichum lindemuthianum* race 1 grown in liquid culture (lane b).

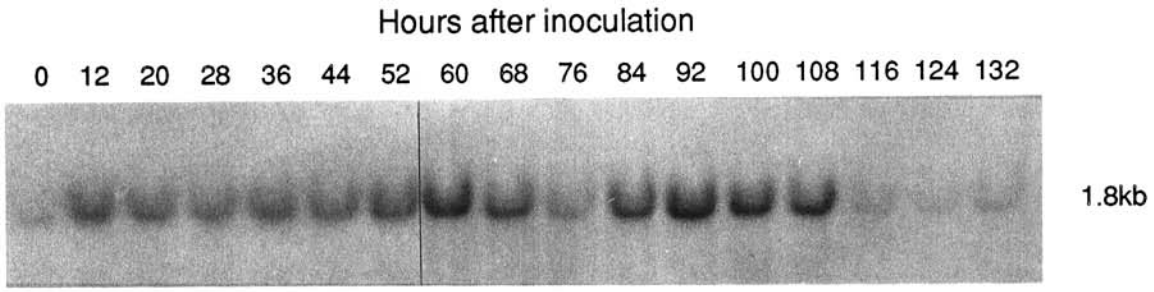

Fig. 2. Time course of translation elongation factor (EF-1α) mRNA accumulation in *Phaseolus vulgaris* '$P_{12}S$' cotyledonary leaves infected by the virulent *Colletotrichum lindemuthianum* race 1. Total cellular RNA (10 μg per lane) was fractionated by electrophoresis in denaturing agarose gels, blotted onto nylon filters, and hybridized to a ^{32}P-labeled bean EF-1α DNA probe.

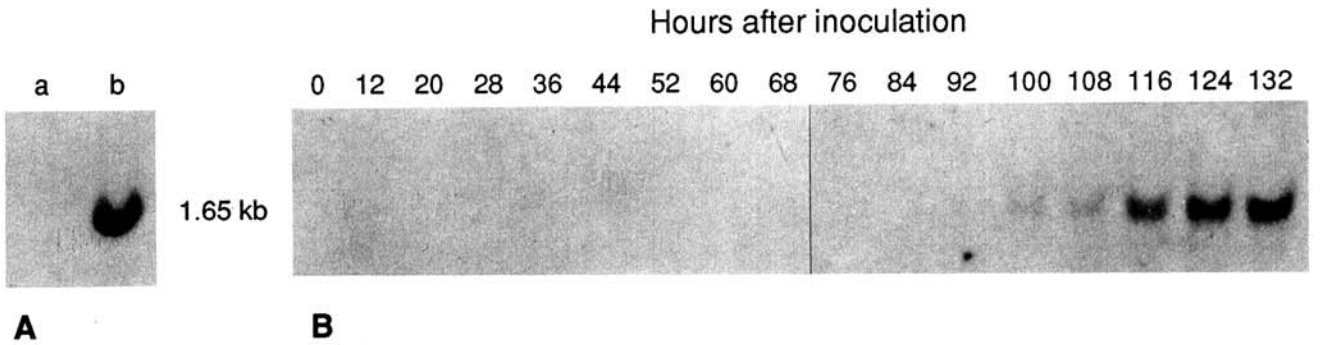

Fig. 3. Northern blot analysis of fungal actin mRNA. **A,** RNA from healthy bean leaves (lane a) and *Colletotrichum lindemuthianum* race 1 grown in liquid culture (lane b). **B,** RNA isolated at the indicated times after inoculation of *Phaseolus vulgaris* '$P_{12}S$' cotyledonary leaves by the virulent *C. lindemuthianum* race 1. The same blots that were previously used for translation elongation factor (EF-1α) mRNA detection (Figs. 1B, 2) were hybridized to a ^{32}P-labeled *Aspergillus nidulans* actin DNA probe.

smaller in *S. cerevisae* (Gallwitz and Seidel 1980) and *D. discoideum* (McKeown and Firtel 1981).

The lack of a detectable signal with bean RNA (Fig. 3A) is probably the result of a low homology between the *A. nidulans* and bean actin sequences. This was confirmed by Southern hybridization experiments carried out under high stringency conditions in which no signal appeared after hybridization of fungal actin probe to bean DNA (data not shown).

Thus, in analyzing the amount of fungal mRNA in infected leaves, the same northern blots, which had been previously used for the EF-1α mRNA analysis, were hybridized with the *A. nidulans* actin probe. As shown in Figure 3B, no fungal actin mRNA could be detected within the first 100 hr after inoculation. Detection of actin mRNA began at 100–108 hr and rapidly increased up to 132 hr after inoculation, when total leaf necrosis is complete. The comparison between bean EF-1α and *C. lindemuthianum* actin mRNA levels in the latest stages of infection makes obvious that the decrease of EF-1α mRNA matches the increase of actin mRNA (Fig. 4).

Based on the analysis of a constant amount of RNA isolated from infected leaves, a decrease in the amount of a constitutively produced plant mRNA in the latest stages of infection when fungal mRNA amount increases was expected. Thus, because the amount of EF-1α mRNA decreases when fungal mRNA appears, it behaves as a plant constitutive mRNA.

Cytological analysis of fungal development during the compatible interaction between bean and *C. lindemuthianum*. The infection process of *C. lindemuthianum* in hypocotyls of French bean has been extensively studied by O'Connell *et al.* (1985). In the present work, the timing of the infection process was examined to correlate our molecular data with the fungus development during the compatible interaction between the susceptible bean cultivar $P_{12}S$ and *C. lindemuthianum* race 1. The results are summarized in Figure 5 with a description of the visual symptoms observed on the leaves. After spore inoculation and for approximately 30 hr, conidia and appressoria were observed on the surface of the leaves (5a). Intracellular primary hyphae emerging from infection vesicles were detected about 60 hr after inoculation (5b). Extensive development

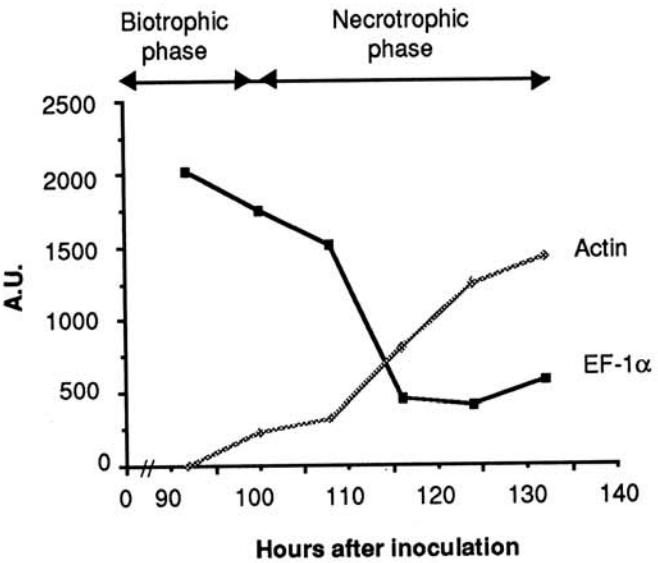

Fig. 4. Changes in amounts of bean translation elongation factor (EF-1α) mRNA and of fungal actin mRNA in *Phaseolus vulgaris* '$P_{12}S$' cotyledonary leaves infected by the virulent *Colletotrichum lindemuthianum* race 1 during the infection course (92–132 hr). The values of A.U. (arbitrary unit) correspond to the integration of each hybridization signal on the autoradiograms (Figs. 2,3) scanned by densitometry (CD 60 DESAGA).

of primary mycelium was established within 100 hr after inoculation (5c). Simultaneously, narrow secondary hyphae branching out of the primary hyphae were visible in brown-colored cells (5d). By 116 hr after inoculation, secondary hyphae were well-developed in the host cells (5e).

Observations of leaves during the fungal development showed that dark brown limited lesions appeared mainly on the veins 92 hr after inoculation (5α) and then propagated. The veins had a brownish color 108 hr after inoculation (5β). Water-soaked lesions and macerated host tissues were observed 132 hr after inoculation (5δ).

A correlation exists between the development of *C. lindemuthianum* within bean tissues and the accumulation of the fungal actin mRNA (Figs. 3,5). Fungal actin mRNA was undetectable during the 92 hr after inoculation and then appeared and accumulated rapidly during the last

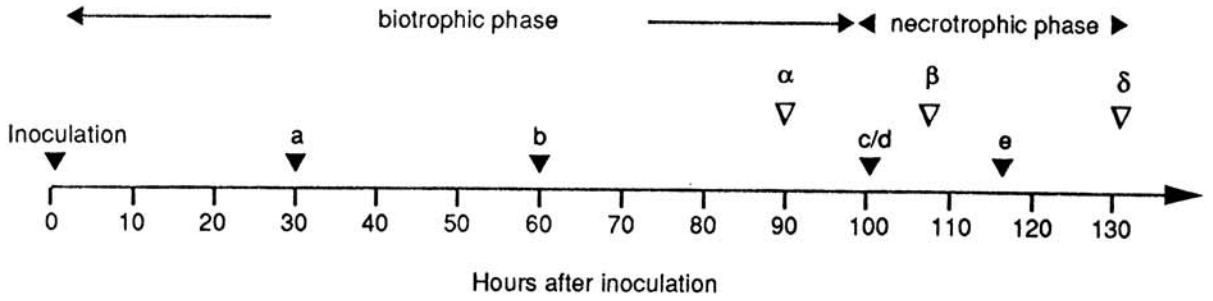

Fig. 5. Timing of the fungal development and appearance of visual symptoms on cotyledonary leaves during the compatible interaction between bean cv. $P_{12}S$ and *Colletotrichum lindemuthianum* race 1. ▼ = cytological observations: a = appressorium; b = primary hyphae emerging from infection vesicles; c = extensive development of primary hyphae; d = appearance of secondary hyphae; e = secondary hyphae well-developed. △ = visual symptoms: α = limited lesions on veins; β = brownish color of the veins; δ = water-soaked lesions and macerated host tissues.

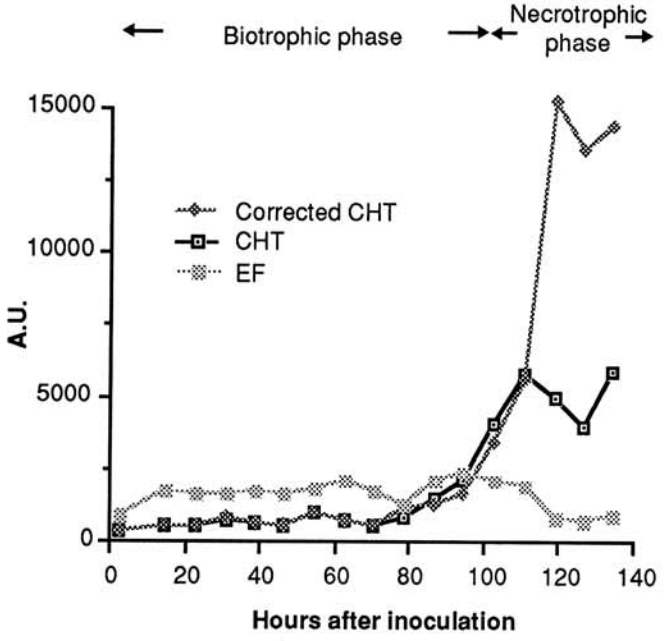

Fig. 6. Kinetics of apparent (-□-), corrected (-◈-), chitinase (CHT) and elongation factor (EF-1α) (···▒···) mRNA accumulation in *Phaseolus vulgaris* '$P_{12}S$' cotyledonary leaves infected by the virulent *Colletotrichum lindemuthianum* race 1. A.U. (arbitrary unit) is as defined in the legend of Figure 4. The corrected CHT curve was obtained by normalizing the values at each point of the apparent CHT curve to a constant value for the amount of EF-1α mRNA.

hours of infection, until complete leaf necrosis occurred. Appearance of actin mRNA corresponded quite well to the differentiation of secondary hyphae (Figs. 3,5).

O'Connell *et al.* (1985) have shown that the fungal growth is biotrophic from conidia germination until primary hyphae establishment and is necrotrophic when secondary hyphae emerge (Fig. 5).

All the data suggest that the decreased level of EF-1α transcripts observed in the latest stages of infection mainly reflects a reduction of the contribution of plant RNA to total RNA because of the increase in fungal RNA. Thus, the amount of plant RNA can be estimated by quantifying EF-1α transcript level in infected bean leaves.

Apparent and corrected quantification of chitinase mRNA induction during the bean $P_{12}S$ and *C. lindemuthianum* race 1 compatible interaction. Changes in chitinase mRNA levels in bean leaves infected by the virulent race 1 of the pathogen were studied by northern blot hybridization. The quantification of mRNA transcripts is presented in Figure 6. The chitinase mRNA induction started 84 hr after inoculation and rapidly increased to reach a maximum level at 108 hr (17-fold over the basal level). This level was maintained until the end of the infection kinetics.

EF-1α mRNA level has been shown to reflect quite well the amount of plant RNA in total RNA isolated from infected leaves. Thus, EF-1α mRNA has been used for the normalization of chitinase mRNA induction to a constant amount of plant RNA. This was achieved for each timepoint of the infection kinetics. Because the plant RNA ratio was shown to be lower in the three last timepoints of the infection kinetics (cf. EF-1α curve in Fig. 6), the maximum level of chitinase mRNA accounted for an increase of 48-fold instead of 17-fold above the basal level (Fig. 6). This important change in the magnitude of chitinase mRNA induction modifies slightly the time at which chitinase transcript accumulation was observed. After adjustment, the maximum of induction was reached at 116 hr instead of 108 hr after inoculation.

DISCUSSION

The aim of this work was to identify a constitutive plant RNA marker and thus estimate the true value of plant RNA in a mixture of plant and fungal RNA resulting from fungal development in plants and monitor the expression of inducible plant defense genes during the infection process.

EF-1α expression can be considered as a specific constitutive plant RNA marker; its level remains almost unchanged in mock-infected plants. In bean plants infected by the virulent *C. lindeminthianum* race 1, when a constant amount of total RNA was analyzed, a substantial decrease of EF-1α mRNA level was observed in the latest stages of the infection. Such a result is expected during a compatible interaction. However, we cannot rule out a differential regulation of EF-1α transcription during the course of the infection process. Exogenous factors such as hormones have been shown to induce changes in the expression pattern of EF-1α genes (Pokalsky *et al.* 1989; Ursin

et al. 1991). Nevertheless, a decline of the EF-1α mRNA as a result of a differential regulation of the EF-1α gene transcription would only be superimposed on the dilution effect of plant RNA by fungal transcripts.

The changes in EF-1α and actin transcript levels were exactly opposite and matched the transition between the biotrophic and the necrotrophic phases of fungal infection. The increase in fungal actin mRNA revealed the complete invasion of the plant tissues by the fungus at the end of infection. After penetration in bean leaves, the fungus completed a life cycle. Thus, it is unlikely that the overall *C. lindemuthianum* actin mRNA level remains stable during this period. Changes in actin gene expression have been reported in different species such as soybean (Hightower and Meagher 1985), rice (McElroy *et al.* 1990), petunia (Baird and Meagher 1987), *D. melanogaster* (Fyrberg *et al.* 1983), and *D. discoideum* (McKeown and Firtel 1981). Nevertheless, fungal actin mRNA level may be used to follow fungal mycelium development within the plant tissue.

EF-1α transcript has been proposed as a convenient internal and specific bean RNA marker during the course of infection. Its quantification was used to normalize bean chitinase mRNA induction kinetics to a constant amount of plant RNA during a compatible interaction between bean and *C. lindemuthianum*.

The chitinase gene used in this work encodes a basic protein, which is known to represent the major chitinase activity during bean AMV (alfalfa mosaic virus) infection or $HgCl_2$ treatment (Awade *et al.* 1989). Activation of the chitinase gene during infection by *C. lindemuthianum* has already been described (Hedrick *et al.* 1988). A similar chitinase induction was observed in our system; however, the time at which maximal induction occured was earlier (108 hr) when compared to the results of Hedrick *et al.* (1988) (170 hr). This discrepancy may be attributed to different genetic material sources (cultivar Kievietsboon Koeckoek and *C. lindemuthianum* race β used by Hedrick *et al.* [1988] versus cultivar $P_{12}S$ and *C. lindemuthianum* race 1 in our case) and different experimental inoculation procedures (inoculation of excised hypocotyls versus leaves harvested after inoculation of plants).

The normalization of the chitinase gene induction by using bean EF-1α as an internal constitutive marker of plant RNA has two effects. First, it shows that the induction of the transcript is much more important than it directly appears from northern blot analysis (48-fold instead of 17-fold above the basal level). No correction needs to be performed during the biotrophic phase (0–100 hr after inoculation), because no change was detected in the level of EF-1α mRNA. Second, the maximum of the chitinase mRNA induction occurs at 116 hr instead of 108 hr after inoculation.

As far as we are aware, this is the first report in which induction of a defense gene is normalized to a constant amount of plant RNA. Usually accumulation of specific transcripts was corrected to a constant RNA quantity by estimating from ethidium bromide staining (Voisey and Slusarenko 1989) or from quantification of a transcript that did not appear to be affected by the infection process (Dong *et al.* 1991). In these cases, normalization leads to the adjustment to a constant RNA level present in the infected tissues. But, in a compatible interaction, total RNA extracted from infected organs is composed of a mixture of plant and pathogen RNA. Thus, such normalization may underestimate the magnitude of the induction of the plant defense gene at the moment of the pathogen development and possibly may alter the time at which the plant defense gene induction is observed.

In conclusion, studies of plant defense gene expression in infected tissues need to account for the extensive pathogen development in the later stages of compatible interactions. Nevertheless, *in situ* analysis will be required to fully understand the significance of plant defense gene induction.

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