European Journal of Plant Pathology **104:** 207–220, 1998.

Fungal and plant gene expression during synchronized infection of tomato leaves by *Botrytis cinerea*

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Accepted 5 December 1997

Key words: actin, fungal biomass, grey mould, necrosis, PR proteins, β-tubulin

Abstract

An inoculation procedure was developed to obtain efficient and synchronous infection on detached tomato leaves by *Botrytis cinerea*. In spray-inoculated leaves incubated at 20 °C, the infection process consisted of three phases: the formation of primary necrotic lesions (until 20 hpi), a quiescent phase (20-72 hpi), and the expansion of a proportion of the primary lesions (from 72 hpi onwards), resulting in full tissue maceration. At 4 °C, the infection progressed slowly but steadily without inducing necrotic responses in the host. The actin and β-tubulin genes of *B. cinerea* were cloned, characterized and used as probes on blots containing RNAs from leaves at various stages of the infection. The genes displayed a similar expression pattern throughout the infection and the hybridization signal reflected the amount of fungal biomass. The actin mRNA accumulated to higher levels than the β-tubulin mRNA. Tomato PR protein mRNAs (chitinase, β-1,3-glucanase and PR-1) were induced during the infection, albeit with different kinetics and to different levels. At 20 °C, β-1,3-glucanase and PR-1 mRNAs were induced more rapidly than chitinase mRNAs. At 4 °C, mRNAs encoding extracellular β-1,3-glucanase and intracellular, as well as extracellular chitinase were hardly induced.

Abbreviations: AOS – active oxygen species; bp – basepairs; hpi – hours post inoculation; kbp – kilobasepairs; nt – nucleotides; PR – pathogenesis-related.

Introduction

Botrytis cinerea is a ubiquitous plant pathogenic fungus with a very wide host range (Jarvis, 1977). Due to their destructive effects on many important crops, *Botrytis* diseases have attracted great efforts in different research disciplines over the last century, aimed at the development of effective control strategies. Histological studies performed on various host plants indicate that *B. cinerea* utilizes different infection mechanisms. Conidial germ tubes can penetrate through wounds or natural openings, and they are also capable of directly penetrating and killing intact, healthy plant tissues, which are subsequently invaded by mycelium spreading from previously colonized, dead plant tissues (Jarvis, 1977). Different types of disease symptoms have been described, such as softening and rot in grapes (McClellan and Hewitt, 1973) or tomato (Verhoeff, 1970), as well as localized necrotic spots on gerbera flowers (Salinas and Verhoeff, 1995) or French bean leaves (Van den Heuvel, 1981). When localized necrotic spots are formed, only a proportion of these develop into spreading lesions, and this may depend on a number of factors (Van den Heuvel, 1981). Presumably, the induction of necrosis and the accompanying defence response in the host effectively restrict fungal growth from primary lesions for a certain period of time. In this respect, the physiology of the host plays an important role in the interaction between *B. cinerea* and its hosts (Elad and Evensen, 1995). Salinas and Verhoeff (1995) reported that the incubation of gerbera flowers, inoculated with *B. cinerea*, at 4 °C resulted in spreading lesions instead of the restricted lesions that are obtained upon incubation at room tem-

perature. Stress imposed on the host at low temperature might abolish or reduce defence responses in favour of *B. cinerea*, which is able to grow in culture and colonize plant tissues at temperatures just above 0 °C. A well characterized defence response that coincides with necrosis involves the local and systemic accumulation of *de novo* synthesized pathogenesis-related (PR) proteins. Some of these have been identified as chitinases and β-1,3 glucanases, proteins with antifungal and/or antibacterial activity (Linthorst, 1991). In tomato, both intracellular (type I) and extracellular (type II) chitinases and β-1,3 glucanases accumulate upon infection by *Cladosporium fulvum* (Joosten and de Wit, 1989; van Kan et al., 1992; Danhash et al., 1993). In addition, two extracellular PR-1 proteins accumulate (Joosten et al., 1990; van Kan et al., 1992) which possess inhibiting activity towards the oomycete pathogen *Phytophthora infestans* (Niderman et al., 1995).

Our aim is to unravel the sequence of events during the infection of plants by *B. cinerea* at the gene expression level. In this context, molecular genetic approaches are undertaken to isolate and characterize fungal genes involved in pathogenicity. The rationale of this strategy is the assumption that fungal genes, that play an important role in pathogenicity, are expressed preferentially during growth of the fungus in the host. Therefore, we have initiated a detailed comparison of the mRNA expression pattern of the fungus *in planta* with its mRNA expression pattern during growth *in vitro* (Benito et al., 1996). To perform such an expression analysis, several tools need to be established. First, a standard inoculation procedure under laboratory conditions is required, that reproducibly results in efficient and synchronous infection. To achieve this, high germination rates and a high rate of infection of host cells by germinated conidia are required. As the infection progresses, the fungal biomass within the host tissue increases. Consequently, the proportion of fungal RNA, present in the total RNA extracted from infected plant tissue ('interaction RNA'), increases with time. In order to estimate the infection progress and quantify fungal gene expression *in planta* in relation to the fungal biomass, genes with a constitutive expression pattern have been used as internal standards (Mahe et al., 1992; Pieterse et al., 1993; Van den Ackerveken et al., 1993). For this purpose, we chose to test the usefulness of genes encoding actin and β-tubulin, highly conserved proteins which are ubiquitously expressed in all eukaryotic cells. They constitute microfilaments and microtubules, respectively, involved in the determination of the cell shape and in cellular and subcellular movements. Actin genes occur in a single copy in the genome of most fungi (Ng and Abelson, 1980; Fidel et al., 1988; Dudler, 1990), whereas β-tubulin is encoded by one gene in *Saccharomyces cerevisiae* (Neff et al., 1983) and *Neurospora crassa* (Orbach et al., 1986) and by two genes in *Aspergillus nidulans* (May et al., 1987) and *Colletotrichum gloeosporioides* (Buhr and Dickman, 1993; Buhr and Dickman, 1994). The *A. nidulans tubC* gene and the *C. gloeosporioides tub1* gene are differentially expressed during conidiation, whereas the *A. nidulans BenA* gene and the *C. gloeosporioides tub2* gene are considered as housekeeping genes (May et al., 1985; Buhr and Dickman, 1993; Buhr and Dickman, 1994).

In this paper we describe inoculation conditions used to obtain high efficiency and synchronicity of infection of *B. cinerea* on detached tomato leaves, both at low and room temperatures. Fungal colonization was followed visually and microscopically over time and the amount of fungal biomass was quantified by RNA blot hybridization, using *B. cinerea* actin and β-tubulin genes as probes. Furthermore, the expression levels of tomato PR protein mRNAs during the infection at different temperatures were monitored over time.

Materials and methods

Inoculations

Botrytis cinerea strain SAS56 was cultured as described by Van der Vlugt-Bergmans et al. (1997a). Conidia were harvested from sporulating plates by washing with 5 ml of sterile water, containing 0.05% Tween 80. The conidia were filtered through glasswool, washed three times by centrifugation (8 min, 800 rpm, 114 x g) and resuspending in sterile water, and finally resuspended in inoculum buffer as specified in the text.

Tomato plants (cultivar Moneymaker genotype *Cf4*) were grown in soil in the greenhouse for approximately 7 weeks. Leaves were cut from the plant with a scalpel knife and inserted with their stems in a block of water-saturated florist's foam. The block was placed in a petridish in a plastic box (30 × 45 cm) with a grid on the bottom, such that the leaf was spread out over the grid. Wet filter paper was placed beneath the grid to obtain high humidity. Leaflets were inoculated with conidial suspensions on the upper side either by pipetting individual droplets or by spraying using a

DeVilbiss atomizer. The leaves were dried at room temperature for 30 min. The box was closed with a plastic transparent cover in order to obtain high humidity, and incubated either at 20 °C with a 16 h photoperiod or at 4 °C in the dark.

Initial experiments performed to determine the effect of inoculum composition on infection efficiency were carried out by applying twenty to fourty 5-μl droplets (10^5 conidia ml^{-1}) of each suspension on one half of a leaflet. On the other half of the leaflet, sterile water was applied as control. Two leaflets were used per type of suspension in each inoculation. Each solution was tested in three independent experiments. The percentage of germination on the leaf surface was determined by counting 100 conidia in five different lesions at 16 or 20 hpi.

The standard inoculation procedure used for the gene expression studies was as follows: conidia were harvested, washed and resuspended at a density of 10^6 ml^{-1} in Gamborg's B5 medium (Duchefa BV, Haarlem, the Netherlands), supplemented with 10 mM sucrose and adjusted to 10 mM potassium phosphate (pH 6). The suspension was preincubated without shaking for 2–3 h at room temperature and inoculated by spraying droplets ($\pm$1-2 μl) onto detached tomato leaves until saturation. Leaves were dried and incubated as described above.

Light microscopy of inoculated tissues

Epidermis strips from inoculated tomato leaves were prepared at different time points, destained in acetic acid:ethanol:chloroform (1:6:3, v/v/v) for one hour, dipped into 0.2% cotton-blue in lacto-phenol for 3 min, washed in ethanol and examined using a Leitz microscope.

Isolation of the B. cinerea tubA *and* actA *genes*

A genomic λEMBL3 library of *B. cinerea* strain SAS56 was screened and positive phages were purified and characterized as described by Van der Vlugt-Bergmans et al. (1997a). The probes used were derived from the *tub2* gene from *N. crassa* (Orbach et al., 1986) and the *actA* gene from *C. fulvum* (van den Ackerveken, unpubl.). DNA manipulations and cloning experiments were performed according to standard procedures (Sambrook et al., 1989).

RNA blot analysis

RNA isolation, electrophoresis and RNA blot hybridization were performed as described by Van der Vlugt-Bergmans et al. (1997a). RNA concentrations were determined spectrophotometrically and 20 μg of total RNA was loaded in each lane. For hybridization, the following DNA fragments were radioactively labeled by random priming in the presence of (α-^{32}P) dATP: a 730 bp *Eco*RI-*Hin*dIII fragment containing most of the fifth exon of the *B. cinerea actA* gene, a 480 bp *Kpn*I-*Bgl*II fragment containing part of the sixth exon of the *B. cinerea tubA* gene, a 1.7 kbp *Eco*RI fragment of the radish 18S rDNA gene (Grellet et al., 1989) and the entire *Eco*RI- *Xho*I inserts of the cDNA clones GLUA, GLUB, P6 (Van Kan et al., 1992), CHI3 and CHI9 (Danhash et al., 1993). The specific activity of the probes was routinely 7–9 $\times$ 10^5 dpm per ng of DNA. Hybridizations were carried out in 10 ml of hybridization solution containing 1.5 -2 $\times$ 10^6 dpm per ml. The blots were washed at 65 °C in 0.2 $\times$ SSC/ 0.1% SDS and exposed to Kodak X-OMAT AR films. The intensities (I) of the signals obtained on the autoradiograms, shown in Figure 3 and in Figure 4A, were quantified using an LKB Ultroscan XL densitometer. The proportion of fungal RNA in the interaction RNA at a given timepoint after inoculation was calculated as follows: I_{actin} (interaction)/I_{actin} (*B.c. in vitro*) multiplied by I_{rDNA} (*B.c. in vitro*)/I_{rDNA} (interaction) x 100%. This proportion was calculated for each timepoint and displayed in a graph in relation to the time after inoculation (Figure 4A, lower part).

Results

Sugar and phosphate are required for successful infection

In laboratory inoculation systems, it has previously been reported that sugars and phosphate are essential components of the inoculum for infection to occur on intact plants (Harper et al., 1981; van den Heuvel, 1981). The effect of sugars and phosphate on the infection of detached tomato leaves by *B. cinerea* was tested in droplet inoculation experiments. Minimal inoculum doses of 500 conidia in 5μl droplets, in solutions containing sucrose and potassium phosphate, were required to induce the formation of spreading lesions on tomato leaves. No necrotic lesions were obtained at all when either sucrose or potassium phosphate alone

were present in the inoculum buffer. Sucrose, mannose and glucose stimulated infection in the presence of phosphate, while inositol did not. The minimal concentrations that yielded reproducible results were 10 mM potassium phosphate and 10 mM of either of the sugars. Concentrations higher than 10 mM did not have a significant additional stimulatory effect. Therefore 10 mM was adopted as standard concentration, in order to limit nutritional effects of excessive supplements.

The first symptoms were observed at 18 h after inoculation (hpi) as necrotic spots of the size of the droplet applied. The lesions started to expand only from 72 hpi onwards. Microscopic observations of epidermal peelings prepared 20 hpi showed that the lesions consist of areas of collapsed epidermal cells. A high proportion of conidia (40%) had not germinated on the leaf surface and the germ tubes in the necrotic areas significantly varied in length. When conidial suspensions were prepared in Gamborg's B5 medium supplemented with sucrose and potassium phosphate (10 mM each) and preincubated at room temperature for 2–3 h prior to inoculation, equally high infection efficiencies were achieved. In this medium, and only upon the preincubation, conidia germinated within a narrow timespan (4–5 hpi) and the percentage of germinated conidia on the leaf surface at 20 hpi increased to 90%. Moreover, the size of the germ tubes was rather uniform (5–8 times the diameter of the conidium). This method of inoculum preparation was used as standard for further experiments.

Standard inoculation procedure

The amount of fungal biomass is the limiting factor for the analysis of fungal gene expression *in planta*, particularly during early stages of infection. A useful inoculation procedure should provide conditions determining the formation of as many lesions as possible. Therefore a foliar spraying inoculation method was used. Conidial suspensions were prepared as described above and sprayed onto detached tomato leaves. Upon incubation at 20 °C the first symptoms are visible 18–20 hpi as small, brownish spots appearing all over the surface of the leaf. During the following 48 h neither the number nor the size of the lesions increased, but the lesions became darker and growth of the fungus in the lesions was arrested. Unlike the case in the single droplet inoculations, not all primary lesions expanded. At 72 hpi, a fraction of the lesions (1-10%) simultaneously started to spread. From these few spreading lesions the fungus was able to colonize the entire leaf. Viable mycelium could be recovered from non-spreading necrotic lesions transferred to water agar in all cases. At 120–144 hpi, total leaf necrosis was observed and the fungus sporulated vigorously on the surface of the macerated tissue. The three distinct phases in the infection process were observed in all subsequent experiments performed as described here. The percentage of lesions that eventually expanded varied from one experiment to the other but never exceeded 20% of the primary lesions. The appearance of primary necrotic lesions and the initiation of lesion expansion occurred at highly similar timepoints in every experiment.

The early stages of the infection were followed microscopically in epidermal peelings. At 16 hpi, before symptom appearance, penetration of epidermal cells by germ tubes was observed and death of the penetrated host cells was manifested by their brownish cell content. Microscopic observations of epidermal peelings taken at 20 hpi showed that the dark brown spots represent necrotic lesions in which a group of collapsed epidermal cells is in contact with *B. cinerea* germ tubes.

B. cinerea *infection progresses differently at low temperature*

Salinas and Verhoeff (1995) observed that low temperature incubation of inoculated gerbera flowers resulted in expanding, soft-rotting lesions instead of restricted, necrotic lesions. We set out to investigate whether similar differences could be observed in inoculated tomato leaves incubated at low temperature. Since tomato is rather sensitive to cold stress, it was expected that the plant tissue would serve as a rather inert, albeit living, substrate. Individual droplets of standard conidial suspensions were inoculated onto detached tomato leaves, which were incubated at 4 °C in darkness for up to 144 hpi. The conidial germination rate on agar plates at 4 °C was $>75\%$. At 20 hpi small water-soaked translucent lesions of the size of the inoculum droplet were observed. No host defence responses, such as the accumulation of dark-colored phenolic compounds, were observed. From each primary lesion, the fungus grew slowly but steadily into the neighbouring tissue. At 120 hpi at 4 °C the lesions had reached a diameter of approximately 7 mm. Each lesion on a particular leaf grew at equal rate. Differences in lesion growth were observed on different leaves and among experiments. Especially the apical leaflet of composite tomato leaves was relatively resistant to infection.

Foliar spray inoculation and subsequent incubation at 4 °C yielded similar symptoms as the single droplet inoculations although the number of lesions was much higher. Unlike the primary lesions developing after spray inoculation at 20 °C, all primary lesions obtained at 4 °C expanded into a soft-rotting lesion and each lesion on a particular leaf grew with approximately equal speed. Due to the large number of primary lesions that expanded, the leaf material degenerated severely around 144 hpi.

The structure of fungal genes used for quantitative estimations of infection progress

Assuming that the proportion of fungal RNA in the total RNA extracted from infected tissues ('interaction RNA') reflects the proportion of fungal biomass at any given moment during an interaction, its determination allows an estimation of fungal ingress and, therefore, provides the basis for normalization of fungal gene expression *in planta*. The proportion of fungal RNA in the interaction RNA can be determined by quantifying the hybridization intensity of constitutively expressed fungal genes and comparing it with the hybridization signal of RNA from *B. cinerea* cultured *in vitro*.

The *B. cinerea tubA* gene encoding β-tubulin and the *actA* gene encoding actin were isolated by heterologous hybridization as described in Materials and methods. Figure 1 shows the sequence of the *B. cinerea tubA* gene and its flanking sequences. Partial sequences of the coding region of the *tubA* gene have been published previously (Yarden and Katan, 1993; Luck and Gillings, 1995). The general structure of the gene is identical to that of the *N. crassa tub2* gene and the *C. gloeosporioides tub2* gene. It comprises a 1341 nt coding region, consisting of seven exons separated by six introns located at identical positions in the three genes. Southern blot analysis using two different restriction enzymes, under moderate stringency hybridization conditions, indicated that the *B. cinerea tubA* gene occurs in a single copy in the genome of SAS56 and nine other *B. cinerea* isolates (not shown). The protein shows more than 85% identity to other fungal β-tubulins. The *B. cinerea* β-tubulin resembles more closely the proteins encoded by the *A. nidulans BenA* gene (May et al., 1987) and the *C. gloeosporioides tub2* gene (Buhr and Dickman, 1994) than those encoded by the *A. nidulans tubC* (May et al., 1987) and the *C. gloeosporioides tub1* (Buhr and Dickman, 1993) genes.

The sequence of the *B. cinerea actA* gene is shown in Figure 2. The gene consists of six exons separated by five introns, and contains a coding region of 1125 nt encoding a protein of 375 amino acids, which shows 99% identity to the *A. nidulans* γ-actin (Fidel et al., 1988). The positions of the five introns of the *B. cinerea actA* gene exactly coincide with the positions of the five introns in the coding region of the *A. nidulans* γ-actin gene. There is a sixth intron located in the 3′ untranslated region of the *A. nidulans* γ-actin gene (Fidel et al., 1988), which is probably also present in the 3′ untranslated region of the *B. cinerea actA* gene since intron processing sequences are found at equivalent positions. As the *B. cinerea tub*A gene, the *actA* gene occurs in a single copy in the genome of strain SAS56, as demonstrated by Southern blot analysis (data not shown).

Estimation of fungal infection progress by RNA blot hybridization

To test whether probes derived from these genes discriminate between plant and fungal β-tubulins and actins, and whether expression of the two fungal genes is detectable at early time points during the interaction, total RNA was extracted from mycelium of *B. cinerea* grown *in vitro*, from healthy tomato leaves and from *B. cinerea*- infected tomato leaves harvested 16 hpi. The upper panel of Figure 3 shows an ethidium bromide-stained gel containing 20μg of each RNA sample and demonstrates that approximately equal amounts of total RNA were loaded in each lane. The gel was blotted and hybridized successively with a *tubA* probe and an *actA* probe. Equivalent exposures of this blot with both probes showed that there is no cross-hybridization with β-tubulin or actin mRNAs from healthy tomato leaves under the conditions used (Figure 3, panels *tubA* and *actA*). Single mRNA species of the predicted sizes (1.7 kb for *tubA* and 1.5 kb for *actA*) were detected in the RNA sample extracted from *B. cinerea* mycelium grown *in vitro*. The hybridization intensity with the *actA* probe was about 4-5 times higher than with the *tubA* probe. The high hybridization intensity of the *actA* mRNA allows easy detection at early stages of the interaction. Longer exposure times were required to detect expression of the *tubA* gene *in planta* at these time points (not shown). The same blot was probed with an 18S rDNA gene from radish (Grellet et al., 1989). This probe hybridized to the same extent with fungal and tomato 18S rRNA (Figure 3, lower panel, lanes B.c. and L.e.). Therefore the hybridization

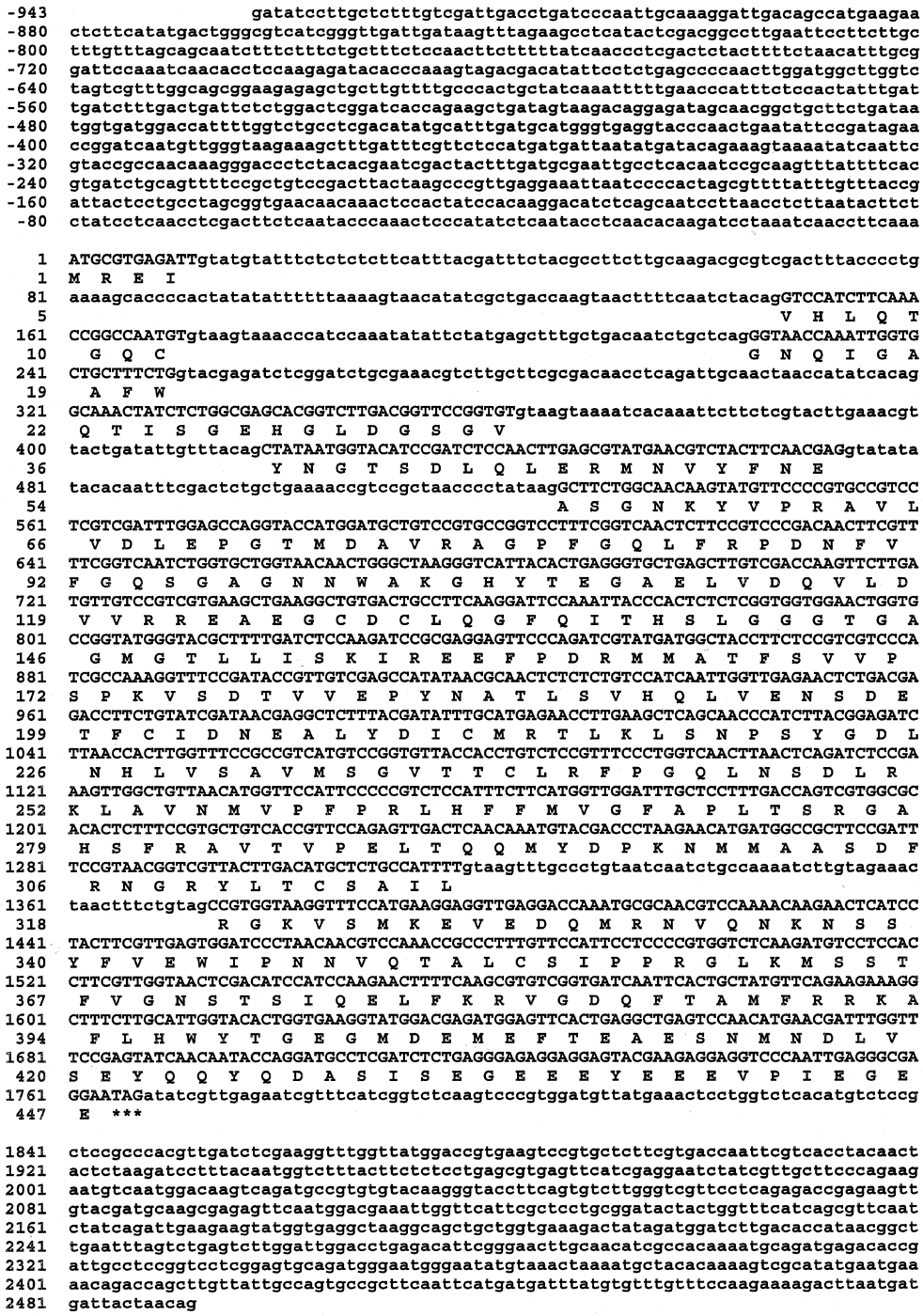

Figure 1. Sequence of the *B. cinerea* β-tubulin (*tubA*) gene. The nt sequence is numbered from the ATG initiation codon. Non-coding sequences are indicated in lower case letter. The sequence of the deduced encoded protein is shown below the nt sequence of exons 1 to 7 (in capitals) and is numbered from the first methionine residue. GenBank accession number Z69263.

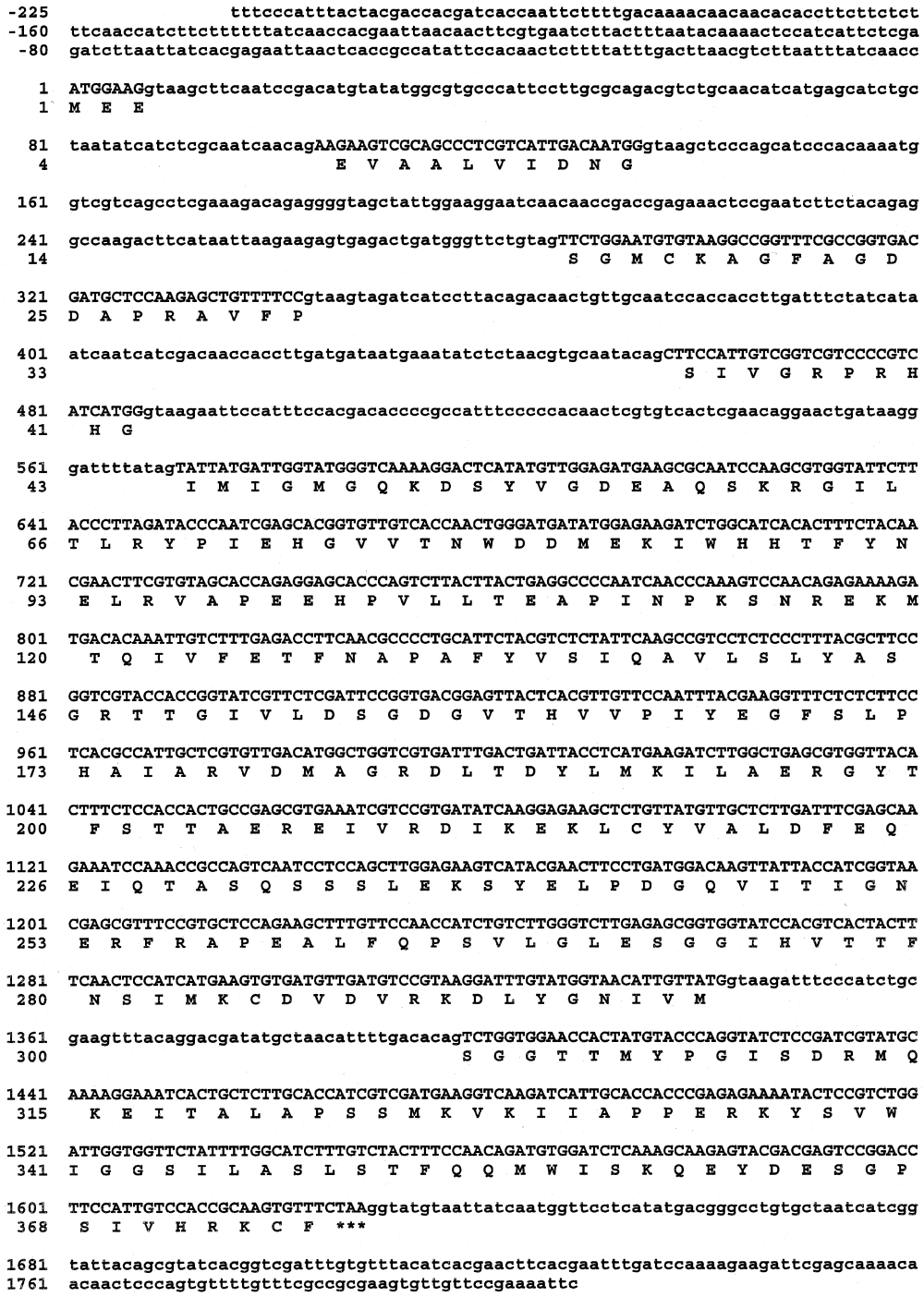

Figure 2. Sequence of the *B. cinerea* actin (*actA*) gene. The nt sequence is numbered from the ATG initiation codon. Non-coding sequences are indicated in lower case letter. The sequence of the deduced encoded protein is shown below the nt sequence of exons 1 to 6 (in capitals) and is numbered from the first methionine residue. GenBank accession number AJ000335.

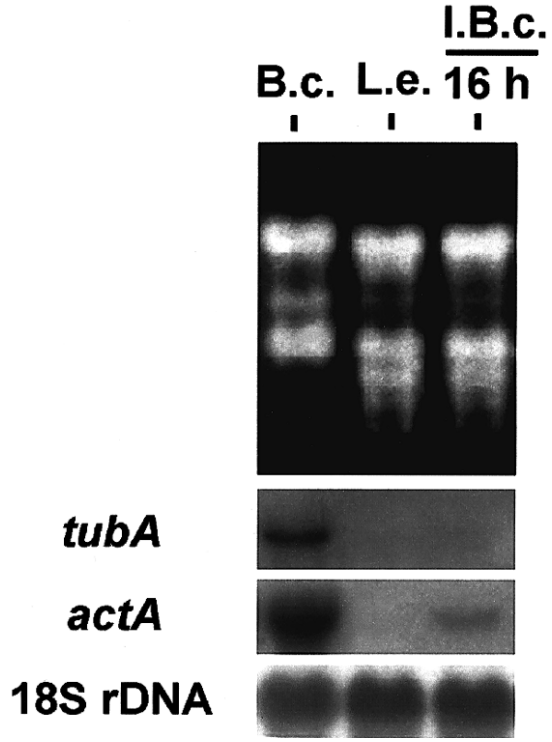

Figure 3. Detection of the *B. cinerea* β-tubulin and actin mRNAs at early stages of the infection process.
Upper panel: ethidium-bromide stained RNA gel containing 20μg of total RNA extracted from *B. cinerea* grown in liquid culture (lane B.c.), from healthy tomato leaves (lane L.e.) and from tomato leaves inoculated with *B. cinerea* collected 16 hpi (lane I.B.c. 16h). Middle and lower panels: autoradiograms of the corresponding RNA blot hybridized with probes derived from the *B. cinerea tubA* gene (*tubA*), the *B. cinerea actA* gene (*actA*) and the radish 18S rDNA gene (18S rDNA). After hybridization, the blots were washed and exposed for 16 h (*tubA* and *actA*) or 5 h (18S rDNA).

intensity of 18S rRNA in interaction samples (e.g. lane I.B.c. 16h) reflect the total sum of plant and fungal RNA loaded in each lane. In consequence, differences in the intensity of this band in different lanes was used to correct for differences in loading between lanes (see below).

The expression *in planta* of the *B. cinerea actA* gene was analyzed in a time course experiment. Detached tomato leaves were inoculated according to standard procedures, incubated at 20 °C or at 4 °C, and harvested at different time points after inoculation. Total RNA was extracted and electrophoresed on a denaturing agarose gel in parallel with RNA extracted from mycelium of *B. cinerea* grown *in vitro* and from healthy tomato leaves. A blot containing equal amounts of total RNA from each sample was hybridized with an *actA* probe and an 18S rDNA probe (Figure 4A, upper panel). The *B. cinerea actA* mRNA is detectable at 16 hpi, remains at a similar level until 72 hpi, and the intensity rapidly increases at 96 and 120 hpi. Hybridization of the same blot with the radish 18S rDNA gene confirmed equal RNA loading.

The intensities of the hybridization signals obtained with the *actA* probe were analyzed densitometrically. The graph in the lower part of Figure 4A represents the ratios between the hybridization intensity obtained at each time point and the intensity of the signal obtained with the RNA sample isolated from *B. cinerea* grown *in vitro*, corrected for equal loading by means of the 18S rDNA hybridization intensity. For a constitutively expressed gene, these ratios provide an estimation of the proportion of fungal RNA in the interaction RNA at each time point. At 20 °C, the ratio remains low and constant during early stages of the infection process, until 72 hpi. From this timepoint onwards there is a rapid increase of the ratio up to 120 hpi. The increase coincides with the infection phase in which colonization and extensive maceration of the plant tissue occurs. At 4 °C, the ratio increased more gradually during the infection process. The slight decrease between 72 hpi and 96 hpi in the experiment shown in Figure 4 was caused by sample-to-sample variation among inoculated leaves (not shown). At 120 hpi the ratio of fungal RNA / interaction RNA was about half of the value determined at the same timepoint at 20 °C (see graph in Figure 4A). Hybridization of the blot with the *tubA* probe yielded very similar results, but the exposure time for autoradiograms had to be increased as compared to the *actA* hybridizations (not shown).

PR protein mRNAs are differentially induced by B. cinerea *infection at different temperatures*

Blots identical to the one shown in Figure 4A were hybridized with tomato cDNA probes encoding intracellular (type I) and extracellular (type II) chitinases and β-1,3 glucanases, as well as extracellular PR-1. The results of the hybridizations are shown in Figure 4B. None of the five probes cross-hybridized to either of the others (not shown). None of the five PR protein mRNAs was detected in uninoculated tomato leaves. At 20 °C all mRNAs were induced at 16 hpi, before any symptoms became apparent, but the hybridization intensities were different. PR-1 mRNA showed a very high, nearly constant, level of expression until 120 hpi. Induction of both β-1,3 glucanase mRNAs was higher and faster than of the two chitinase

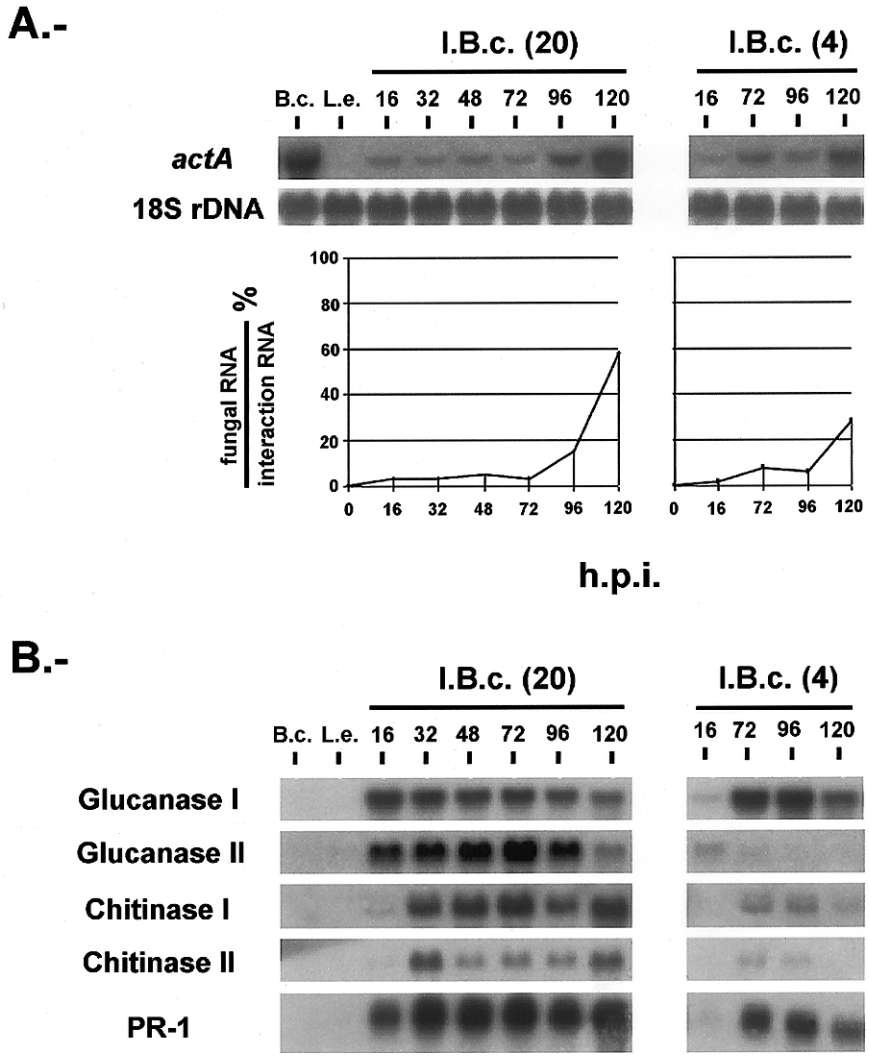

Figure 4. (A) Estimation of the infection progress of *B. cinerea* on tomato leaves. Autoradiograms of an RNA blot containing total RNA extracted from *B. cinerea* grown *in vitro* (B.c.), from healthy tomato leaves (L.e.) and from tomato leaves inoculated with *B. cinerea* collected at the indicated timepoints (hpi) after incubation at 20 °C (I.B.c. (20)) or at 4 °C (I.B.c. (4)). The blot was successively hybridized with a *B. cinerea actA* probe *(actA*) and a radish 18S rDNA probe (18S rDNA). The graphs below represent the proportion of fungal RNA in the interaction RNA at a given timepoint after inoculation, calculated as follows: I_{actin} (interaction)/I_{actin} (*B.c. in vitro*) multiplied by I_{rDNA} (*B.c. in vitro*)/I_{rDNA} (interaction) x 100%. This proportion was calculated for each timepoint and is displayed graphically in relation to the hpi.
(B) Analysis of PR protein mRNAs expression during the infection process. Autoradiograms of blots containing the same RNA samples as in panel 4A, hybridized with probes derived from the tomato intracellular type I β-1,3-glucanase clone GLUB (panel Glucanase I), the extracellular type II β-1,3-glucanase clone GLUA (panel Glucanase II), the intracellular type I chitinase clone CHI9 (panel Chitinase I), the extracellular type II chitinase clone CHI3 (panel Chitinase II) and the extracellular PR-1 clone P6 (panel PR-1). Exposure times were 16 h except for the 18S rDNA and PR-1 blots which were exposed 5 h.

mRNAs. The level of the type I β-1,3 glucanase mRNA was constantly high between 16 and 72 hpi and slowly declined later on. The level of the type II β-1,3 glucanase mRNA was comparable to the expression of the type I β-1,3-glucanase at 16 hpi but it increased slightly until 72 hpi and declined at later time points. Both chitinase mRNAs were transiently induced during the interaction but the kinetics of their accumulation were slightly different. For the type I chitinase, the mRNA level was maximal at 72 and 120 hpi while for the type II chitinase maximal mRNA levels were reached at 32 and 120 hpi.

At 4 °C, the mRNA expression patterns were clearly different (Figure 4B). Both β-1,3-glucanase mRNAs were weakly induced at 16 hpi, whereas induction of the two chitinases and the PR-1 mRNAs could not be observed. The type I β-1,3-glucanase mRNA accumulated strongly at later time points, from 72 hpi, whereas the type II β-1,3-glucanase mRNA declined. The type I and type II chitinase mRNAs were induced to relatively low levels at 4 °C as compared to their induction at 20 °C and the induction occurred rather late in the infection. As the type I β-1,3-glucanase mRNA, the PR-1 mRNA accumulated to high levels at 4 °C at later time points. When inoculated leaves were incubated at 4 °C for 72 h and subsequently transferred to 20 °C, the expression of all five PR proteins was strongly induced at 48 h upon transfer (not shown).

Discussion

In this paper we describe experimental conditions to obtain highly efficient and synchronized infections of detached tomato leaves with *B. cinerea*. Preincubation of conidia in an appropriate medium (Gamborg's B5 medium supplemented with sucrose and phosphate) prior to spray inoculation was important for obtaining uniform germ tube sizes and synchronous penetration of host tissue. In foliar spray inoculations incubated at 20 °C, the infection process develops in three distinct phases. The first phase involves adhesion, germination and penetration into epidermal cells and the occurrence of host cell death, leading to the formation of primary lesions within a short time lapse, between 18 and 20 hpi. During the second phase, between 20 and 72 hpi, growth of the fungus is restricted and no new primary lesion is formed. In the third phase, starting 72 hpi, a proportion of the primary lesions is able to grow out into spreading lesions. The three-phase infection process in tomato leaves strongly resembles the observations made in French bean leaves under similar experimental conditions (Van den Heuvel, 1981). However, no attempts were made to synchronize the infection in French bean leaves as we have described here. The similarities between the results obtained with bean and tomato leaves suggest that the synchronized infection method might be applicable to leaf tissue of a range of other host plants. On the contrary, at 4 °C all primary lesions developed into spreading lesions, slowly but steadily, as was also observed in gerbera flowers by Salinas and Verhoeff (1995). The successful host tissue colonization by *B. cinerea* at low temperatures might be a general phenomenon on a range of host plants, since *B. cinerea* is renowned for its destructive post-harvest effects, even under low temperature storage conditions. Post-harvest colonization by *B. cinerea* may result from the inability of the host to activate effective defense mechanisms at low temperatures.

The strict requirement of sugars and phosphates in the inoculum to stimulate *B. cinerea* infection was described earlier by several groups (Harper et al., 1981; van den Heuvel, 1981; Edlich et al., 1989). Nutrients in the inoculum may mimic the situation during natural infections starting via wounded or dead tissue. It has been proposed that phosphates stimulate the production of pectolytic enzymes (van den Heuvel and Waterreus, 1985; Leone, 1990) and that sugars provide energy for superficial growth and the formation of prepenetration structures (Akutsu et al., 1981; Clark and Lorbeer, 1976). Edlich et al. (1989), however, presented evidence that sugars were not required as nutrients, but rather as substrates for oxidation by fungal glucose and/or xylose oxydases. Such an oxidation generates H_2O_2, which can be converted to superoxide (O_2^-) and hydroxyl ($OH^\cdot$) radicals, active oxygen species (AOS) capable of destroying relatively inert material such as cutin and membrane lipids. H_2O_2 is also able to diffuse across cell membranes and exert toxic effects on neighbouring plant cells (Levine et al., 1994). Recently, Von Tiedemann (1997) detected both H_2O_2 and $OH^\cdot$ radicals during the infection of bean leaf disks by *B. cinerea*. The aggressiveness of the fungal isolate was related to the amount of AOS produced (Von Tiedemann, 1997). Such experiments do, however, not discriminate between AOS generated directly by extracellular fungal enzymes or by host tissue in response to the perception of fungal constituents such as sterols (Granado et al., 1995), chitin fragments (Felix et al., 1993), phytotoxins (Rebordinos et al., 1996) or other, as yet unidentified molecules. The involvement of AOS in the infection by *B. cinerea* is in agreement with the observed protective effect of antioxidants (Elad, 1992). The production of AOS at the host-pathogen interface may be responsible for induction of tomato catalase mRNA from the moment of necrotic lesion appearance onwards (Van der Vlugt-Bergmans et al., 1997b). The observation that, at the same time, the *B. cinerea* catalase mRNA is not induced *in planta* (Van der Vlugt-Bergmans et al., 1997b), suggests that the oxidative stress imposed by AOS is not experienced equally severely by both organisms.

After penetration and primary lesion induction at 20 °C, a relatively long quiescent period is observed during which no lesion expansion or fungal growth is observed. Quiescent infections have mainly been described for various fruits (reviewed by Williamson, 1994; Prusky, 1996). The succesful restriction of fungal growth has been ascribed to substances present prior to infection (e.g. the saponin tomatine (Verhoeff and Liem, 1975) or polygalacturonase inhibiting proteins (Johnston et al., 1994), or synthesized in response to pathogen invasion. It was demonstrated that the growth of *B. cinerea in planta* could be inhibited by the grapevine phytoalexin resveratrol, when overproduced in transgenic tobacco (Hain et al., 1993). In the lesions that developed on tomato leaves, the darkening of lesions during the quiescent phase (20–72 hpi) is indicative of the accumulation of *de novo* synthesized phenolic compounds, some of which presumably have fungistatic activity. The quiescent period may be required for the fungus to produce enzymes that either degrade growth inhibiting compounds (Pezet et al., 1991) or secrete these compounds from the mycelium by ABC transporter activity (De Waard, 1997). Plants also respond to fungal infection by producing PR proteins (Figure 4B), several of which have hydrolytic activity towards fungal cell wall components (Linthorst, 1991). Although tomato PR proteins possess antifungal activity (Joosten et al., 1995) it is questionable whether they contribute to (temporary) fungal growth restriction, since *B. cinerea* is insensitive to chitinases from various plants (Broekaert et al., 1988).

During the quiescent phase, *B. cinerea* is not killed within the lesion. It could be argued that only 1-5% of the primary lesions develops into a spreading lesion because the plant defense mechanism successfully kills the invading fungus in the other lesions. However, viable mycelium could be recovered from all primary lesions, including the non-spreading lesions. Moreover, if the pathogen were killed within the necrotic lesions, hyphae would degenerate and fungal mRNAs would become exposed to nucleases, resulting in a decreased transcript level of actin and β-tubulin. Since the *actA* mRNA level remained constant until the end of the quiescent phase (Figure 4A) without the development of new primary lesions, it can be concluded that the fungus is alive within the lesion and probably needs a signal to enter into the third, expansion phase. It remains to be determined whether such a signal is released from host tissue or whether the fungus needs time to degrade and/or excrete growth inhibiting compounds before being able to resume hyphal growth (Pezet et al., 1991; De Waard, 1997).

The infection of tomato leaves by *B. cinerea* induced all PR protein mRNAs analysed. Remarkably, the mRNA induction kinetics are different from those induced by the biotrophic pathogen *Cladosporium fulvum*. Both in compatible and incompatible interactions with *C. fulvum*, all intracellular, type I PR protein mRNAs display very similar induction kinetics, as do the extracellular, type II PR protein mRNAs. Among the two types, there is a distinct timing of induction (Van Kan et al., 1992; Danhash et al., 1993). In the tomato-*B. cinerea* interaction, the two β-1,3-glucanase mRNAs are induced at the same rate and to equally high levels at 20 °C but not at 4 °C. The slow and transient induction of the type I and type II chitinase mRNAs at 20 °C is particularly intriguing, since these mRNAs are the first to be induced during HR, trigerred by injection of race-specific elicitors of *C. fulvum* into appropriate tomato genotypes (Wubben et al., 1996). At both temperatures the mRNA encoding the extracellular protein PR-1 has induction kinetics most similar to the intracellular type I β-1,3-glucanase mRNA. The extracellular, type II chitinase and β-1,3-glucanase mRNAs were hardly induced at 4 °C and this coincides with the absence of host tissue necrosis. It will be very interesting to investigate why host cell death induced by the necrotrophic pathogen *B. cinerea* leads to clearly different PR protein induction kinetics than HR-associated host cell death induced by an avirulent race of a biotrophic pathogen, or its purified race-specific elicitors. Whether the generation of ethylene (Elad, 1990) or salicylic acid in response to the infection plays a role in the induction of the various PR protein mRNAs remains to be determined. Most tomato PR protein mRNAs investigated here are not induced by ethephon or only to low levels, and type I chitinase mRNA is not induced by salicylate (Van Kan et al., 1995).

As a measure for the progress of the infection, we used the hybridization intensity of the *B. cinerea* actin and β-tubulin mRNAs. Several parameters have been used for estimating the fungal biomass of a pathogenic fungus growing within its host: ergosterol (Padgett and Posey, 1994), chitin (Ekblad and Nasholm, 1996), the activity of reporter genes such as β-glucuronidase (GUS) (Couteaudier et al., 1993; Oliver et al., 1993), and the accumulation of constitutively expressed fungal mRNAs (Mahe et al., 1992; Pieterse et al., 1993; van den Ackerveken et al., 1993). Quantification of ergosterol and chitin in infected host tissue by chem-

ical detection methods provides the advantage that it discriminates between fungus and plant. It does, however, not discriminate between dead and living fungal hyphae. Moreover, the induction of chitinase activity in infected host tissue (Figure 4B) might lead to partial chitin hydrolysis and consequently, to underestimation of the fungal biomass. The quantification of GUS reporter gene activity as a parameter for *B. cinerea* biomass has been investigated. Histochemical staining for GUS activity was succesfully used in early stages of the infection of gerbera flowers and tomato fruits by *B. cinerea* (Van Kan et al., 1997), but it proved very irreproducible in necrotic tomato leaf tissue (Van 't Klooster and van Kan, unpubl.) for several reasons: first, it is difficult to infiltrate necrotic tissue with aqueous solutions containing the substrate for GUS; second, the structure of necrotic leaf tissue was disintegrated to a larger extent than the infected tomato fruit or gerbera flower tissue (Van Kan et al., 1997); third, the activity of GUS is irreversibly inhibited by the presence of oxidizing agents released during and after the collapse of host cells (Pineiro et al., 1994). Therefore we decided to determine the progress of fungal infection by measuring the proportion of fungal actin and β-tubulin mRNAs in a constant amount of total 'interaction RNA'. This proportion (Figure 4A) correlated well with the development of symptoms as they were visually observed over time. In previous work, the expression of the *tubA* gene was used as internal standard to estimate fungal infection progress (Benito et al., 1996; Van der Vlugt-Bergmans et al., 1997a and 1997b). Here we show that the quantification of the expression *in planta* of the *actA* gene is even more useful because of its higher hybridization signals. The expression profile in time course experiments was highly similar with both probes suggesting that indeed both genes are constitutively expressed throughout the fungal growth phases considered in this study.

The proportion of fungal RNA in the interaction RNA might not be entirely accurate as an absolute measure for the fungal biomass in infected host tissue for two reasons. First, the amount of RNA per cell or mass unit might differ among plant and fungus (by differing water content). Second, the efficiency of RNA extraction might differ among plant cells and fungal hyphae. On the other hand, the quantification of actin mRNA in each RNA sample corrects for sample-to-sample variation. It provides an excellent marker for the relative amount of living, metabolically active mycelium in the plant tissue. Hence it can be used as internal standard in quantifying the mRNA level of other fungal genes, in order to assess whether a particular gene is induced at a particular stage of the infection process (Benito et al., 1996). Our future interest lies in the characterization of changes in the gene expression pattern in *B. cinerea* during two stages of the infection process: at the penetration of the host cell (16 hpi) and at the onset of the formation of spreading lesions (60–70 hpi). We have developed tools for the analysis of fungal gene expression *in planta* by differential display RT-PCR (Benito et al., 1996), using the inoculation method described here. Furthermore, this bioassay can be used to determine the relative pathogenicity of *B. cinerea* gene disruption mutants in comparison with the wild type isolate (ten Have and van Kan, unpubl.).

Acknowledgements

EPB was recipient of an EC postdoctoral fellowship in the framework of the Human Capital and Mobility Programme (Contracts ERBCHBICT941179 and ERBFMBICT960969). AtH is supported by the Technology Foundation (STW). We are grateful to our colleagues Cécile van der Vlugt-Bergmans, Elly Jansen, Lia Wagemakers and Theo Prins for their contributions to parts of the experimental work and the stimulating discussions. Dr. M.A. de Waard and Dr. M.H.A.J. Joosten are acknowledged for critical reading of the manuscript. Prof. P. Tudzynski and his coworkers are acknowledged for the continuous stimulating discussions during bilateral group meetings over the past few years. Discussions with Prof. A. von Tiedemann and Dr. C.B. Lawrence have been helpful during preparation of the manuscript.

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