

Activation of a Bean Chitinase Promoter in Transgenic Tobacco Plants by Phytopathogenic Fungi

Dominique Roby,¹ Karen Broglie, Robert Cressman, Phyllis Biddle, Ilan Chet,² and Richard Broglie³

E.I. Du Pont de Nemours & Company, Agricultural Products Department, Experimental Station, P.O. Box 80402, Wilmington, Delaware 19880

The temporal and spatial expression of a bean chitinase promoter has been investigated in response to fungal attack. Analysis of transgenic tobacco plants containing a chimeric gene composed of a 1.7-kilobase fragment carrying the chitinase 5B gene promoter fused to the coding region of the *gus A* gene indicated that the chitinase promoter is activated during attack by the fungal pathogens *Botrytis cinerea*, *Rhizoctonia solani*, and *Sclerotium rolfsii*. Although induction of β -glucuronidase activity was observed in tissues that had not been exposed to these phytopathogens, the greatest induction occurred in and around the site of fungal infection. The increase in β -glucuronidase activity closely paralleled the increase in endogenous tobacco chitinase activity produced in response to fungal infection. Thus, the chitinase 5B-*gus A* fusion gene may be used to analyze the cellular and molecular details of the activation of the host defense system during pathogen attack.

INTRODUCTION

During the course of fungal attack, plants are induced to express a number of polypeptides that function either passively or actively to combat infection by the pathogenic organism. Among the proteins induced are chitinase and β -1,3-glucanase, lytic enzymes that catalyze the hydrolysis of the chitin and β -1,3-glucan components, respectively, of the fungal cell wall. Chitinases have been purified from a variety of sources, including a number of economically important crop plants (Molano et al., 1979; Pegg and Young, 1982; Boller et al., 1983; Leah et al., 1987; Legrand et al., 1987; Shinshi et al., 1987; Fink et al., 1988; Nasser et al., 1988). In bean plants, the major chitinase activity is known to be associated with a basic, 30-kD protein that is localized in the vacuolar compartment (Boller et al., 1983; Boller and Vögeli, 1984). In addition, two of the four acidic pathogenesis-related proteins have been identified as chitinase enzymes (Awade et al., 1989). In healthy, uninfected bean plants, chitinase levels are low or undetectable. However, an induction of enzyme activity is observed upon treatment with the phytohormone ethylene (Boller et al., 1983) or with oligosaccharide elicitors (Kurosaki et al., 1986; Roby et al., 1986) or upon infection by fungal pathogens (Pegg and Vessey, 1973; Roby and Esquerre-Tugaye, 1987; Joosten and De Wit, 1989). We

have isolated full-length cDNA clones encoding ethylene-induced chitinases from bean and have used these as hybridization probes to isolate the corresponding nuclear genes. In bean, chitinase is encoded by a small multigene family consisting of three members; the expression of at least two of these genes is known to be transcriptionally regulated by ethylene or elicitor treatment (Broglie et al., 1986; Hedrick et al., 1988).

We are interested in understanding the mechanism(s) by which plants perceive and respond to pathogen attack. Toward this end, we have been studying the regulation of the chitinase 5B gene from bean. Expression of this gene in transgenic tobacco plants has been shown previously to be dependent upon the application of either exogenous ethylene or oligosaccharide elicitors. DNA sequences responsible for mediating this response have been shown to be situated upstream of the transcription initiation site. Two regions necessary for optimal gene expression have been identified: a quantitative region required for maximal gene expression and a region required for induction of gene expression by ethylene (Broglie et al., 1989).

In this study we have focused our attention on the spatial and temporal aspects of chitinase gene activation during pathogen attack. These studies were facilitated by using a chimeric gene consisting of the chitinase 5B gene promoter fused to the reporter *gus A* gene of *Escherichia coli*. The *gus A* gene product, β -glucuronidase, can be easily assayed by spectrophotometric, fluorometric, and histochemical techniques (Jefferson et al., 1986; Jefferson, 1987; Jefferson et al., 1987). Analysis of transgenic plants

¹ Current address: Laboratoire de Biologie Moléculaire des Relations Plantes-Microorganismes, CNRS/INRA, Chemin de Borderouge, BP27, 31326 Castanet Tolosan Cedex, France.

² Permanent address: Hebrew University, Otto-Warburg Center for Biotechnology, Faculty of Agriculture, Rehovot, Israel.

³ To whom correspondence should be addressed.

infected with either foliar or soil-borne fungal pathogens revealed that the strongest induction of the chitinase gene promoter occurred at the site of fungal infection and in the region immediately surrounding it, although some activation was evident in tissues not exposed to fungal pathogens. The expression pattern of the chitinase 5B gene promoter was found to parallel the activation of endogenous tobacco chitinase enzyme activity during fungal infection.

RESULTS

To study the activation of the bean chitinase gene promoter during fungal infection, a chimeric gene was constructed utilizing β -glucuronidase (GUS) as a reporter gene. Details of the construction of pCG2226 and its introduction into tobacco plants by *Agrobacterium*-mediated transformation have been described previously (Brogliè et al., 1989). Briefly, a 1.7-kb fragment containing the upstream region of the bean chitinase 5B gene was fused to the coding sequence of β -glucuronidase followed by the 3'-untranslated region of nopaline synthase. This construct, in the binary vector pBI101.2, was introduced into *Agrobacterium tumefaciens* LBA4404 by conjugation, and the resultant bacteria were used to infect tobacco leaf discs. Primary transformants were assayed for GUS expression after treatment of the plants with 50 ppm ethylene. Based on this analysis, plant 41C, which displayed a 30-fold induction of GUS activity, was selected for the fungal infection experiments.

Activation of Bean Chitinase Promoter in Fungus-Infected Transgenic Plants

Plants homozygous for the chitinase-GUS fusion gene were initially subjected to infection with the foliar pathogen *Botrytis cinerea*, a sclerotinaceous ascomycete commonly referred to as "grey mold." Three-week-old tobacco plants were inoculated by placing 10- μ L aliquots of a suspension of *Botrytis* conidia on one-half of the surface of the third leaf. Plants were first incubated in a dew chamber for 24 hr to allow germination of the conidia and were then transferred to a growth chamber. Within 24 hr after inoculation, symptoms of the disease began to appear as brown spots on the surface of the inoculated leaf. Figure 1A shows the appearance of typical lesions 48 hr after the start of the infection. The lesions continued to increase in size with time and reached a maximum 4 days post-infection. By 7 days, the lesions appeared as restricted necrotic areas at the site of fungal inoculation.

The induction of the chitinase promoter was monitored during the course of infection by staining infected leaf tissue with the chromogenic GUS substrate 5-bromo-4-

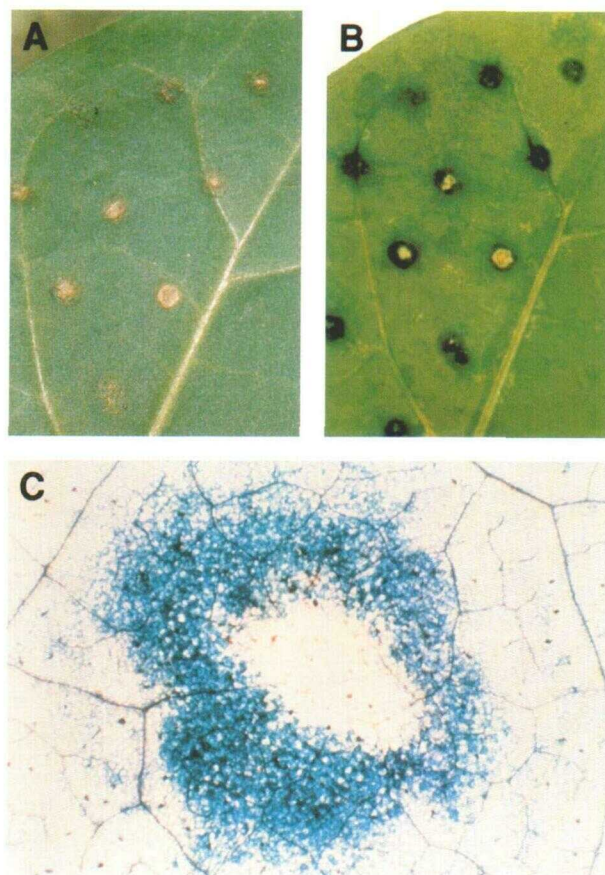


Figure 1. Histochemical Staining of β -Glucuronidase Activity in Transgenic Tobacco Leaves Infected with *B. cinerea*.

(A) Typical appearance of lesions produced by *B. cinerea* infection of transgenic tobacco plants. The lesions pictured are 2 days post-inoculation.

(B) Histochemical staining of infected tissue for GUS activity 48 hr post-infection.

(C) Histochemical staining of infected tissue for GUS activity 72 hr post-infection. In **(C)** tissue samples were fixed and cleared of chlorophyll by boiling in ethanol for 5 min and visualized under a stereomicroscope (magnification $\times 12$).

chloro-3-indolyl glucuronide (X-gluc). At the earliest time-point investigated, 24 hr after inoculation, β -glucuronidase activity appeared in a narrow region of cells at the periphery of the developing lesion. Staining was also evident within the lesion itself. As the infection progressed, the region showing positive staining increased in size as well as intensity, reaching a maximum 3 days after inoculation (Figures 1B and 1C). At these late timepoints, a diffuse blue staining pattern could also be discerned between the individual lesions and on the uninoculated half of the infected leaf.

The staining pattern displayed by the infected transgenic plants is an accurate reflection of the induction of the chitinase promoter. This was shown by the following experiments: (1) Inoculation with yeast extract alone showed no detectable staining with X-gluc. (2) To allow better penetration of the substrate, an infected leaf was sliced before staining. This resulted in the expected appearance of a stained ring of cells around the lesion; however, a thin light-blue line was apparent around the cut edge as a result of wounding. (3) Staining of transgenic tobacco plants harboring a chimeric GUS gene under the control of the cauliflower mosaic virus 35S promoter resulted in an even staining pattern over the entire surface of the leaf, with no preferential staining around the individual lesions. Thus,

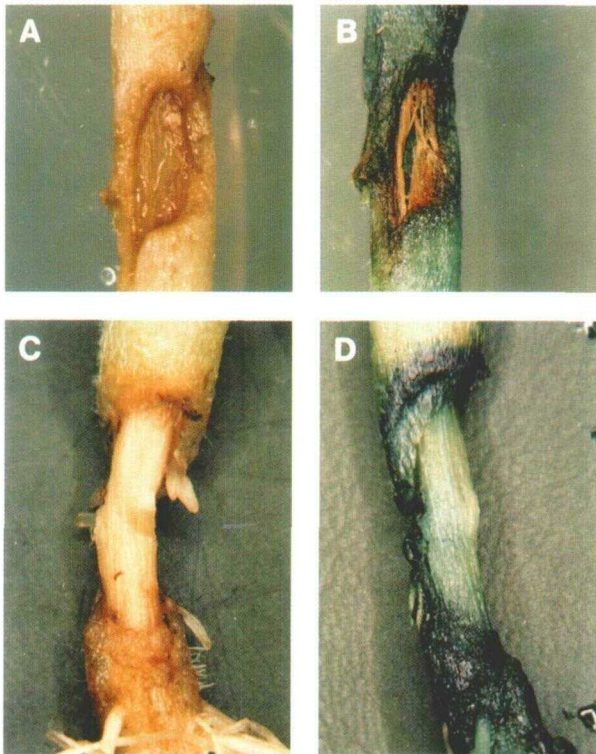


Figure 2. Histochemical Localization of β -Glucuronidase Activity in Transgenic Tobacco Plants Infected with the Soil-Borne Pathogens *R. solani* and *S. rolfsii*.

- (A) Lesions formed on transgenic tobacco by *R. solani*.
 (B) Histochemical localization of GUS activity in *R. solani*-infected transgenic tobacco plants.
 (C) Lesion produced by *S. rolfsii* infection of transgenic tobacco plants.
 (D) Histochemical localization of GUS activity in *S. rolfsii*-infected transgenic tobacco plants.

we can conclude that the bean chitinase promoter is activated during fungal attack, with the highest level of induction concentrated at the site of infection.

The induction pattern observed for the bean chitinase promoter in *Botrytis*-infected plants was not restricted to this pathogen. Qualitatively similar results were obtained when 41C transgenic tobacco plants were infected with two soil-borne pathogens, *Rhizoctonia solani* and *Sclerotium rolfsii*. Tobacco seedlings were infected with the root rot pathogen *R. solani* by transplanting into fungus-infested soil. After 5 days to 6 days, symptoms of the disease were observed by the appearance of typical lesions on the root neck, as shown in Figure 2A. Under the inoculation conditions used in this experiment, death of the plants usually did not occur. When the infected seedlings were assayed for β -glucuronidase enzyme activity, an intense staining reaction was evident, with the darkest staining tissue concentrated in the vicinity of the lesion (Figure 2B). In addition, GUS activity could also be detected beyond the lesion itself; however, this reaction was less intense and gradually declined as the distance from the lesion increased.

Although tobacco is not a typical host plant for *S. rolfsii*, infection by this fungus could be achieved by placing a mycelium-containing agar disc on the surface of the soil near the crown of the plant. Under these conditions, the hyphae grew on the root neck and produced a lesion, as shown in Figure 2C. When these plants were incubated with X-gluc, rapid staining of the infected tissue occurred. In this case, the highest level of GUS enzyme activity was found in the region immediately surrounding the lesion (Figure 2D). The results obtained with the *Rhizoctonia* and *Sclerotium* infection are consistent with those derived from the *Botrytis*-infected plants and provide further evidence for a localized, high-level activation of the bean chitinase 5B promoter at the site of fungal infection.

A quantitative measure of the level of chitinase promoter activity during *Botrytis* infection was made using 4-methylumbelliferyl glucuronide as the substrate for β -glucuronidase. As shown in Figure 3, the highest level of enzyme activity was found within the necrotic region itself; however, activation of the chitinase promoter was also seen in the region surrounding the lesion, albeit at a much reduced level. Tissue sampled 0 mm to 3 mm away from the lesion showed a 12-fold lower level of GUS activity. At 3 mm to 6 mm away, GUS activity underwent a further sevenfold reduction and was only 2 times the background value. Control experiments indicated that the enzyme activity detected by the fluorescence assay was due to GUS expression by the plant cells. Little or no enzyme activity could be detected in extracts of fungus grown on culture media or in extracts of *Botrytis*-infected wild-type tobacco plants. In addition, microscopic visualization of X-gluc-stained tissue sections confirmed that the GUS activity was confined to the plant cells.

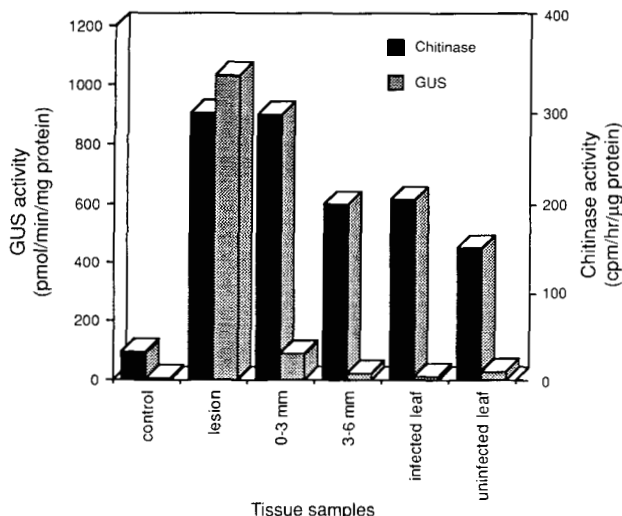


Figure 3. Activation of Bean Chitinase Promoter and Endogenous Chitinase Activity in Transgenic Tobacco Plants Infected with *B. cinerea*.

Analysis of β -glucuronidase enzyme activity and endogenous chitinase activity in transgenic tobacco plants infected with *B. cinerea*. The following tissue samples were assayed: control, leaves of plants inoculated with yeast extract alone; lesions, necrotic tissue resulting from *Botrytis* infection; 0–3 mm, apparently healthy tissue 0 mm to 3 mm surrounding the lesion; 3–6 mm, apparently healthy tissue 3 mm to 6 mm surrounding the lesion; infected leaf, remaining tissue from the fungus-inoculated leaf half; uninfected leaf, tissue sampled from the uninoculated half of the *Botrytis*-infected leaf.

Systemic Induction of Bean Chitinase Promoter Activity in Transgenic Plants

To determine the systemic level of chitinase promoter induction, fungus-infected plants were divided into different parts and assayed for β -glucuronidase activity. The results of this experiment for *Botrytis*-infected plants are summarized in Figure 4. The highest level of GUS activity was found on the inoculated half of the leaf, where a 25-fold induction was evident 24 hr after inoculation. GUS enzyme activity in this portion of the leaf continued to increase for 2 days to 3 days after inoculation and reached a maximum 200-fold to 400-fold stimulation over control values (Figure 4A). A small but significant induction of GUS activity was also seen on the uninoculated half of the same leaf, where an eightfold to 17-fold induction above control values was observed 3 days to 4 days post-infection (Figure 4B). At distances further removed from the inoculated leaf, no induction of GUS enzyme activity above control values could be detected (Figure 4C).

Assays of protein extracts prepared from *R. solani*-infected and *S. rolfii*-infected tobacco plants yielded results similar to those obtained with *Botrytis*-infected plants.

As shown in Figure 5B, GUS activity was strongest in the region immediately surrounding lesions produced by *Rhizoctonia* infection. However, enzyme activity was also found to a lesser extent in the adjacent lower stem and in the upper stem as well. Similar results were observed with

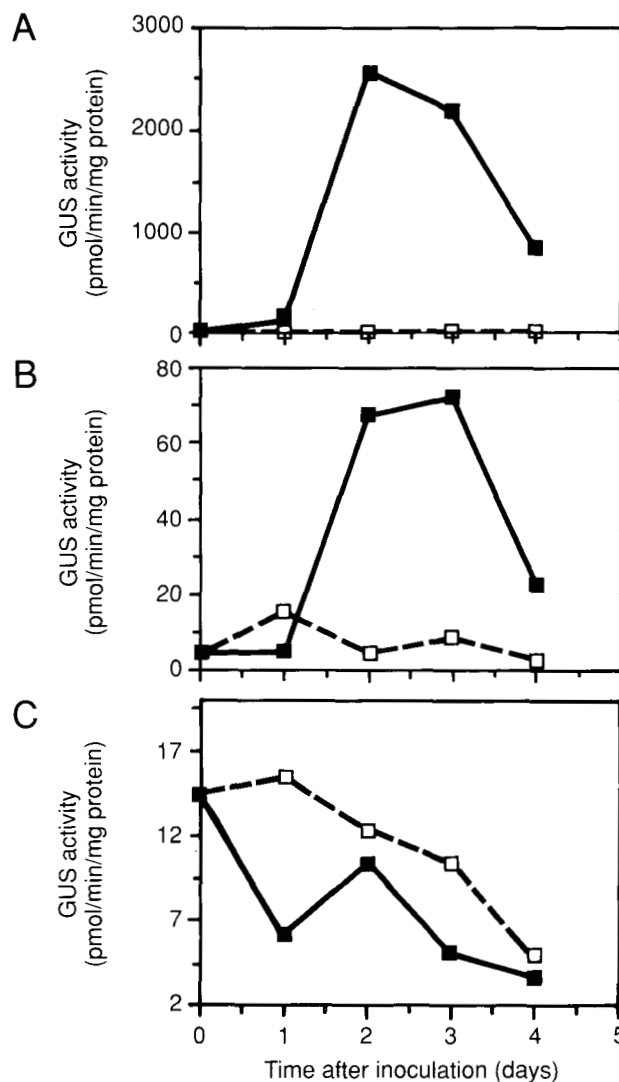


Figure 4. Quantitative Analysis of β -Glucuronidase Activity in Healthy and Fungus-Infected Transgenic Tobacco Plants.

(A) Induction of β -glucuronidase activity in the infected half of a transgenic tobacco leaf after inoculation with a suspension of *Botrytis conidia* (10 μ L containing 2×10^6 conidia/mL).

(B) Induction of β -glucuronidase activity in the uninoculated half of the *Botrytis*-infected leaf.

(C) β -Glucuronidase activity in the younger, uninoculated leaves of a *Botrytis*-infected plant. Tissue was collected at 24-hr intervals post-inoculation, and GUS activity was determined using the fluorescence substrate 4-MUG.

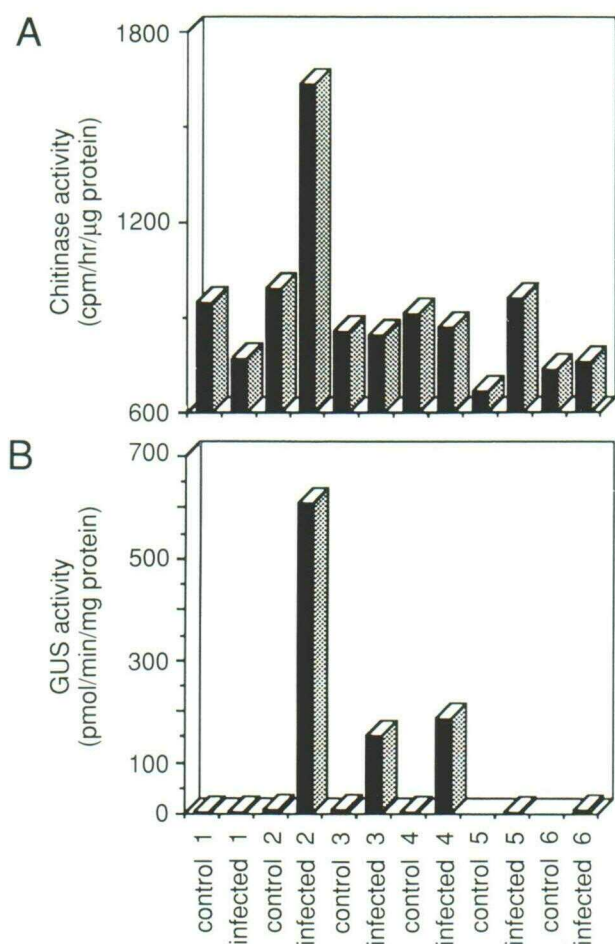


Figure 5. Analysis of β -Glucuronidase and Endogenous Chitinase Activity in *Rhizoctonia*-Infected Transgenic Tobacco Plants.

Transgenic tobacco plants were infected with *R. solani* as described in Methods. After 6 days of growth in infested soil, plants were harvested and the following tissue samples collected: roots (1); infected stem region (2); lower, uninfected stem (3); upper, uninfected stem (4); primary leaves (5); and true leaves (6).

(A) Chitinase activity was determined using tritiated chitin as the substrate (Molano et al., 1977).

(B) GUS activity was determined using the fluorescence substrate 4-MUG (Jefferson, 1987).

S. rolfisii-infected plants. With both of these pathogens, no GUS enzyme activity could be detected in crude mycelial extracts.

Activation of the Chitinase 5B Gene Promoter Parallels the Induction of Tobacco Chitinase Activity in Fungus-Infected Plants

To relate the observed activation of the chitinase gene promoter to the induction of the host defense response,

endogenous chitinase activity was measured in healthy and infected transgenic tobacco plants. As shown in Figures 4 and 5, chitinase enzyme activity is increased during infection of tobacco plants with the fungal pathogens *R. solani* and *B. cinerea*. The induction of enzyme activity is greatest at the site of infection; a 10-fold and a 1.6-fold increase is observed upon *Botrytis* and *Rhizoctonia* infection, respectively. The level of chitinase activity decreases as the distance from the site of infection is increased. This expression pattern is similar to the pattern of β -glucuronidase activity displayed in the fungus-infected 41C transgenic tobacco plants.

Localization of *B. cinerea* in Infected Transgenic Tobacco Plants

Measurements of GUS enzyme activity during fungal infection have indicated that induction of the chitinase 5B gene promoter is greatest at and immediately around the site of fungal infection and decreases significantly as the distance from the point of infection increases. Assays of endogenous chitinase activity have additionally shown that the spatial expression pattern of the bean chitinase promoter parallels that for the tobacco chitinase gene(s). We were interested in determining the localization of the fungus within the infected tissue and in determining whether fungal colonization of the plant cells was necessary for activation

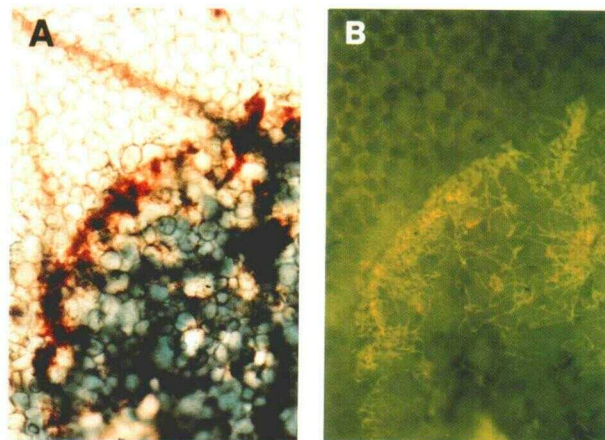


Figure 6. Localization of *B. cinerea* Hyphae in and around Lesions Stained for GUS Activity.

(A) Visualization of cells expressing GUS activity in *B. cinerea*-infected transgenic tobacco plants. The area shown represents the outer edge of GUS-positive tissue surrounding a lesion. Samples were observed using a light microscope equipped with differential interference contrast optics.

(B) Fluorescence detection of fungal hyphae stained with FITC-conjugated wheat germ agglutinin in the same area.

of the bean chitinase 5B promoter, or whether activation instead occurred in advance of fungal growth. To examine this, *Botrytis*-infected tissue was fixed after the X-gluc reaction. Fungal hyphae were stained with fluorescein isothiocyanate-conjugated wheat germ agglutinin and then visualized under a fluorescence microscope equipped with differential interference contrast optics. A high concentration of fungal mycelia and conidia was found within the lesion itself. As shown in Figure 6, significantly fewer hyphae were seen in the stained ring of cells surrounding the lesion. Further observation indicated that these hyphae were restricted to the upper surface of the leaf or were found intercellularly in the first few upper cell layers. No fungal mycelia were visible beyond the intensely stained band of cells.

DISCUSSION

An important component of the plant defense system is the induction of a number of pathogenesis-related proteins. Among these is the enzyme chitinase (Legrand et al., 1987; Kombrink et al., 1988; Awade et al., 1989; Joosten and De Wit, 1989), which has been shown by *in vitro* experiments to partially hydrolyze isolated fungal cell walls (Boller et al., 1983) and to inhibit the growth of certain phytopathogenic fungi (Schlumbaum et al., 1986; Mauch et al., 1988). In the present paper, we have used β -glucuronidase as a marker enzyme to investigate the activation of the bean chitinase gene promoter during fungal infection of transgenic tobacco plants. We have observed a high-level localized induction of chitinase promoter activity in response to infection by the phytopathogens *B. cinerea*, *S. rolfsii*, and *R. solani*. In the case of *B. cinerea* and *S. rolfsii*, infection was achieved by the local inoculation of plant tissue. For *R. solani*, the infection was initiated by transplanting seedlings into fungus-infested soil. In this case, only low levels of GUS activity were observed in the roots themselves. The strongest induction of GUS enzyme activity was evident in the infected tissues of the root neck. This indicates that promoter induction is not only dependent on exposure to the pathogen but also requires fungal penetration and/or tissue damage that occurs as a result of fungal attack.

Antoniw and White (1986) have analyzed the distribution of the pathogenesis-related protein PR1a in local lesions of *Nicotiana tabacum* cv Xanthi-nc infected with tobacco mosaic virus (TMV). PR1a protein levels were found to be greatest in the region immediately surrounding the viral lesion. Only low levels of the protein were present in the lesion itself. More recently, Ohshima et al. (1990) have studied induction of the PR1a promoter in transgenic tobacco plants utilizing β -glucuronidase as a reporter gene. GUS enzyme activity was found to be increased by treatment with salicylic acid, by wounding, and by TMV-asso-

ciated local lesion formation. Histochemical staining of TMV-infected tissues showed a localized induction of GUS activity that was consistent with the distribution of endogenous PR1a protein found by Antoniw and White (1986). Failure to observe β -glucuronidase activity within the TMV lesions was attributed to the degradation of the protein in the necrotic cells. A similar explanation may be invoked here to explain the sharp decrease in lesion-associated GUS enzyme activity that occurs 4 days post-infection.

The localized induction of the bean chitinase promoter suggests that the signal that mediates promoter activation is concentrated at the site of fungal infection. In the case of infection by *B. cinerea*, the intensity of this signal decreases sharply with increasing distance. Measurement of GUS activity in tissues 0 mm to 3 mm away from the lesion showed a 12-fold decrease in chitinase promoter activity. No significant expression of GUS could be detected in leaves above or below the inoculation site. However, a signal that activates the bean chitinase promoter must be propagated to regions of the leaf that have not been exposed to the pathogen because a small but significant induction of GUS enzyme activity was observed on the uninoculated half of the infected leaf. Although maximal GUS activity in this portion of the leaf was only 12% of the level in the infected half, the kinetics of induction were similar in both cases. In *R. solani*-infected plants, GUS activity was evident in portions of the stem approximately 2 cm away from the infection site, although the level of activity was only 15% of that seen in the vicinity of the lesion itself.

A localized induction of chitinase enzyme activity has been reported for infection of cucumber plants by *Colletotrichum lagenarium* and tobacco necrosis virus (Mettraux and Boller, 1986). With both treatments, a weaker systemic induction of chitinase activity was apparent at distances removed from the inoculation site. Chitinase activity has also been found to be locally and systemically induced in melon plants during fungal infection or upon treatment with elicitors (Roby et al., 1988). These former studies involved the measurement of endogenous enzyme activity contributed by several different chitinase isozymes. Here, we have been able to investigate the response of a single bean chitinase promoter to fungal infection. Our inability to detect significant induction of GUS enzyme activity at distances far removed from the infection site may reflect the shape of the signal gradient in the infected transgenic tobacco plants. Alternatively, this may be a characteristic feature of the response of the chitinase 5B promoter. In this regard, it is interesting to note that although the induction of endogenous tobacco chitinase activity follows a similar pattern of distribution, the response is not as sharply defined as that for the induction of the chitinase 5B promoter.

The utilization of β -glucuronidase as a reporter of chitinase promoter activity has enabled us to examine the response of this promoter to fungal infection at the cellular

level. In *B. cinerea*-infected plants, the spatial pattern of GUS expression was similar to the distribution of fungus within the infected tissue. The highest levels of GUS enzyme activity were found 2 days to 3 days after fungal inoculation when large amounts of fungal mycelia were visible within the lesion. In the surrounding regions, both the expression of GUS and the concentration of fungus were reduced. However, the level of GUS enzyme activity in this area was sufficient to produce blue-stained cells when infected tissues were subjected to the histochemical staining procedure. Microscopic visualization of fungal growth in this region revealed that the mycelial concentration was reduced in amount and restricted to a few cell layers on the upper surface of the leaf. Beyond this region, GUS expression decreased to twice the background level and no fungal structures could be detected. This system may thus be used to provide a better understanding at the cellular level of the timing, mode of action, and efficiency of the defense response of plants to fungal attack.

METHODS

Generation of Transgenic Tobacco Plants

The construction of pCG2226 and its introduction into *Nicotiana tabacum* cv Xanthi has been described in detail elsewhere (Brogie et al., 1989). Primary tobacco transformants were screened by assaying β -glucuronidase activity after treatment of the plants with 50 ppm ethylene. Homozygous seed stocks of two transformants, 10 and 41C, were generated and used for the fungal infection experiments. Both plants showed a similar pattern of GUS gene expression during fungal infection with *Botrytis cinerea*. However, because the level of GUS enzyme activity was fourfold higher in 41C, this transformant was used in all subsequent work.

Infection of Tobacco Plants with Fungal Pathogens

Tobacco plants were grown in controlled environmental chambers maintained for a 12-hr, 24°C day and for a 12-hr, 20°C night cycle, with approximately 80% relative humidity, under mixed cool-white fluorescent and incandescent lights. *B. cinerea* was grown on potato dextrose agar/yeast extract medium at 20°C in the dark. Conidia were harvested from a 7-day-old to 8-day-old culture and suspended at a density of 10^6 conidia/mL in sterile 1% yeast extract. Inoculation of plants with *B. cinerea* was performed by applying 10- μ L droplets of the conidia suspension to one-half of the surface of the third or fourth leaf of 3-week-old plants. Controls were inoculated in an identical manner with sterile yeast extract only. Inoculated and control plants were incubated in a humidity chamber under reduced lighting for 24 hr (relative humidity, 100%; temperature, 23°C to 24°C). After this time, the plants were removed and transferred to a growth chamber maintained as described above. At intervals after the start of the infection, tissue was harvested from the plants and either used directly or stored at -80°C.

Inoculum for infection of tobacco plants with *Rhizoctonia solani* (AG-4) was prepared by growing the fungus on a sand/cereal

medium consisting of 500 mL of quartz sand, 40 mL of cream of wheat, 40 mL of corn meal, and 75 mL of water. Potting soil (Metromix 300) was mixed with a level of fungal inoculum empirically determined to yield about 50% disease incidence. Transgenic tobacco seedlings were transferred 20 days after sowing into the *R. solani*-infested soil and then incubated under the growth conditions defined above. After 5 days to 6 days, brown lesions typical of *Rhizoctonia* infection appeared on the root neck of surviving plants. At this time, plants were uprooted, washed thoroughly with water, and either divided into separate sections for assay of GUS or chitinase activity or the entire seedling was stained for GUS activity by the histochemical method.

Sclerotium rolfsii was grown on potato dextrose agar medium at 20°C. Agar discs were excised from *S. rolfsii* cultures and placed on the soil surface adjacent to 20-day-old tobacco seedlings. After 5 days to 6 days, lesions appeared on the root neck of infected plants.

Detection of β -Glucuronidase Activity

β -Glucuronidase activity was determined in infected and noninfected plants by histochemical staining and by a fluorometric assay. For quantitative determination of GUS activity, plants were divided into different sections and soluble protein was isolated essentially as described by Jefferson et al. (1987). Assays of GUS enzymatic activity were performed in 1 mL of extraction buffer [50 mM NaH₂PO₄ (pH 7.0), 10 mM EDTA, 0.1% Triton X-100, 0.1% sodium lauryl sarcosine, 10 mM mercaptoethanol] containing 1 mM 4-methylumbelliferyl glucuronide (4-MUG). At 0 min, 30 min, and 60 min, 200 μ L of the assay mixture was diluted into 800 μ L of 0.2 M Na₂CO₃. The fluorescence at 455 nm was determined on a Perkin-Elmer model LS-3B spectrofluorometer using an excitation wavelength of 365 nm. The protein concentration of plant extracts was determined using the Bradford dye reagent (Bio-Rad Chemical Co).

Histochemical assays were performed using X-gluc (5-bromo-4-chloro-3-indolyl- β -D-glucuronide, Clontech Laboratories) as the substrate (Jefferson et al., 1987). Inoculated or control leaves were excised, placed in a solution consisting of 1 mg/mL X-gluc in 0.1 M NaH₂PO₄ (pH 7.0), 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, and 10 mM EDTA, and vacuum infiltrated for 2 min. The GUS enzymatic reaction was then allowed to proceed at 37°C overnight. For some experiments, samples were fixed in 1% glutaraldehyde, then cleared of pigment by boiling for 5 min in ethanol and stored in chloral hydrate until use. Staining of *R. solani*-infected and *rolfsii*-infected and control plants was accomplished in a similar fashion, except that the entire seedling was incubated in the assay buffer and the GUS reaction allowed to proceed at 37°C for approximately 5 hr.

Chitinase Enzyme Assays

Tobacco chitinase activity was determined by a radiochemical enzyme assay using regenerated tritiated chitin as the substrate (Molano et al., 1977). Total soluble protein was extracted from control and infected plant tissues in 0.1 M sodium citrate buffer (pH 5.0) according to the procedure of Boller et al. (1983). The chitinase enzyme assay was initiated by the addition of protein to a 4 mg/mL suspension of tritiated chitin in 20 mM sodium phos-

phate buffer (pH 6.5) in a final volume of 0.25 mL. The reaction was stopped after 1 hr at 30°C by the addition of 0.25 mL of 1 M trichloroacetic acid. The suspension was then centrifuged and the released radioactivity measured by liquid scintillation counting of 0.3 mL of the supernatant. Because product formation was nonlinear with enzyme concentration, several aliquots of the extract were assayed and the activity was determined for an enzyme concentration approaching zero.

Localization of *B. cinerea* in Infected Plants

B. cinerea was visualized in infected tissues using fluorescein isothiocyanate-conjugated wheat germ agglutinin (FITC-WGA, Polysciences, Inc., Warrington, PA). Fungal cell walls were specifically stained by first washing samples for 1 hr in 10 mM Tris-HCl (pH 7.2), 0.5 M NaCl, 50 mM MgCl₂, and 0.1% polyethylene glycol; staining for 30 min to 60 min with FITC-WGA in 50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM MnCl₂, 0.5% polyethylene glycol; and then rinsing twice in the same buffer. Samples were then observed with a Zeiss Axiophot light microscope equipped with differential interference contrast optics and viewed at a magnification of $\times 100$.

ACKNOWLEDGMENTS

We are grateful to Todd Jones and Rick Howard for their help with the microscopy. We would also like to thank Barbara Mazur for her critical reading of the manuscript. D. R. was supported in part by a National Science Foundation-Centre National de la Recherche Scientifique fellowship.

Received July 23, 1990.

REFERENCES

- Antoniw, J.F., and White, R.F. (1986). Changes with time in the distribution of virus and PR protein around single local lesions of TMV infected tobacco. *Plant Mol. Biol.* **6**, 145–149.
- Awade, A., DeTapia, M., Didierjean, L., and Burkard, G. (1989). Biological function of bean pathogenesis-related (PR3 and PR4) proteins. *Plant Sci.* **63**, 121–130.
- Boller, T., and Vögeli, U. (1984). Vacuolar localization of ethylene-induced chitinase in bean leaves. *Plant Physiol.* **74**, 442–444.
- Boller, T., Gehri, A., Mauch, F., and Vögeli, U. (1983). Chitinase in bean leaves: Induction by ethylene, purification, properties and possible function. *Planta* **157**, 22–31.
- Brogliè, K.E., Gaynor, J.J., and Brogliè, R.M. (1986). Ethylene-regulated gene expression: Molecular cloning of the genes encoding an endochitinase from *Phaseolus vulgaris*. *Proc. Natl. Acad. Sci. USA* **83**, 6820–6824.
- Brogliè, K.E., Biddle, P., Cressman, R., and Brogliè, R. (1989). Functional analysis of DNA sequences responsible for ethylene regulation of a bean chitinase gene in transgenic tobacco. *Plant Cell* **1**, 599–607.
- Fink, W., Liefland, M., and Mendgen, K. (1988). Chitinases and β -1,3-glucanases in the apoplastic compartment of oat leaves (*Avena sativa* L.). *Plant Physiol.* **88**, 270–275.
- Hedrick, S.A., Bell, J.N., Boller, T., and Lamb, C.J. (1988). Chitinase cDNA cloning and mRNA induction by fungal elicitor, wounding and infection. *Plant Physiol.* **86**, 182–186.
- Jefferson, R.A. (1987). Assaying chimeric genes in plants: The GUS gene fusion system. *Plant Mol. Biol. Rep.* **5**, 387–405.
- Jefferson, R.A., Burgess, S.M., and Hirsh, D. (1986). β -Glucuronidase from *Escherichia coli* as a gene fusion marker. *Proc. Natl. Acad. Sci. USA* **83**, 8447–8451.
- Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS gene fusions: β -Glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**, 3901–3907.
- Joosten, M.H.A.J., and De Wit, P.J.G.M. (1989). Identification of several pathogenesis-related proteins in tomato leaves inoculated with *Cladosporium fulvum* (syn. *Fulvia fulva*) as 1,3- β -glucanases and chitinases. *Plant Physiol.* **89**, 945–951.
- Kombrink, E., Schroder, M., and Hahlbrock, K. (1988). Several pathogenesis-related proteins are 1,3- β -glucanases and chitinases. *Proc. Natl. Acad. Sci. USA* **85**, 782–786.
- Kurosaki, F., Tashirs, N., and Nishi, A. (1986). Induction of chitinase and phenylalanine ammonia-lyase in cultured carrot cells treated with fungal mycelial walls. *Plant Cell Physiol.* **27**, 1587–1591.
- Leah, R., Mikkelsen, D., Mundy, J., and Svendsen, I. (1987). Identification of a 28,000 dalton endochitinase in barley endosperm. *Carlsberg Res. Commun.* **52**, 31–37.
- Legrand, M., Kauffman, S., Geoffroy, P., and Fritig, B. (1987). Biological function of pathogenesis-related proteins: Four tobacco pathogenesis-related proteins are chitinases. *Proc. Natl. Acad. Sci. USA* **84**, 6750–6754.
- Mauch, F., Mauch-Mani, B., and Boller, T. (1988). Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and β -1,3-glucanase. *Plant Physiol.* **88**, 936–942.
- Metraux, J.P., and Boller, T. (1986). Local and systemic induction of chitinase in cucumber plants in response to viral, bacterial, and fungal infections. *Physiol. Mol. Plant Pathol.* **28**, 161–169.
- Molano, J., Duran, A., and Cabib, E. (1977). A rapid and sensitive assay for chitinase using tritiated chitin. *Anal. Biochem.* **83**, 648–656.
- Molano, J., Polacheck, I., Duran, A., and Cabib, E. (1979). An endochitinase from wheat germ. *J. Biol. Chem.* **254**, 4901–4907.
- Nasser, W., Tapia, M., Kauffmann, S., Montasser-Kouhsari, S., and Burkard, G. (1988). Identification and characterization of maize pathogenesis related proteins. Four maize PR proteins are chitinases. *Plant Mol. Biol.* **11**, 529–538.
- Ohshima, M., Itoh, H., Matsuoka, M., Murakami, T., and Ohashi, Y. (1990). Analysis of stress-induced or salicylic acid-induced expression of the pathogenesis-related 1a protein in transgenic tobacco. *Plant Cell* **2**, 95–106.
- Pegg, G.F., and Vessey, J.C. (1973). Chitinase activity in *Lycopersicon esculentum* and its relationship to the in vivo lysis

- of *Verticillium albo-atrum* mycelium. *Physiol. Plant Pathol.* **3**, 207–222.
- Pegg, G.F., and Young, D.H.** (1982). Purification and characterization of chitinase enzymes from healthy and *Verticillium albo-atrum*-infected tomato plants and from *V. albo-atrum*. *Physiol. Plant Pathol.* **21**, 389–409.
- Roby, D., and Esquerre-Tugaye, M.T.** (1987). Induction of chitinases and of translatable mRNA for these enzymes in melon plants infected with *Colletotrichum lagenarium*. *Plant Sci.* **52**, 175–185.
- Roby, D., Toppan, A., and Esquerre-Tugaye, M.T.** (1986). Cell surfaces in plant microorganism interactions. VI. Elicitors of ethylene from *Colletotrichum lagenarium* trigger chitinase activity in melon plants. *Plant Physiol.* **81**, 228–233.
- Roby, D., Toppan, A., and Esquerre-Tugaye, M.T.** (1988). Systemic induction of chitinase activity and resistance in melon plants upon fungal infection or elicitor treatment. *Physiol. Mol. Plant Pathol.* **33**, 409–417.
- Schlumbaum, A., Mauch, F., Vögeli, U., and Boller, T.** (1986). Plant chitinases are potent inhibitors of fungal growth. *Nature* **324**, 365–367.
- Shinshi, H., Mohnen, D., and Meins, F.** (1987). Regulation of a plant pathogenesis-related enzyme: Inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissues by auxin and cytokinin. *Proc. Natl. Acad. Sci. USA* **84**, 89–93.