Expression and Localization of Two *in Planta* Induced Extracellular Proteins of the Fungal Tomato Pathogen *Cladosporium fulvum*

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Expression of two *in planta* induced genes of the biotrophic fungal tomato pathogen *Cladosporium fulvum* and accumulation of their products, extracellular proteins (ECPs), were studied in time and space during pathogenesis. Immunogold localization revealed that proteins ECP1 and ECP2 accumulated abundantly in extracellular material in the vicinity of fungal and host cell walls. Expression of the genes encoding ECP1 and ECP2 was studied in transformants carrying the reporter gene *Gus* fused to promoter sequences of the *ecp* genes. In germinated conidia on the leaf surface no expression of gene *ecp*1 and only low expression of gene *ecp*2 could be detected. Expression of both *ecp*1 and *ecp*2 was strongly induced during colonization of the intercellular space between tomato mesophyll cells. The highest expression was observed in hyphae growing near vascular tissue. Expression levels were low in newly formed conidia on leaves. Possible functions of ECP1 and ECP2 for *C. fulvum* during pathogenesis are discussed.

Additional keywords: β-glucuronidase, immunogold labeling, pathogenicity.

Most plant-pathogenic fungi are specialized and capable of infecting only one or a few plant species. These fungi are able to circumvent the induction of a defense response and depend upon pathogenicity factors which may be involved in the formation of infection structures, such as appressoria (Talbot *et al.* 1993) or haustoria; degradation of host cell walls (Maiti and Kolattukudy 1979; Dickman *et al.* 1989); or detoxification of phytoalexins (Schäfer *et al.* 1989) or plant toxins (Osbourn *et al.* 1991). Two putative pathogenicity factors that have been studied in detail are cutinase and pisatin demethylase produced by the pea pathogen *Nectria haematococca* (Maiti and Kolattukudy 1979; Dickman *et al.* 1989; Schäfer *et al.* 1989). Recent data indicate that the genes encoding cutinase and pisatin demethylase are not strictly required for pathogenicity, as disruption of these genes did not result in a detectable decrease in pathogenicity (Stahl and Schäfer 1992; VanEtten *et al.* 1993). These results indicate that a directed functional approach to estimate the importance of single genes in the pathogenicity of fungal pathogens is often not successful. An unbiased approach was followed by Talbot *et al.* (1993) in studying the pathogenicity of the rice blast fungus, *Magnaporthe grisea;* using differential screening in a search for genes induced *in planta,* they cloned the *MPG1* gene. Disruption of this gene resulted in reduced pathogenicity of *M. grisea* on rice, possibly as a result of impaired appressorium formation.

We are investigating the interaction between the plant-pathogenic fungus *Cladosporium fulvum* Cooke (syn. *Fulvia fulva* (Cooke) Cif.) and its only host, tomato (*Lycopersicon esculentum* Mill.). *C. fulvum* is a biotrophic pathogen which grows in the intercellular space of the leaf without penetrating the mesophyll cells (Lazarovits and Higgins 1976a,b; De Wit 1977; De Wit and Flach 1979). Extensive studies of molecular aspects of the gene-for-gene relationship between *C. fulvum* and tomato have resulted in cloning and characterization of two fungal avirulence genes (Van Kan *et al.* 1991; Van den Ackerveken *et al.* 1992; Joosten *et al.* 1994). The corresponding resistance genes in tomato have not yet been cloned. Several defense-related responses are activated after initial contact between avirulent races of *C. fulvum* and resistant tomato plants, one of which is the accumulation of pathogenesis-related proteins. Several of the pathogenesis-related proteins from tomato have been purified to homogeneity (Joosten *et al.* 1989, 1990a), and genes encoding these proteins have been cloned (Danhash *et al.* 1993; Linthorst *et al* 1991; Van Kan *et al.* 1992). The role of these pathogenesis-related proteins in the defense of tomato against *C. fulvum* is still unclear.

Pathogenicity factors of *C. fulvum* have been studied in less detail. In search of these factors we examined the proteins present in the intercellular space of *C. fulvum*–infected tomato leaves. Several low molecular weight proteins (20 kD or less), of supposed fungal origin, have been found in apoplastic fluid of *C. fulvum*–infected leaves of susceptible tomato plants, but not in culture filtrate of *C. fulvum* grown *in vitro* (De Wit *et al.* 1989). Three extracellular proteins (ECPs) have been purified to homogeneity, polyclonal antibodies have been raised (Joosten and De Wit 1988; M. H. A. J. Joosten, unpublished), and the genes encoding ECP1 and ECP2 have been cloned from race 5 of *C. fulvum* (Van den Ackerveken *et al.* 1993). These genes, *ecp*1 and *ecp*2, appear to be highly expressed *in planta,* whereas their expression is low or undetectable in the fungus grown *in vitro* (Van den Ackerveken *et al.* 1993).

Here we describe the purification of ECP2 and present data on the *in situ* localization of both ECP1 and ECP2 in *C.*

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fulvum–infected tomato leaves. The expression of the *ecp*1 and *ecp*2 genes in time and space during pathogenesis was studied in transformants of *C. fulvum* carrying *ecp* promoter–*Gus* fusions. Possible functions of ECP1 and ECP2 for *C. fulvum* during pathogenesis on tomato are discussed.

RESULTS

Purification of the ECPs.

The purification of the protein ECP1 was described previously (Joosten and De Wit 1988). The protein ECP2 from apoplastic fluid from tomato genotype Cf5 in a compatible interaction with race 5 of *C. fulvum* was purified to homogeneity by gel filtration followed by anion exchange chromatography (Fig. 1A, lane 4). From the first anion exchange run, following fractionation on Sephadex G-50, two peaks containing ECP2 were obtained: one eluting around 110 mM NaCl and the other eluting around 160 mM NaCl. Rechromatography of each fraction containing ECP2 resulted again in the same two peaks. After analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), a single protein band of 17 kD was present in both peaks, while in native, high-pH PAGE the ECP2 protein resolved into two separate bands (results not shown). The protein probably exists in two conformations which reversibly change under nondenaturing conditions.

Western blot analysis, using polyclonal antibodies raised against the purified ECP2, revealed that the protein was present in apoplastic fluids obtained from tomato in a compatible interaction with *C. fulvum* (Fig. 1B, lanes 2 and 3). ECP2 was not detected in culture filtrate of *C. fulvum* grown *in vitro* (results not shown), in apoplastic fluids obtained from tomato in an incompatible interaction with *C. fulvum* (Fig. 1B, lane 1), or in healthy tomato plants (results not shown).

Immunolocalization of ECP1 and ECP2 in infected tomato leaves.

Immunolocalization experiments were performed on *C. fulvum*–inoculated tomato leaves in compatible and incompatible interactions at 7 and 12 days postinoculation. The polyclonal antibodies used were raised against the purified ECP1 and ECP2 proteins. Accumulation of ECP1 and ECP2 in the apoplast of *C. fulvum*–infected tomato leaves was observed at 12 days postinoculation. Both proteins accumulated at similar locations in *C. fulvum*–infected tomato leaves in a compatible interaction at 12 days postinoculation, primarily in extracellular material present in the vicinity of fungal and host cell walls (Fig. 2A and C). The proteins were detected in low quantities in the cytoplasm of growing hyphae (Fig. 2A–C) and in the extracellular matrix between leaf mesophyll cells (results not shown). However, ECP1 and ECP2 were never detected inside tomato mesophyll cells (Fig. 2A–C). Occasionally, ECP1 and ECP2 were detected in and near intact mycelium in incompatible interactions (results not shown).

Characterization of transformants of *C. fulvum* containing the P*ecp*-*Gus* constructs.

The cloning of the *ecp*1 and *ecp*2 genes from *C. fulvum* was described previously (Van den Ackerveken *et al.* 1993). The regulation of the expression of *ecp*1 and *ecp*2 during infection of tomato was studied with transformants of *C. fulvum* containing the coding region of the reporter gene *Gus* (*uidA*), encoding β-glucuronidase (GUS), fused to the promoter sequence of *ecp*1 or *ecp*2 (P*ecp*1 or P*ecp*2, respectively). The construction of the P*ecp*1-*Gus* and P*ecp*2-*Gus* fusions is depicted in Figure 3. Perfect fusions of the *ecp* promoter fragments with *Gus* were made by introducing an *Nco*I site at the ATG start codon of the *ecp* genes with polymerase chain reaction (PCR). This *Nco*I site was subsequently used for the fusion to the coding region of the *Gus* gene (*uidA*) in pCF20. The *ecp* promoter–*Gus* fusion constructs were introduced into *C. fulvum* race 5 by cotransformation using the vector pAN7-1, containing the hygromycin B resistance gene as a selection marker. Cotransformation was assessed by screening different hygromycin-resistant P*ecp*-*Gus* transformants for GUS activity *in vitro* (grown on potato-dextrose agar) and *in planta* (grown on susceptible tomato seedlings) (results not shown). Several transformants of *C. fulvum* were used for Southern analysis. All transformants which revealed induced GUS activity *in planta* contained at least one intact copy of the P*ecp*1-*Gus* or P*ecp*2-*Gus* fusion construct (results not shown). Four representative transformants, which revealed an increased GUS activity *in planta* and contained a low number of intact insertions of the particular P*ecp*-*Gus* construct, were analyzed by northern blotting (P*ecp*1-*Gus* transformants ecp1-6 and ecp1-21 and P*ecp*2-*Gus* transformants ecp2-26 and ecp2-40). Transcript levels of *uidA*, *ecp*1, and *ecp*2 were compared after culture of the transformants *in vitro* and *in planta*. In both the P*ecp*1-*Gus* and the P*ecp*2-*Gus* transformants, the level of *uidA* transcript was similar to that of endogenous *ecp*1 and *ecp*2 genes (Fig. 4A, ecp1-6 and ecp1-21, and Fig. 4B, ecp2-26 and ecp2-40). As controls, similar studies were performed with a transgenic race 4 of *C. fulvum* containing the *uidA* gene fused to the constitutive *gpd* promoter of *Aspergillus nidulans* (P*gpd*-*Gus*) (Fig. 4, gpd-gus) and with an untransformed race 5 of *C. fulvum* (Fig. 4, race 5). In the P*gpd*-*Gus* transformant grown *in vitro*, a high level of *uidA* transcript was observed, while in the P*ecp*1-*Gus* transformants grown under similar conditions no *uidA* transcript was detected (Fig. 4A, lanes F), and in the P*ecp*2-*Gus*

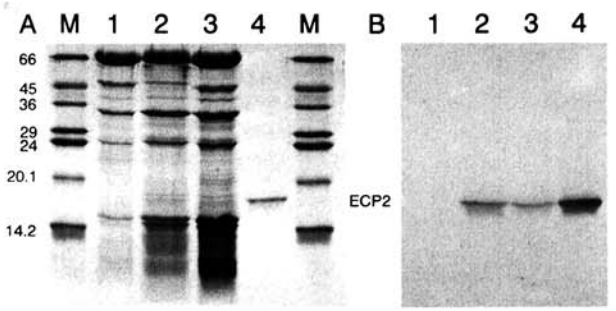

Fig. 1. A, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of 50-µl samples of apoplastic fluid obtained from tomato genotype Cf4 in an incompatible interaction with *Cladosporium fulvum* race 5 (lane 1), from genotype Cf4 in a compatible interaction with race 4 (lane 2), and from genotype Cf5 in a compatible interaction with race 5 (lane 3), at 14 days after inoculation, and of purified ECP2 (lane 4). The two outer lanes (M) contain molecular weight markers (kD). **B,** Immunoblot of a gel similar to the one in **A,** incubated with antibodies raised against ECP2. The antibodies have a high affinity to the purified protein (lane 4) and detect ECP2 only in apoplastic fluid from compatible interactions (lanes 2 and 3), but not in that from an incompatible interaction (lane 1).

transformants only low amounts of *uidA* transcript were observed (Fig. 4B, lanes F). The P*gpd-Gus* transformant, however, showed reduced *ecp*1 and *ecp*2 expression *in planta,* compared to the P*ecp-Gus* transformants and the untransformed *C. fulvum* race 5 (Fig. 4, lanes 4, 7, and 11). This may be due to a difference in the genetic backgrounds of the two types of transformants, which relates to the reduced growth of race 4, compared to race 5 (Joosten *et al.* 1990b). In untransformed race 5 of *C. fulvum* no *uidA* transcript could be detected, while the transcript level of *ecp*1 and *ecp*2 was similar to that of the P*ecp-Gus* transformants (Fig. 4, race 5). The constitutively expressed *C. fulvum* actin gene was used to quantify the amount of fungal RNA in each sample (Van den Ackerveken *et al.* 1993). The actin transcript was abundantly present in the fungus grown *in vitro* (Fig. 4, lanes F). Increasing levels of actin transcript were observed *in planta* (Fig. 4, lanes 4, 7, and 11), indicating that the amount of fungal RNA in the samples increased as the infection proceeded. The P*gpd-Gus* transformant grown *in planta* contained very low actin mRNA levels, indicating that the growth of this transformant (race 4) *in planta* is much less heavy than the growth of the other transformants (race 5) of *C. fulvum.*

Expression of the *ecp*1 and *ecp*2 genes during pathogenesis.

The P*ecp-Gus* transformants of *C. fulvum* were used to study the expression of the *ecp*1 and *ecp*2 genes in time and space during pathogenesis. GUS activity was estimated at different stages of infection of 3-wk-old tomato seedlings that had been inoculated with the different transformants of *C. fulvum* (Table 1). In the P*ecp*1-*Gus* transformants, no GUS activity was observed in conidia, germ tubes, and runner hyphae on the surface of inoculated tomato leaves at early stages of infection (Fig. 5A). However, in the P*ecp*2-*Gus* transformants, several conidia, germ tubes, and runner hyphae on leaves showed a low level of GUS activity (Table 1). Once the fungus had entered the tomato leaf through stomata around 4 days postinoculation, an increase in GUS activity in the thickened hyphae during growth in the intercellular space was observed in both the P*ecp*1-*Gus* transformants (Fig. 5B) and the P*ecp*2-*Gus* transformants (results not shown). Between 5 and 10 days postinoculation abundant fungal growth was observed near vascular tissue, which was correlated with high GUS activity in both the P*ecp*1-*Gus* transformants (Fig. 5C) and the P*ecp*2-*Gus* transformants (Fig. 5D). Ten days

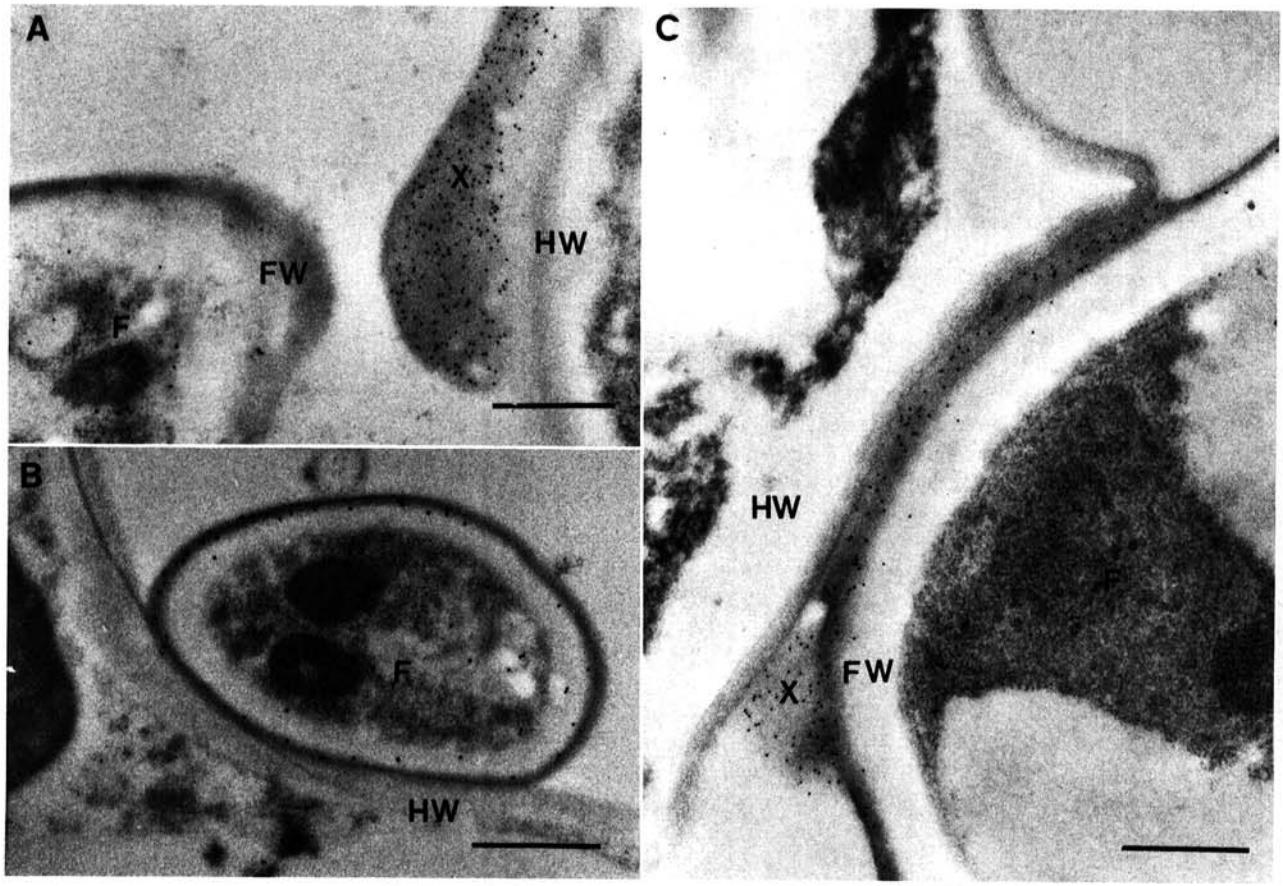

Fig. 2. Immunocytological localization of ECP1 and ECP2 in tomato leaves inoculated with *Cladosporium fulvum.* Immunogold labeling of ECP1 (**A**) and ECP2 (**B** and **C**) was performed on ultrathin sections of leaf material from tomato genotype Cf5 in a compatible interaction with *C. fulvum* race 5, at 12 days after inoculation. Gold particles are visible in fungal hyphae (F) and in extracellular material (X) between the fungal cell wall (FW) and the tomato mesophyll cell wall (HW). In control experiments, performed on uninoculated tomato leaves with the specific antibodies and on inoculated leaves with preimmune serum, no labeling was observed (results not shown). Bar = 0.5 μm.

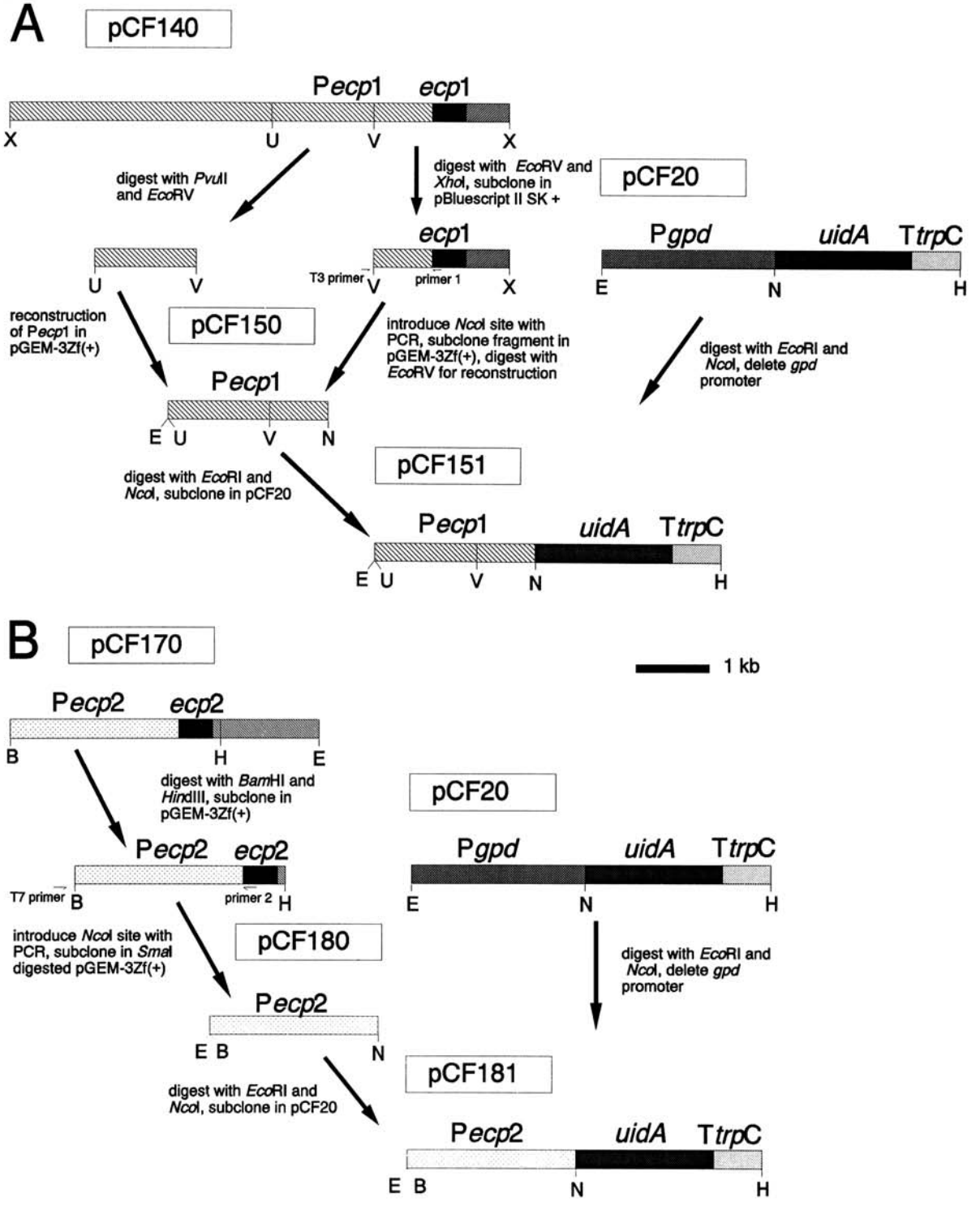

Fig. 3. Construction of transformants of *Cladosporium fulvum* containing the coding region (*uidA*) of the reporter gene G*us* fused to promoter sequences of the *ecp*1 and *ecp*2 genes. **A,** To construct the *ecp*1 promoter–*Gus* fusion, the *gpd* promoter (P*gpd*) present in pCF20 (Van den Ackerveken *et al.* 1994) was deleted by digestion with *Eco*RI and *Nco*I and replaced by a 2.2-kb *ecp*1 promoter fragment (P*ecp*1) isolated from pCF140 (Van den Ackerveken *et al.* 1993). An *Nco*I site was introduced at the ATG start codon of the *ecp*1 gene with polymerase chain reaction (PCR); and after reconstruction of the promoter in pGEM-3Zf(+) (pCF150), this plasmid was digested with *Eco*RI and *Nco*I, and the 2.2-kb P*ecp*1 region was isolated and ligated to the *uidA* coding region in pCF20 (pCF151). **B,** A similar approach was used for the fusion of the promoter of *ecp*2 with the *Gus* coding region. The *gpd* promoter (P*gpd*) in pCF20 was replaced by a 2.3-kb *ecp*2 promoter fragment (P*ecp*2) from pCF170 (Van den Ackerveken *et al.* 1993). With PCR, an *Nco*I site was introduced at the ATG start codon of the *ecp*2 gene, and this PCR product was subcloned in pGEM-3Zf(+) (pCF180). The 2.3-kb P*ecp*2 fragment was isolated from pCF180 by digestion with *Eco*RI and *Nco*I and ligated to the *uidA* gene in pCF20 to obtain pCF181. B = *Bam*HI, E = *Eco*RI, H = *Hin*dIII, N = *Nco*I, U = *Pvu*II, V = *Eco*RV, and X = *Xho*I.

postinoculation, young conidiophores originating from the substomatal primordia and emerging through stomata showed high GUS activity (Fig. 5E), while the newly formed conidia showed much less GUS activity or none at all, as can be seen in P*ecp1-Gus* transformants (Fig. 5F). The transformant of *C. fulvum* containing the *uidA* gene controlled by the constitutive *gpd* promoter showed a consistently high level of GUS activity in conidia and during all growth stages *in planta* (results not shown), confirming that the *uidA* gene is constitutively expressed and that the availability of substrate (X-Gluc) was not a limiting factor in the GUS assays.

DISCUSSION

Here we describe the isolation of the fungal protein ECP2 and localization of both ECP1 and ECP2, which are produced by *C. fulvum* during pathogenesis on tomato. The proteins were purified from apoplastic fluid originating from *C. fulvum*–infected tomato leaves. Upon anion exchange chromatography ECP2 eluted in two different fractions, which after analysis by SDS-PAGE were shown to contain one and the same protein. ECP2 likely occurs in two conformations, a dimeric and a monomeric form, which are in equilibrium.

The localization of ECP1 and ECP2 proteins and *in situ* expression of *ecp*1 and *ecp*2 genes during pathogenesis were studied to obtain further information about a possible role for these proteins in the interaction between *C. fulvum* and tomato. Immunolocalization experiments revealed that ECP1 and ECP2 accumulated predominantly in extracellular material in close contact with fungal and host cell walls at late stages of infection. Compared to the accumulation of the ECPs in the apoplast, less accumulation of ECP1 and ECP2 was detected in the fungal hyphae themselves. Therefore, these proteins must be released from hyphae into the intercellular space. Most likely, this is achieved by secretion, as predicted from the presence of a signal sequence in the preprotein of both ECP1 and ECP2 (Van den Ackerveken *et al.* 1993). Aside from ECP1 and ECP2, tomato PR-1b, unesterified pectin, and arabinogalactan proteins were also detected in this extracellular material by immunogold labeling (J. P. Wubben, unpublished), indicating that it consists of a complex network of fungal and plant macromolecules.

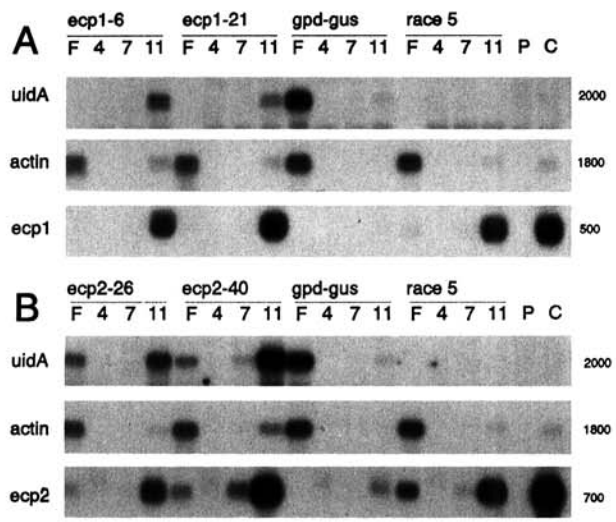

Fig. 4. Northern blot analysis of total RNA (10 µg) isolated from tomato seedlings infected by *Cladosporium fulvum* at 4, 7, and 11 days after inoculation (lanes 4, 7, and 11, respectively) and from *C. fulvum* grown in liquid shake culture (lane F in each series). Blots of the transformants ecp1-6 and ecp1-21 of *C. fulvum*, with the P*ecp1-Gus* construct (**A**), and of the transformants ecp2-26 and ecp2-40, with the P*ecp2-Gus* construct (**B**), were hybridized with *ecp*1 and *ecp*2, respectively, and with the *uidA* and actin genes. RNA of the constitutively expressing GUS transformant (gpd-gus) and of the untransformed *C. fulvum* race 5 was blotted as well. Lane P contains RNA from leaves of uninoculated tomato plants, and lane C contains RNA from leaves of infected susceptible tomato (genotype Cf5 infected by race 5), isolated at 14 days after inoculation.

Previously, GUS assays have been used to follow the growth of *C. fulvum in planta* (Oliver *et al.* 1993; Roberts *et al.* 1989). Here, transformants of *C. fulvum* containing fusions of the promoter of *ecp*1 or *ecp*2 with the coding region of the *Gus* reporter gene (*uidA*) were used to study the expression of *ecp*1 and *ecp*2 during colonization of tomato. The transformants with intact insertions of P*ecp-Gus* constructs all showed induced GUS activity *in planta*. No GUS activity was found in transformants which lacked the P*ecp-Gus* constructs. Expression studies, using northern blot analyses, revealed that the P*ecp*1-*Gus* and P*ecp*2-*Gus* transformants accumulated *uidA* transcripts at levels similar to those of the *ecp*1 and *ecp*2 transcripts in untransformed race 5 of *C. fulvum*. Therefore, the GUS activity of the P*ecp*1-*Gus* and P*ecp*2-*Gus* transformants *in planta* is a representative measure of *ecp*1 and *ecp*2 expression during pathogenesis.

The histochemical GUS assays as performed with X-Gluc staining on tomato seedlings inoculated with the P*ecp-Gus* transformants clearly demonstrated that transcription of *ecp*1 and *ecp*2 does not increase immediately after the fungus has entered a leaf through stomata. This is in contrast to transcription of the avirulence gene *avr*9 of *C. fulvum,* which starts immediately after the fungus penetrates a tomato leaf (Van den Ackerveken *et al.* 1994). Increased expression of *ecp*1 and *ecp*2 is observed especially when the fungus is growing near vascular tissue. The high expression could be due to a high metabolic activity of the fungus in that area of

Table 1. β-Glucuronidase (GUS) activity of the P*ecp-Gus* transformants of *Cladosporium fulvum* grown *in vitro* or in susceptible tomato seedlings[a]

Growth stage	Days after inoculation	P*ecp*1-*Gus*	P*ecp*2-*Gus*
Fungus grown *in vitro* on potato-dextrose agar	...	−	+
Conidia on leaf surface	...	−	+
Germ tubes	1–2	−	+
Runner hyphae	2–4	−	+
Penetration hyphae	4–5	−	+
Thickened hyphae in mesophyll	5–10	++	++
Branched hyphae near vascular tissue	5–10	++	++
Substomatal primordia	10–14	++	++
Conidiophores	10–16	++	++
Conidia	12–16	−	+

[a]GUS activity was estimated by studying three or four leaflets of infected 3-wk-old tomato seedlings, stained for GUS activity as described in Materials and Methods, at 4, 6, 10, 12, and 16 days postinoculation. −, No GUS activity observed (Fig. 5A and F). +, Low GUS activity observed in some hyphae (approximately one out of 10). ++, Consistent strong GUS activity in all hyphae (Fig. 5B–E).

the plant. However, most likely, specific environmental conditions in the intercellular space or particular plant signals are required to initiate transcription of the *ecp*1 gene and to increase transcription of the *ecp*2 gene, since no such high levels of expression were found when the fungus was grown *in vitro*. As in *Rhizobium*- and *Agrobacterium*-plant interactions (Peters *et al.* 1986; Stachel *et al.* 1985), plant factors might be involved in the activation of the *ecp* genes. Low nitrogen concentration was found to trigger the expression of avirulence gene *avr*9 *in vitro* (Van den Ackerveken *et al.* 1994) but did not induce *ecp*1 or *ecp*2 gene expression (G. F. J. M. Van den Ackerveken, unpublished). Since *ecp*1 and *ecp*2 gene expression was low in runner hyphae on leaves and in newly formed conidia, a role for these proteins during pathogenesis is likely to be restricted to intercellular growth in tomato leaves. It is known that, upon infection, the fungal biomass increases substantially, and leaves develop symptoms of nutrient limitation. *C. fulvum* significantly influences the carbohydrate metabolism of the tomato plant (Joosten *et al.* 1990b) and withdraws sugars and other nutrients from tomato leaves, primarily near vascular tissue, the area where the expression of *ecp*1 and *ecp*2 is highest.

The nucleotide sequences and amino acid sequences of ECP1 and ECP2 did not reveal known enzymatic or structural functions (Van den Ackerveken *et al.* 1993). However, a significant similarity was observed in a repeat pattern of cysteines in ECP1 and tumor necrosis factor receptors from mammalian systems (Bazan 1993). This author suggested a role for ECP1 as a soluble receptor, of which the host target might be a protein mediator of the plant defense response (Bazan 1993), a hypothesis which is currently under investigation.

Although the exact role of ECP1 and ECP2 during pathogenesis of *C. fulvum* on tomato remains to be elucidated, the localization of the proteins and the high level of expression of their encoding genes *in planta* suggest functions related to the growth of the fungus inside the tomato leaf. Presently, experiments are in progress in which the *ecp*1 and *ecp*2 genes will be disrupted separately or in combination, in order to study their role in pathogenicity.

MATERIALS AND METHODS

Purification of ECP2 and production of polyclonal antibodies.

For the purification of ECP2, 300 ml of apoplastic fluid was isolated from a compatible interaction between tomato genotype Cf5 and race 5 of *C. fulvum* at 14 days after inoculation. Proteins were precipitated by adjusting the apoplastic fluid to 60% (v/v) acetone, followed by incubation at –20° C for 3 hr and centrifugation at 1,000 *g* for 5 min. The pellet was air-dried and resuspended in 7.5 ml of elution buffer (50 mM Tris-HCl and 100 mM NaCl, pH 7.5). After centrifugation for 10 min at 1,500 *g* the proteins present in the supernatant were subjected to gel filtration on a Sephadex G-50 column as described previously (Joosten *et al.* 1990a). Fractions were combined according to the UV absorption profile (280 nm) of the eluate and analyzed by SDS-PAGE. Fractions containing the 17-kD ECP2 protein were dialyzed against H_2O, freeze-dried, and dissolved in 30 mM Tris-HCl buffer, pH 8.5 (buffer A). Aliquots of 200 µl, containing about 3 mg of protein, were subjected to high-resolution liquid chromatography (Bio-Rad, Richmond, CA) on an MA 7Q anion exchange column (Bio-Rad) equilibrated with buffer A. After the sample was loaded, the column was washed for 3 min with buffer A at a flow rate of 1.5 ml min^{-1}, and the bound proteins were subsequently eluted in buffer A, with a linear NaCl gradient from 0 to 200 mM in 15 min, followed by a linear gradient from 200 to 400 mM in 2.5 min, at a flow rate of 1.5 ml min^{-1}. Fractions (0.75 ml) were collected and analyzed by SDS-PAGE and native high-pH PAGE as described by Joosten *et al.* (1990a). Fractions containing ECP2 were freeze-dried, dialyzed against H_2O, and rechromatographed under similar conditions, except that 30 mM Tris-HCl, pH 9.3, was used. This resulted in about 300 µg of pure ECP2. Polyclonal antibodies against the ECP2 protein were raised in rabbits as described previously (Joosten and De Wit 1988).

Immunogold localization of ECP1 and ECP2.

Immunolocalization experiments were performed as described previously (Wubben *et al.* 1992). Leaf discs of *C. fulvum*–inoculated tomato, obtained at 7 and 12 days postinoculation, were fixed with formaldehyde and glutaraldehyde and subsequently embedded in LR Gold resin (Agar Scientific, Stansted, England) at low temperature (–20° C). Ultrathin sections were cut and incubated with antibodies. Detection of the antibodies was performed with protein A–gold (7 nm). For each experiment, 10–20 sections of five leaf samples each were labeled. The sections were stained with uranyl acetate and lead citrate and were then examined with a Philips CM 12 transmission electron microscope at 80 kV.

Construction of *ecp*1 and *ecp*2 promoter–GUS fusions.

All recombinant DNA techniques were performed essentially according to Sambrook *et al.* (1989). Fusions were made between promoter sequences of *ecp*1 or *ecp*2 and the *Gus* (*uidA*) reporter gene to study the expression of these genes in *C. fulvum* during colonization of tomato (Fig. 3). For both constructs, a fragment of at least 2 kb, upstream of the ATG start codon of the gene, was used.

For the *ecp*1 promoter (Fig. 3A), a 1.8-kb *Eco*RV/*Xho*I fragment of pCF140, containing the *ecp*1 gene (Van den Ackerveken *et al.* 1993), was subcloned in pBluescript II SK+ (Stratagene, La Jolla, CA). An *Nco*I site was introduced at the ATG start codon of the gene with PCR using primer 1 (5′-CGGGATCCATGGTGGAGGGAAGTGGG-3′), which contains an additional *Bam*HI site at the 5′ end, and the T3 primer of pBluescript II SK+. The PCR product was digested with *Bam*HI, and the resulting fragment of 0.8 kb was subcloned in pGEM-3Zf(+) (Promega, Madison, WI). A *Pvu*II/*Eco*RV fragment of the *ecp*1 promoter region from pCF140 was subcloned at the *Eco*RV site of the PCR fragment, which resulted in the reconstruction of a 2.2-kb *ecp*1 promoter fragment in pGEM-3Zf(+) (pCF150). The *gpd* promoter in front of the *uidA* gene in pCF20 was replaced by the *ecp*1 promoter fragment (Van den Ackerveken *et al.* 1994), using the *Eco*RI and *Nco*I sites (pCF151).

Cloning of the *ecp*2 promoter fragment in front of the *uidA* coding region was performed essentially the same as for the P*ecp*1-*Gus* construct (Fig. 3B). A 2.9-kb *Hin*dIII/*Bam*HI fragment of pCF170 containing the *ecp*2 gene (Van den Ackerveken *et al.* 1993) was subcloned in pGEM-3Zf(+). An *Nco*I

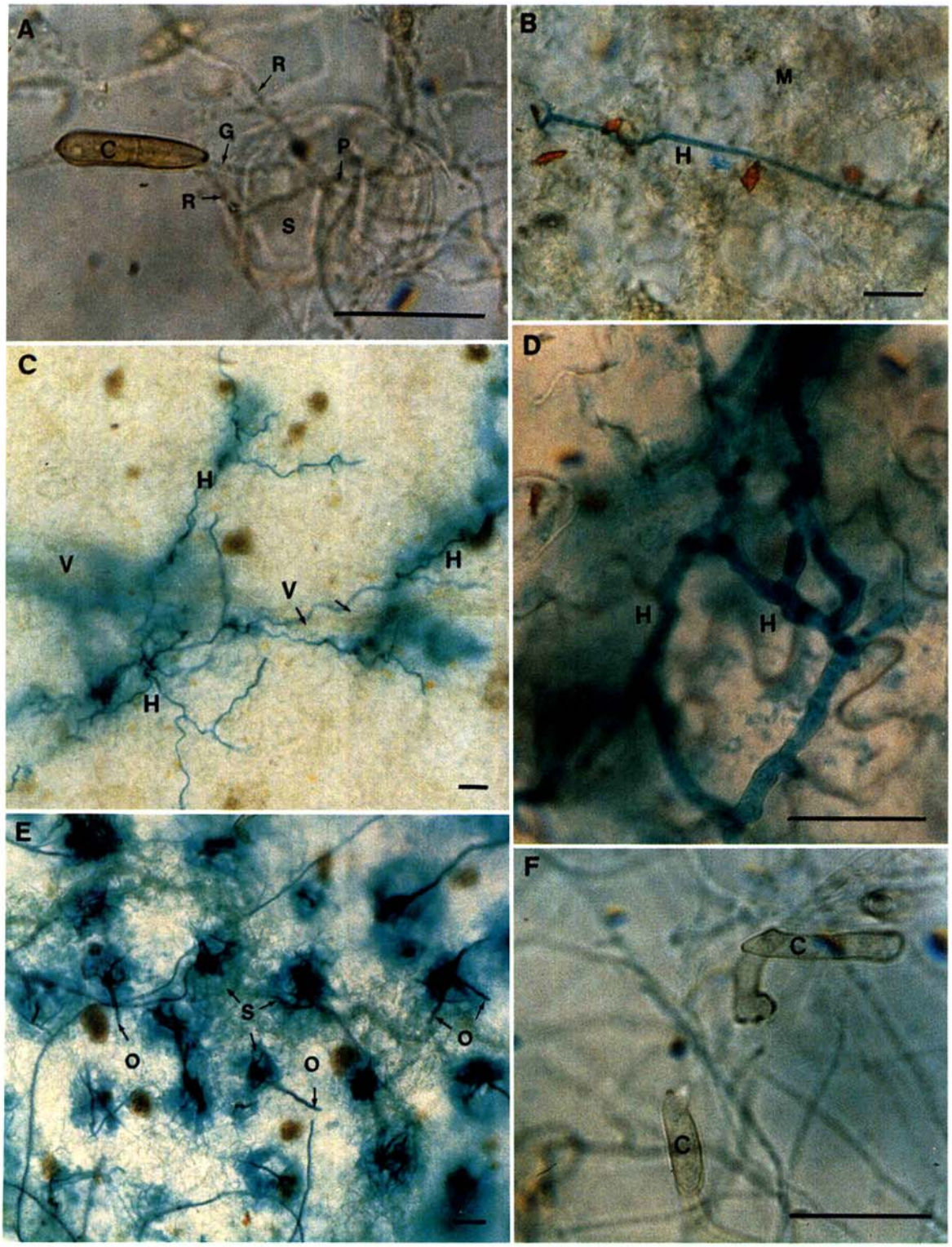

Fig. 5. Histochemical localization of β-glucuronidase (GUS) activity in susceptible tomato seedlings inoculated with the *ecp* promoter–*Gus* transformants of *Cladosporium fulvum*. Inoculated leaflets of tomato seedlings were incubated in X-Gluc and subsequently destained in 70% (v/v) ethanol. GUS activity is visible as a clear blue stain. **A,** Germinated conidium (C) with germ tube (G) and several runner hyphae (R) on the lower epidermis of a tomato leaf are visible as well as penetration of a stoma (S) by penetration hyphae (P). **B,** After penetration of a leaf, a long hypha (H) is visible in the intercellular space between mesophyll cells (M). **C,** Abundant growth of hyphae (H) can be seen near vascular tissue (V). **D,** High GUS activity is visible in branched hyphae (H) near vascular tissue. **E,** Around 12 days after inoculation, conidiophores (O) emerge from stomata (S). **F,** The conidiophores eventually produce conidia (C) on the lower side of the leaf. The transformants shown are ecp1-6 at 6 days after inoculation (**A–C**), ecp2-40 at 8 and 10 days after inoculation (**D** and **E,** respectively), and ecp1-21 at 16 days after inoculation (**F**). Bar = 25 μm.

site was created at the ATG start codon of the gene with PCR using primer 2 (5′–CGGGATCCATGGTGAAGCTGGTTG-TATGG-3′) and the T7 primer from pGEM-3Zf(+). A 2.3-kb PCR fragment was subcloned in a *Sma*I-digested pGEM-3Zf(+) vector (pCF180) as described by Holton and Graham (1991). The *gpd* promoter in front of the *uidA* gene in pCF20 was subsequently replaced by the 2.3-kb *Eco*RI/*Nco*I promoter fragment of *ecp*2 (pCF181).

Fungal protoplast isolation and transformation.

Isolation of protoplasts and transformation of *C. fulvum* were performed according to the procedures described by Harling *et al.* (1988) and Oliver *et al.* (1987) with the modifications of Van den Ackerveken *et al.* (1992). Cotransformation of *C. fulvum* was performed by adding 4 μg of DNA (*ecp* promoter–*Gus* fusion constructs pCF151 and pCF181) and 2 μg of pAN7-1 as a selection marker (Punt *et al.* 1987) to protoplasts of race 5 of *C. fulvum*.

GUS activity assays.

GUS activity assays were performed as described by Jefferson *et al.* (1987). In transformants, GUS activity was assayed on conidia germinated and grown on filter paper placed on potato-dextrose agar. The filter paper with germinated conidia was incubated overnight with X-Gluc (0.5 mg ml^{-1}) (Clontech, Palo Alto, CA) in 50 mM phosphate buffer, pH 7.0, 1 mM KFeCN, and 0.05% (v/v) Triton X-100, and GUS activity was estimated visually by the development of blue color in the colonies. The GUS activity of the fungus grown *in planta* was determined in homogenized leaf tissue. Ten volumes (v/w) of extraction buffer (50 mM NaH_2PO_4, pH 7.0, 10 mM EDTA, 0.1% [v/v] Triton X-100, 0.1% [w/v] SDS, and 10 mM β-mercaptoethanol) were added to the infected leaflets, and the material was homogenized with a pestle in a 1.5-ml reaction vial. After centrifugation for 1 min at 15,000 *g*, 25 μl of the supernatant was transferred to a single well of a 96-well microtiter plate. Aliquots of 25 μl of 2 mM 4-methylumbelliferyl glucuronide (Research Organics, Cleveland, OH) in extraction buffer were added to each well, and the plate was incubated at 37° C for 2 hr. GUS activity was estimated visually on a UV transilluminator. Both these activity assays were used only to screen for cotransformation and to select for transformants which showed induced GUS activity *in planta*. Transformants containing the GUS gene fused to the constitutive *gpd* promoter were used as an internal standard.

Southern blot analysis.

DNA was extracted from freeze-dried mycelium as described by Van Kan *et al.* (1991). A 5-μg sample of DNA was digested with restriction endonucleases, separated on a 1% agarose gel, and blotted onto Hybond-N^+ (Amersham, 's Hertogenbosch, Netherlands) by alkali blotting. Filters were hybridized with random-primed ^{32}P-labeled DNA fragments (Hodgson and Fisk 1987). For P*ecp*1-*Gus* transformants the blot was hybridized with a probe containing a 1.4-kb *Eco*RV/-*Hin*dIII *uidA-trpC* fragment or a 0.4-kb *Eco*RI/*Hin*dIII *ecp*1 promoter fragment. For P*ecp*2-*Gus* transformants a 2.9-kb *Eco*RI/*Eco*RV fragment of pCF181, containing a part of the promoter of *ecp*2 and a part of the coding sequence of *uidA*, was used as a probe.

Northern blot analysis.

RNA was isolated from freeze-dried mycelium of *C. fulvum* and *C. fulvum*–infected seedlings as described by Van Kan *et al.* (1991). A 10-μg sample of total RNA was separated on a 1.5% denaturing formaldehyde-agarose gel and transferred onto Hybond-N^+ membranes as described by Sambrook *et al.* (1989). Filters were hybridized with random-primed ^{32}P-labeled DNA fragments obtained from *ecp*1 and *ecp*2 cDNA clones and *uidA* and actin genomic clones.

Histochemical GUS assay.

Primary leaves of the inoculated seedlings were infiltrated at reduced pressure with X-Gluc (0.5 mg ml^{-1}) in 50 mM phosphate buffer, pH 7.0, 1 mM KFeCN, and 0.05% (v/v) Triton X-100 and incubated overnight at 37° C. The tissue was subsequently decolorized with 70% (v/v) ethanol and mounted on microscope slides in 50% (v/v) glycerol in phosphate-buffered saline. For histochemical GUS assays two individual transformants of each construct were used, respectively, for inoculation of leaves of six 3-wk-old tomato seedlings. The experiment was repeated two times. The stained leaves were examined with a Zeiss Axioscope microscope, and photographs were taken with a Zeiss MC-100 camera unit.

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