Cf Gene-Dependent Induction of a β-1,3-Glucanase Promoter in Tomato Plants Infected with *Cladosporium fulvum*

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Tomato *Cf* genes confer resistance to specific races of the leaf mold pathogen *Cladosporium fulvum*. The *Cf* gene-dependent induction of a *Nicotiana plumbaginifolia* β-1,3-glucanase (*gn1*) promoter *-GUS* reporter gene fusion was monitored following either inoculation with race 0 *C. fulvum* or leaf injections with race-specific elicitors. In incompatible interactions, intense foci of GUS activity appeared at infection sites 1–4 days after hyphae entered the leaf through stomata. The time of appearance of these foci and their relative final size was different on *Cf-2, Cf-4, Cf-5,* or *Cf-9*-containing near-isogenic lines (NILs) of the cultivar Moneymaker. In the compatible interaction, *gn1:GUS* induction was later and occurred just prior to sporulation. Co-localization of GUS activity and fungal hyphae revealed that the spatial patterns of induction in relation to fungal hyphae differed between the various incompatible interactions studied. Following race-specific elicitor injection, increased GUS activity was only detected in *Cf* gene-containing NILs. Induction was detected in the *Cf-9* containing NIL within 12 hr and in those carrying *Cf-2* or *Cf-5* within 24 hr. RNA gel blot analysis showed that the induction kinetics of the *gn1* promoter resembled that of an endogenous acidic β-1,3-glucanase gene.

Additional keywords: Lycopersicon esculentum, pathogenesis-related protein, tomato leaf mold.

Disease resistance (*R*) genes have long been deployed by plant breeders and studied by geneticists, but for many interactions, little is known about how they lead to effective plant defense. A wide array of inducible plant defense responses exist, including the accumulation of phytoalexins (Darvill and Albersheim 1984), proteinase inhibitors (Ryan 1990), and pathogenesis-related (PR) proteins (Bowles 1990; Linthorst 1991), and the development of the hypersensitive response (HR) (Klement 1982). Often these responses are associated with the transcriptional activation of defense-related genes (Dixon and Harrison 1990).

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Plant defense genes which are induced more rapidly at the site of the incompatible host-pathogen interface than the compatible one are likely to have a causal role in pathogen containment. A few examples of this type have been found. In a study of the bean-*Colletotrichum lindemuthianum* interaction, tissue dissection and RNA gel blot analysis was used to show that phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), and hydroxyproline-rich glycoproteins (HRGPs) accumulate earlier during incompatible, than compatible, interactions. Induction was confined predominantly to the inoculated areas in the incompatible interactions (Showalter *et al.* 1985; Bell *et al.* 1986). In the bean-*Pseudomonas syringae* pv. *phaseolicola* interaction, using the same experimental approach, the rapid and differential induction of PAL, CHS, and a chitinase gene was revealed to occur only within the infection site (Meier *et al.* 1993). In the same interaction, a lipoxygenase gene was induced only in non-infected tissue surrounding incompatible infections but was induced in both the infected and surrounding non-colonized tissues in compatible infections. In another study *in situ* hybridizations were used to localize defense gene induction during interactions between potato and *Phytophthora infestans* (Cuypers *et al.* 1988). However, in all the examples given above the differential interactions studied were generated using non-isogenic host plant cultivars and/or pathogen races. Potentially, therefore, genotypic differences other than at the resistance and avirulence gene loci could have contributed to the differential responses observed.

Other studies have demonstrated defense gene induction strictly dependent on the presence of an *R* gene, but did not attempt to localize the spatial pattern of this induction. Kiedrowski *et al.* (1992) exploring the *Arabidopsis-Pseudomonas syringae* interaction, used F_3 families either homozygous for the resistance gene *RPM1* or homozygous for the recessive allele *rpm1*, to reveal the rapid activation of a novel defense gene *ELI3* to be *RPM1* dependent. Similarly, Davidson *et al.* (1988) used near-isogenic susceptible and resistant barley lines to *Erysiphe graminis* f. sp. *hordei* to reveal *R*-gene-dependent induction of two mRNAs in lines carrying the *Mla, Mlp,* or *Mlk* resistance genes.

The interaction between tomato and *Cladosporium fulvum* is an excellent system with which to investigate defense gene induction mediated by single, genetically defined resistance genes. *Cf* resistance genes in the plant are matched by a corresponding range of genetically defined races (Day 1956; de Wit *et al.* 1987). The cloning of the *C. fulvum avr9* gene (Van Kan *et al.* 1991; Van den Ackerveken *et al.* 1992) has con-

firmed that this interaction conforms to Flor's "gene-for-gene" hypothesis in that incompatibility occurs when resistance (*R*) genes in the plant are matched by corresponding avirulence (*avr*) genes in the pathogen (Flor 1955). Studies are further facilitated by the availability of near-isogenic lines (NILs) of tomato which differ only in the *Cf* gene that they carry (Tigchelaar 1984). Some *Cf* genes (e.g., *Cf-2*) confer a strong resistance in which the pathogen is contained shortly after penetrating the stomata (Lazarovits and Higgins 1976b; de Wit 1977; Hammond-Kosack and Jones 1994). Other *Cf* genes (e.g., *Cf-3*) confer a resistance in which extensive fungal growth is permitted before resistance is expressed (Lazarovits and Higgins 1976b; Oliver *et al.* 1993). In addition, a correlation has been found between the resistance phenotype of each *Cf* gene and its genetic location (Hammond-Kosack and Jones 1994). The closely linked *Cf-2* and *Cf-5* genes located on chromosome 6 (Dickinson *et al.* 1993; Jones *et al.* 1993), appear to condition a resistance phenotype which involves the localized swelling of mesophyll cells immediately surrounding the penetrated substomatal cavity. This cellular response is not apparent until later in incompatible interactions conditioned by the closely linked *Cf-4* and *Cf-9* genes, located on chromosome 1 (Jones *et al.* 1993). In these infections, mesophyll cell swelling was always restricted to behind the hyphal front (Hammond-Kosack and Jones 1994).

A further attribute of this system for studying defense gene induction is the availability of race-specific elicitors (SEs) of fungal origin. These elicitors, which are presumed to be the fungal avirulence gene products, are found in the intercellular washing fluids (IF) of susceptible tomato leaves infected with virulent races of *C. fulvum* (de Wit and Spikman 1982; de Wit *et al.* 1984). When injected into healthy tomato leaves these elicitors induce necrosis or chlorosis only when the appropriate *Cf* resistance gene is present (de Wit and Spikman 1982). Recent experimental evidence has confirmed that the elicitors present in IF which are active in *Cf-4*- and *Cf-9*-containing tomato lines are indeed the products of the corresponding fungal avirulence genes, *avr4* and *avr9*, respectively (Van den Ackerman *et al.* 1992; Joosten *et al.* 1994). Physiological responses known to be induced by IF in a *Cf* gene-dependent manner include increases in electrolyte leakage, lipoxygenase activity, lipid peroxidation, and the generation of active oxygen species (Peever and Higgins 1989; Vera-Estrella *et al.* 1992).

The defense response of tomato to *C. fulvum* hyphae has been correlated with the deposition of callose, a hypersensitive response, and the accumulation of phytoalexins and PR proteins (Lazarovits and Higgins 1976a,b; de Wit 1977; de Wit and Flach 1979; de Wit and Kodde 1981; de Wit *et al.* 1986). At least two of the PR proteins that accumulate were found to be β-1,3-glucanases; an acidic extracellular form and a basic intracellular form (Joosten and de Wit 1989; Van Kan *et al.* 1992). Recently, cDNA clones corresponding to two tomato β-1,3-glucanases have been isolated and their expression patterns compared during compatible and incompatible interactions with *C. fulvum* by RNA gel blot analysis (Van Kan *et al.* 1992). Transcripts for a basic isoform accumulated very rapidly in both types of interaction, while those coding for an acidic isoform were detectable earlier in incompatible interactions.

We are interested in identifying the important components of a successful defense response against *C. fulvum* and in determining whether different *Cf* resistance genes trigger effective defense by activating any of the known classes of plant defense gene promoter at the site of infection. We also wish to ascertain whether the different *Cf* genes condition qualitatively dissimilar or similar resistance reactions. As a first step towards these goals the induction of a fusion between the *Nicotiana plumbaginifolia* β-1,3-glucanase *gn1* gene promoter and the *E. coli* β-glucuronidase (*uidA*) gene (Castresana *et al.* 1990) was used to investigate the patterns of *gn1* promoter induction conditioned by four different *Cf* resistance genes after inoculation with *C. fulvum*. At the time these experiments were initiated, no promoters to endogenous tomato glucanase genes had been isolated. The promoter to the *gn1* gene was selected because it was already known to be inducible upon pathogen infection and the gene originated from a plant species closely related to tomato.

A stock lacking *Cf* genes (Cf0) was transformed with the *gn1:GUS* fusion, which was then introduced into near-isogenic lines (NILs) of tomato carrying different *Cf* resistance genes from primary transformants by conventional genetic crosses. The NILs were produced by six backcrosses of *Cf2, Cf4, Cf5,* or *Cf9* to the cultivar Moneymaker (Tigchelaar 1984). Each NIL shows very few RFLP differences from Moneymaker except at loci closely linked to the *Cf* genes (Jones *et al.* 1993; Dickinson *et al.* 1993; Balint-Kurti *et al.* 1994). This strategy standardized any chromosomal insertion effects acting on the transgene. The histochemical stain for GUS activity enabled us to ask whether the *gn1* promoter was induced specifically where the pathogen was attempting ingress. A sequential staining strategy using acid fuchsin was developed to localize the induction of the fusion in relation to the invading fungal hyphae. The induction of the fusion was

Table 1. Summary of tomato–*Cladosporium fulvum* interactions[a] investigated

		Races of *C. fulvum*		
		Race 0	Race 4	Race 5
		Genotypes of races		
Tomato line	*Cf* gene carried	*avr 2, 4, 5, 9*	*avr 2, 5, 9*	*avr 2, 4, 9*
Cf0	None	C	C	C
Cf2	*Cf-2*	I	I	I
Cf4	*Cf-4*	I	C	I
Cf5	*Cf-5*	I	I	C
Cf9	*Cf-9*	I	I	I

[a] C = Compatible interaction, I = incompatible interaction.

Table 2. Quantification of the growth of the GUS-expressing *Cladosporium fulvum* race 4 during compatible and incompatible interactions with tomato[a]

Days after inoculation	Cf0	Cf2	Cf4	Cf5	Cf9
6	109	104	ND	86	111
8	832	128	ND	86	125
10	3,187 a	72	ND	79	98
12	9,426 a	132	ND	113	175

[a] Values are given in picomoleof MU per milligram of protein per minute.
[b] Values are significantly different at the 5% level (Tukey's D).

also investigated after the injection of race-specific *C. fulvum* elicitors (IF) into leaves of tomato plants either carrying or lacking a *Cf* resistance gene. This permitted a synchronous challenge which was used to define the kinetics of *gn1:GUS* induction mediated by different *Cf* resistance genes.

RESULTS

Growth of a transgenic *GUS*-expressing *C. fulvum* isolate in leaves during compatible and incompatible interactions with tomato.

A transgenic race 4 of *C. fulvum* which constitutively expresses the *GUS* gene (Oliver *et al.* 1993) was used to compare the resistance conferred by different *Cf* resistance genes and to quantify fungal growth. *C. fulvum* race 4 lacks a functional *avr4* gene but expresses functional *avr2, avr5,* and *avr9* genes, which confer incompatible phenotypes on tomato lines containing the corresponding resistance genes *Cf-2, Cf-5,* and *Cf-9*, respectively (Table 1). In all experiments the *Cf* genes were in the heterozygous state. The levels of GUS activity recorded after the inoculation of Cf0, 2, 5, and 9 tomato plants with the *GUS*-expressing *C. fulvum* race 4 are shown in Table 2. In the incompatible interactions mediated by *Cf-2, 5,* or *9*, no statistically significant increases in GUS activity were recorded over the 12 days of the time-course. In the compatible interaction with Cf0, increased GUS activity was first observed 8 days after inoculation and accumulated rapidly thereafter. The GUS activity recorded 12 days after inoculation in the compatible interaction was over 50 times greater than for any of the incompatible interactions.

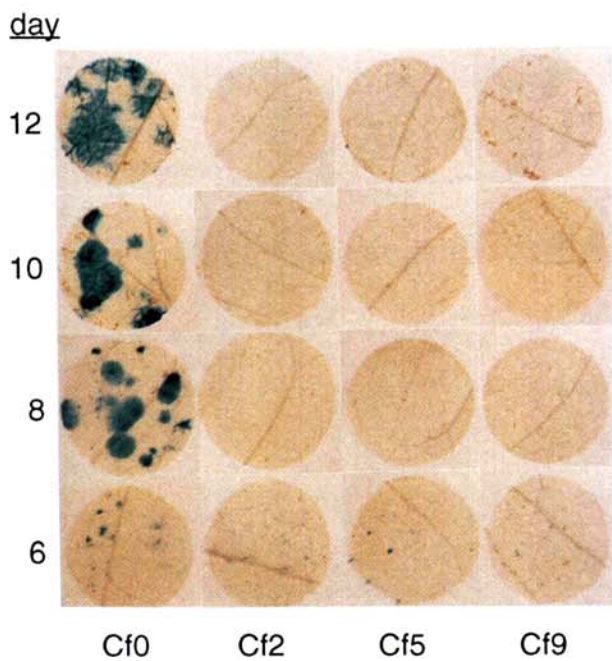


Fig. 1. Influence of *Cf* resistance genes on *Cladosporium fulvum* hyphal growth in tomato leaves. Histochemical localization of GUS activity in leaf disk samples (1 cm in diameter) cut from the leaves of near-isogenic lines of tomato infected with a transgenic, *C. fulvum* race 4 isolate which constitutively expresses β-glucuronidase. Shown is the compatible interaction on Cf0 and the incompatible interactions mediated by *Cf-2, Cf-5,* and *Cf-9.*

Histochemical localization of the GUS activity generated during the growth of the transgenic *C. fulvum* race is shown in Figure 1. Faint staining was detected in all interactions by 6 days postinoculation (p.i.), and this staining was associated with fungal germ tube growth on the surface of the leaves (data not shown). By day 8 the compatible *C. fulvum* infections on Cf0 were seen as macroscopic blue patches in the interveinal areas of the leaf. These blue patches increased in size over the remainder of the time-course. In the three incompatible interactions macroscopic staining was rarely detectable from day 8 onwards. However, in some experimental replicas small flecks of GUS activity were observed on Cf9 at later time-points (data not shown). These results confirm the effectiveness of the *Cf* genes in preventing fungal growth (Hammond-Kosack and Jones 1994) and facilitated the interpretation of the induction patterns of the *gn1:GUS* fusion.

The *gn1:GUS* transgenic tomato lines.

In total 15 independent transgenic tomato plants were recovered from the transformation experiments that carried the *gn1:GUS* fusion. After preliminary characterization of the basal pattern of expression during normal tomato development, two transformants (1623A and R) were selected. Each line contained a single T-DNA integration site, as judged from kanamycin resistance segregation ratios, and exhibited a similar pattern of expression to that originally reported for *N. plumbaginifolia* (Castresana *et al.* 1990). Both transformants were used for experiments outlined below and the results obtained from each were very similar.

Induction of the *gn1:GUS* fusion during compatible and incompatible interactions with *C. fulvum.*

Induction of the *gn1:GUS* fusion was monitored during compatible and incompatible interactions with *C. fulvum*. The experiment included uninoculated Cf0 control plants, the Cf0-race 0 compatible interaction and incompatible interactions with race 0 mediated by the *Cf-2, 4, 5,* and *9* resistance genes. Under the environmental regime used, stomatal penetration by *C. fulvum* germ tubes commenced in all interactions from day 5 onwards. The histochemical localization of GUS activity generated by *gn1:GUS* induction in leaf disk samples cut from infected plants is shown in Figure 2. Induction was observed in both compatible and incompatible interactions, though the patterns of induction differed.

With the compatible interaction on Cf0 some *gn1:GUS* induction occurred at day 10 onward and by day 12, when numerous conidiophores were emerging from the lower leaf surface, large patches of induction were observed. In the incompatible interactions, *gn1* induction was first observed between 6 and 10 days p.i. and took the form of intense foci of GUS activity. These foci increased in number and size over the duration of the time-course. The average size of the foci 12 days p.i. was dependent on which *Cf* gene was present. The largest foci of induction were observed in the *Cf-4*-mediated incompatible response (914 ± 73 μm in diameter), followed by *Cf-2* (566 ± 43 μm) and *Cf-9* (473 ± 41 μm), and then *Cf-5* (204 ± 38.6 μm). There was usually negligible induction of the *gn1:GUS* fusion in uninoculated control leaves harvested from young plants grown under the same high humidity conditions (data not shown).

To assess the spatial pattern of the *gn1:GUS* fusion induction relative to *C. fulvum* hyphae, leaf disks already stained to visualize GUS activity were restained with acid fuchsin (McBryde 1936). This latter dye stained hyphae and host cells deep and pale pink, respectively. In both compatible and incompatible interactions the areas of GUS staining were usually restricted to the sites of *C. fulvum* penetration or growth, although occasionally staining was observed that could not be attributed to the presence of the pathogen. Selected time-points from each interaction are shown in Figure 3.

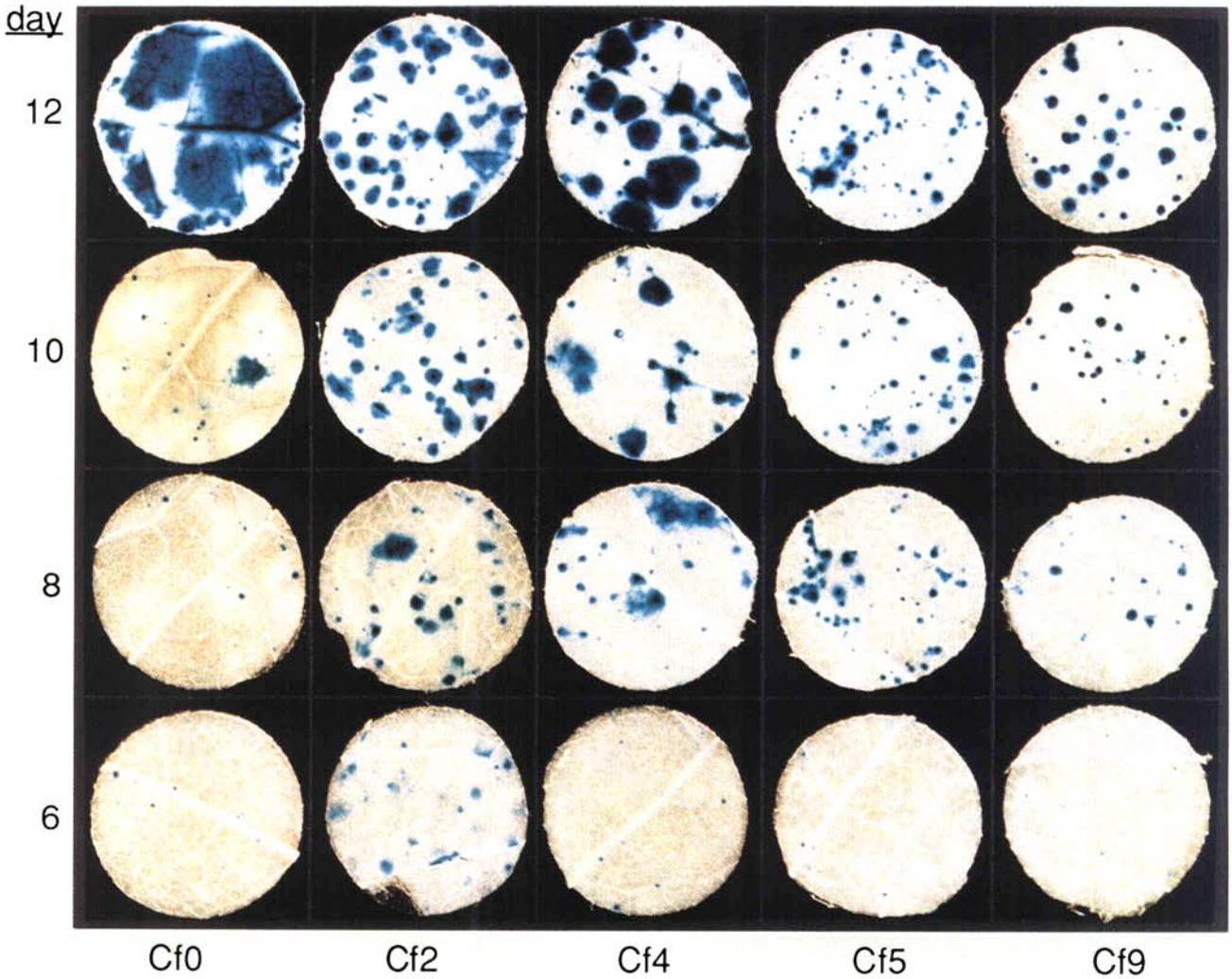
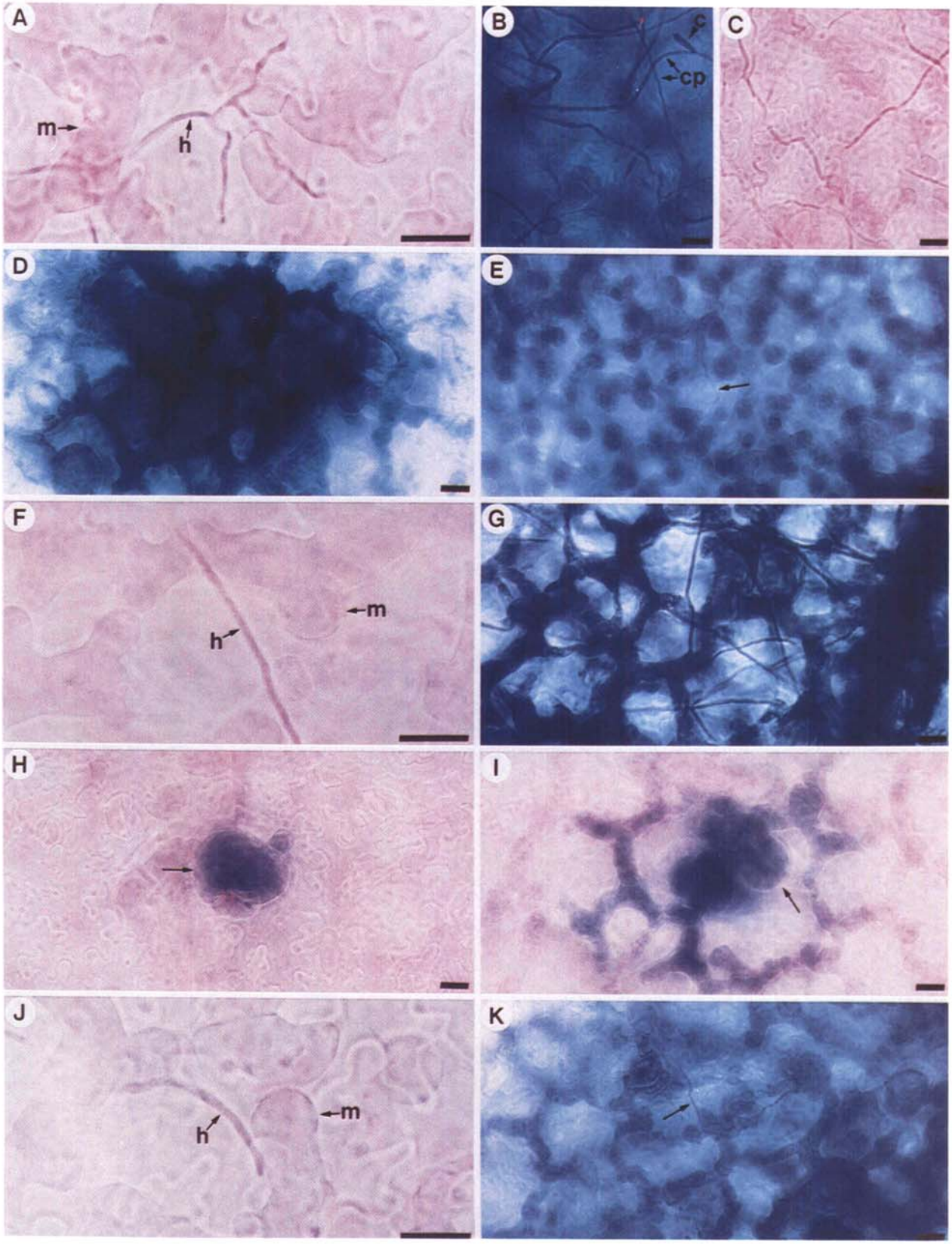


Fig. 2. Histochemical localization of *gn1:GUS* fusion induction during compatible (Cf0) and incompatible (Cf2, 4, 5, 9) interactions between tomato and *Cladosporium fulvum* race 0. GUS activity was localized in leaf disk samples (1 cm in diameter) cut from the leaves of near-isogenic lines of tomato heterozygous for the *gn1:GUS* fusion after inoculation with *C. fulvum* race 0. Induction was compared in a line lacking any *Cf* genes (Cf0) and in those heterozygous for the *Cf-2, Cf-4, Cf-5,* and *Cf-9* resistance genes.

Fig. 3. Microscopic examination of *gn1:GUS* fusion induction in relation to fungal growth during compatible (Cf0) and incompatible (Cf2, Cf4, Cf5, and Cf9) interactions between tomato and *C. fulvum* race 0. Tomato plants carrying the *gn1:GUS* fusion were inoculated with *C. fulvum* race 0. The samples shown were taken at 8 days (left hand panel) and 12 days (right hand panel) after inoculation. Sequential staining with X-Gluc and acid fuchsin revealed GUS activity (blue), plant cells (light pink), and fungal hyphae (dark pink). Fungal hyphae in close proximity to host cells expressing high GUS activities appear blue due to uptake of the excess X-Gluc product. The scale bars are equal to 20 μm. **A,** Compatible interaction in Cf0 (day 8) showing fungal hyphae [h] in contact with plant mesophyll cells [m] in which the fusion is not induced. **B,** Compatible interaction in Cf0 (day 12) showing conidia [c] and conidiophores [cp] associated with *gn1:GUS* fusion induction in the underlying plant tissue. **C,** Compatible interaction in Cf0 (day 12) showing extensive hyphal growth outside the areas of *gn1:GUS* fusion induction. **D and E,** Incompatible interactions in Cf2 at days 8 and 12, respectively, showing extensive induction associated with sites of hyphal penetration (arrowed). **F and G,** Incompatible interactions in Cf4 at days 8 and 12, respectively. Day 8; a fungal hyphae [h] colonizing the lower mesophyll layer without inducing the fusion in neighboring mesophyll cells [m], and at day 12 with extensive hyphal growth ramifying in the lower mesophyll layer now exhibiting extensive induction of the fusion. **H and I,** Incompatible interactions in Cf5 at days 8 and 12, respectively. Fusion induction in a single (H), and in a small cluster (I), of lower mesophyll cells associated with fungal penetrations. The first cell to respond is characteristically swollen and rounded (arrowed). **J and K,** Incompatible interactions in Cf9 at days 8 and 12, respectively. Day 8; limited hyphal growth [h] in contact with lower mesophyll cells [m] in which the fusion is not induced, and at day 12 with a small increase in hyphal growth (arrowed) and extensive induction of the fusion.

After stomatal penetration in the compatible interaction with Cf0 the fungal hyphae proliferated extensively between the plant mesophyll cells as described previously (de Wit 1977; Lazarovits and Higgins 1976b), hyphae accumulating preferentially in the vicinity of the vascular tissue (Hammond-Kosack and Jones 1994). Typically, no induction was observed in the early stages of infection, even in host cells actually in contact with the fungal hyphae (Fig. 3A). By day



10, *gn1* induction was observed in mesophyll cells associated with the colonized veins. These sites accounted for 88 ± 1.8% of the total number of sites of *gn1* induction at 10 days p.i.; the remaining induction was observed in the center of infections in the interveinal areas. By day 12 extensive and strong GUS staining was found in the colonized mesophyll layers above where conidiophores were emerging through stomata (Fig. 3B). At the margins of these lesions, where abundant vegetative hyphae were present, the fusion was still not induced (Fig. 3C).

On Cf0, hyphal growth occasionally stopped shortly after stomatal entry and did not lead to the establishment of a successful compatible interaction. Such penetrations were often associated with induction of the fusion in one, or several, neighboring plant cells (data not shown).

The *Cf-2*-mediated resistance was characterized by a rapid inhibition of fungal growth after stomatal penetration, the hyphae growing less than 1 epidermal cell length. In this interaction the fusion was induced within 1 day of stomatal penetration (Fig. 3D); up to 2 days earlier than in the other incompatible interactions. The induction was very intense by day 12 p.i. and involved a large number of plant cells surrounding the site of fungal penetration (Fig. 3E).

Cf-4 conferred the weakest resistance of the four resistance genes studied. By 12 days p.i. there was significant hyphal growth and an individual mycelium sometimes extended over 20 epidermal cell lengths with hyphae penetrating even the deeper mesophyll layers. However, the growth was considerably less than that seen in the compatible interaction and there was no sporulation. After stomatal penetration the fungus grew for up to 5 days without inducing the fusion (Fig. 3F). After this initial period of growth there was a progressive induction of the fusion in the center of the infection. By day 12 p.i. most of the hyphal growth was contained within regions of induction (Fig. 3G), although some of the younger parts of the mycelium sometimes grew ahead of the induction front (data not shown). At infection sites where hyphae reached the vascular tissue, *gn1* induction also extended into the uninfected mesophyll cells adjoining vascular tissue extending from the infection sites. This phenomenon is visible on the day 12-Cf4 sample in Figure 2.

Cf-5 conferred a slightly weaker resistance than *Cf-2*. It was rare for the hyphae to advance more than one or two epidermal cell lengths from the stomatal entry point. However, with *Cf-5* the induction pattern was characterized by the fusion being induced in only one or a small number of plant cells surrounding the penetration point within 3 days of stomatal entry (Fig. 3H and I). Often the first responding cells had also lost the asymmetrical shape typical of lower mesophyll cells and had become rounded in appearance and considerably swollen in size (arrowed cell in Fig. 3H).

Cf-9 conferred a resistance of intermediate strength. Some hyphal growth was permitted, but this was sparse and occurred with reduced side-branching. Slow growth continued up to 12 days p.i. (the last time-point taken) when individual infection loci were up to six epidermal cell lengths in diameter. As with the resistance conferred by *Cf-4* the fungus grew for up to 5 days in the mesophyll without inducing the fusion (Fig. 3J). However, by 12 days p.i. the fungal mycelium were completely contained within areas in which the fusion was strongly induced (Fig. 3K). Only rarely did any hyphal growth extend beyond these foci of induction.

Significant increases in total GUS activity in the infected leaves of plants carrying the *gn1:GUS* fusion were detected from 10 days p.i. during both compatible and incompatible interactions as shown in Table 3. By day 12 the highest levels of GUS activity were found in the compatible interaction. At this time-point in the four incompatible interactions, higher GUS activity was associated with *Cf-2, Cf-4,* and *Cf-9* dependent resistance, than with *Cf-5*. These data in combination with the cytological observations on hyphal ontogeny in each interaction, indicate that the induction of the defense gene promoter per unit fungal growth was considerably higher in the incompatible than in the compatible interactions.

Table 3. Quantification of the induction of the *gn1:GUS* fusion during compatible and incompatible interactions with *Cladosporium fulvum* race 0[a,b]

Days after inoculation	Cf0	Cf2	Cf4	Cf5	Cf9	Uninoculated
6	463	769	620	335	336	228
8	590	747	750	849	765	559
10	1,374	1,731	1,422	739	1,337	281
12	5,834[a]	3,053[b]	4,258[b]	1,752[c]	3,920[b]	341[d]

[a] Values are given in picomoles of MU per milligram of protein per minute.

[b] The values with difffering letters are significantly different at the 5% level (Tukey's D).

Induction of the *gn1:GUS* fusion by intercellular fluid (IF) containing *C. fulvum* race-specific elicitors.

The induction of the *gn1:GUS* fusion by IF was investigated by injecting the reagent into the apoplast of healthy tomato leaves. We wished to determine whether 1) the fusion was induced in a *Cf* gene-dependent manner, 2) the various *Cf* genes differed in their temporal pattern of *gn1:GUS* in-

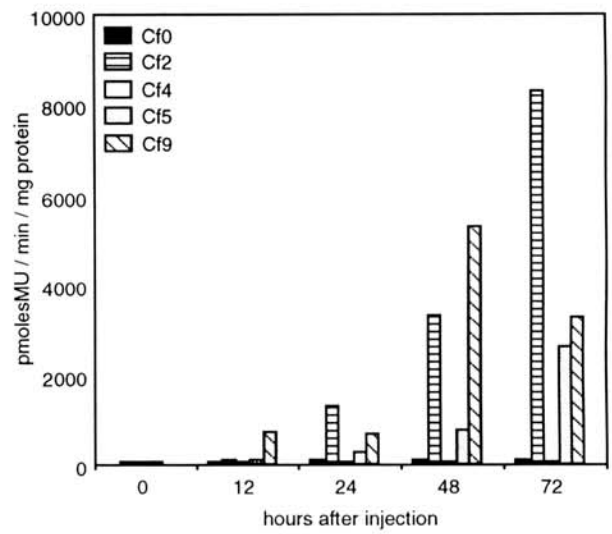


Fig. 4. *gn1:GUS* induction after the injection of intercellular fluid (IF) containing *Cladosporium fulvum* race-specific elicitors into tomato leaves. IF (Cf0-race 0) was injected into tomato NILs heterozygous for the *gn1:GUS* fusion and possessing either no *Cf* gene (Cf0) or heterozygous for the *Cf-2, Cf-4, Cf-5,* or *Cf-9* resistance genes. After various times, GUS activity was quantified in leaf disk samples (1 cm in diameter) cut from the injected panels. The least significant difference at the 5% level = 1,315.

duction, and 3) the pattern of induction of the heterologous *N. plumbaginifolia gn1* promoter reflected the pattern of induction of characterized tomato acidic or basic β-1,3-glucanase genes.

In response to the interveinal panel injection with IF (Cf0-race 0), discrete patches of gray necrosis were first visible 24 hr after injection (a.i.) on Cf9 and subsequently extended throughout the injected area by 72 hr a.i. Chlorosis developed on Cf2 by 72 hr. These responses were restricted to the injected panels as has been reported previously (de Wit and Spikman 1982). Under the environmental conditions chosen and IF preparations used, no macroscopic symptoms were observed on Cf4 or Cf5 plants prior to 72 hr a.i.

Cf0, 2, 4, 5, and 9 tomato plants heterozygous for the *gn1:GUS* transgene were injected with IF from the Cf0-race 0 compatible interaction (containing the products of the *avr2, avr4, avr5,* and *avr9* genes) and the induction of the fusion monitored over the following 3 days. The time-course of *gn1:GUS* induction is shown in Figures 4 and 5. In the absence of a *Cf* gene (Cf0), no significant induction of the fusion was observed. The magnitude and kinetics of induction in *Cf* gene-containing genetic backgrounds varied according to the resistance gene present. Strong induction occurred in the presence of the *Cf-2* and *Cf-9* genes, weak induction with *Cf-5*, and no induction with *Cf-4*. This lack of induction on Cf4 plants could indicate the *Cf-4* gene conditions only a low sensitivity to the *AVR4* gene product or alternatively the concentration of the *AVR4* in the IF preparation used was significantly lower than that of the other *C. fulvum* AVR products. In the Cf9 stock, the induction peaked at 48 hr post-injection before decreasing. The loss in GUS activity was correlated with the progressive necrosis associated with this interaction (see arrowed area on Fig. 5). In the Cf2 and Cf5 backgrounds the GUS activity continued to increase throughout the time-course. Forty-eight hours after inoculation the level of *gn1:GUS* induction was at least 40 times higher on Cf9 than on Cf0 plants. Seventy-two hours after inoculation the corresponding values for Cf2 and Cf5 were 40- and 4-fold, respectively. *Cf-9* mediated induction was detectable by 12 hr a.i.; *Cf-2* and *Cf-5* by 24 hr a.i.

Histochemical localization of the GUS activity, shown in Figure 5, was in agreement with the quantification data. Again it was apparent that the induction was *Cf-* gene-dependent. Occasionally a slight wound induction of the fusion was visible in Cf0 plants where the tissue had been damaged during the injection process (data not shown). The induction was always confined to the vicinity of the injected panels and examination of the boundary between injected and non-injected regions revealed a rapid transition between induced and non-induced tissue.

To determine whether the *gn1:GUS* induction elicited by IF was also race-specific, IFs were prepared from the Cf0-race 0 and Cf0-race 5 (lacking *avr5*) compatible interactions, and their inducing activities compared. The induction elicited by the two IFs after 3 days in Cf2 and Cf5 is shown in Figure 6. A series of dilutions of each IF were injected to allow a comparison of their relative titers. In Cf2 both IF preparations induced the fusion strongly and the dilution series demonstrated that they were of similar titer. In Cf5 only the IF from the Cf0-race 0 interaction had inducing activity. Neither IF induced GUS activity in Cf0 (data not shown). These data are consistent with the view that the induction of the *gn1:GUS* fusion is regulated under the control of a "gene-for-gene" type interaction.

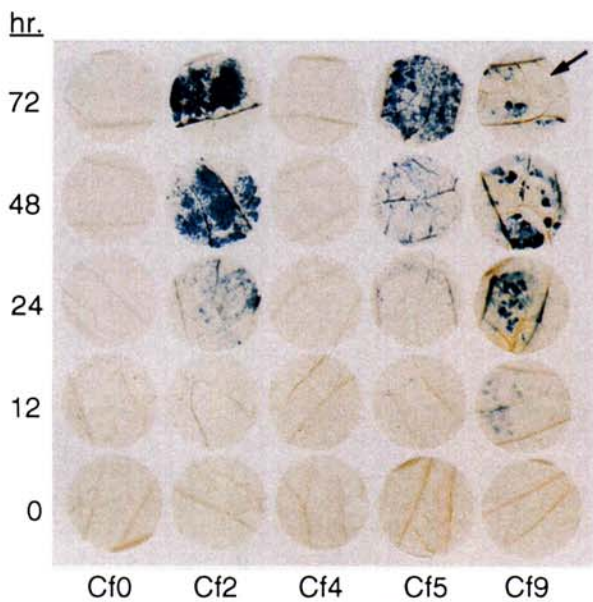


Fig. 5. Histochemical localization of *gn1:GUS* induction after injection of intercellular fluid (IF) containing *Cladosporium fulvum* race-specific elicitors. GUS activity was localized in leaf disk samples (1 cm in diameter) cut from tomato leaves injected with IF (Cf0–race 0). The tomato genotypes used are as described in Figure 4. On Cf9 necrosis (arrowed) is visible from 24 hr onwards.

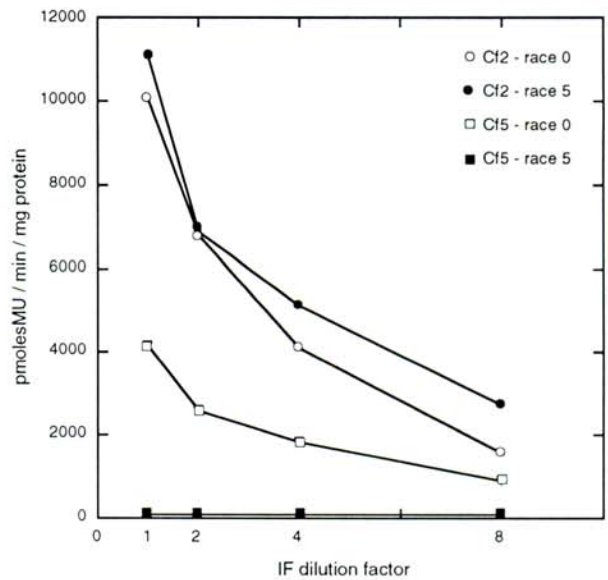


Fig. 6. A comparison of the induction of the *gn1:GUS* fusion in *Cf-2* and *Cf-5* containing tomato lines after the injection of IF isolated from compatible interactions involving *Cladosporium fulvum* race 0 or race 5. Various dilutions of each IF were injected into tomato NILs heterozygous for the *gn1:GUS* fusion and for the *Cf-2* or *Cf-5* resistance genes. After 3 days GUS activity was quantified in leaf disks cut from the injected panels. Key Cf2–race 0 = IF from the Cf0/race 0 interaction injected into Cf2; Cf2–race 5 = IF from the Cf0/race 5 interaction injected into Cf2; Cf5–race 0 = IF from the Cf0/race 0 interaction injected into Cf5; Cf5–race 5 = IF from the Cf0/race 5 interaction injected into Cf5. The IF dilution factor is expressed as a reciprocal value. The least significant difference at the 5% level = 1,713.

A comparison of the induction of two endogenous tomato β-1,3-glucanase genes and the *gn1:GUS* fusion by race-specific elicitor preparations (IF).

To ascertain whether the induction pattern of the *N. plumbaginifolia gn1:GUS* transgene by IF (described above) resembled that of either the acidic (*gluA*) or basic (*gluB*) tomato β-1,3-glucanase genes previously reported by Van Kan *et al.* (1992), the following experiment was undertaken. Entire leaves of Cf0, Cf2, and Cf9 plants heterozygous for the *gn1:GUS* fusion were injected with IF (Cf0-race 0). Total mRNA populations were harvested at 0, 6, 12, 24, and 48 hr a.i. The results of the RNA gel blot hybridizations using probes to the transgene (GUS) and the two endogenous genes *gluA* and *gluB* are shown in Figure 7. In uninjected leaves none of the three transcripts were detectable. Following IF injection, significant accumulation of all three gene transcripts was evident in Cf2 and Cf9, but not Cf0 plants, from 6 hr onwards. These data indicate the induction of each gene was *Cf-* gene-dependent. Interestingly, the accumulation of all three transcripts was again detected earlier in Cf9 than in Cf2. The small amount of *gluB* transcript detectable in Cf0 plants at 12 hr suggests this gene was also inducible by wounding caused during the injection procedure and/or by the transient flooding of the leaf's air spaces with IF. When the overall patterns of induction were compared, the changes in steady-state levels of *GUS* and *gluA* mRNAs resembled each other more closely than the changes to the *gluB* transcript levels. The latter transcript reached a maximal abundance 12 hr a.i. and then declined, whereas the abundance of the *GUS* and *gluA* mRNAs continued to steadily increase up to the final time-point at 48 hr a.i. These data indicate the heterologous *gn1:GUS* fusion used in the main study had an induction pattern which resembles that of an endogenous tomato acidic β-1,3-glucanase gene.

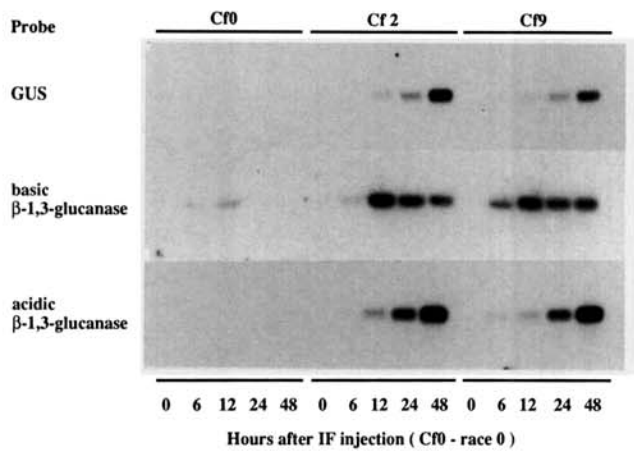


Fig. 7. *Cf* gene-dependent increases in the steady state mRNA levels of the GUS transgene and endogenous acidic and basic β-1,3-glucanase genes in response to challenge with IF containing *Cladosporium fulvum* race-specific elicitors. IF (Cf0–race 0) was injected into the leaves of tomato NILs heterozygous for the *gn1:GUS* fusion and either possessing no known *Cf* genes (Cf0) or heterozygous for the *Cf-2* or *Cf-9* genes. RNA gel blots were hybridized with probes corresponding to the *GUS, gluA* (acidic β-1,3-glucanase), and *gluB* (basic β-1,3-glucanase) genes. The transcript sizes are 1,008 b (*gluA*), 1,080 b (*gluB*), and 1,840 b (*GUS*).

DISCUSSION

In this paper we demonstrate *Cf* resistance gene-dependent induction of a β-1,3-glucanase (*gn1*) gene promoter-*GUS* fusion. Strikingly, induction of the fusion in incompatible interactions was tightly localized to the site of fungal infection and was activated 2–4 days earlier than in compatible interactions. The intensity of induction per cell around the infection sites in the incompatible interactions was much higher than the value obtained by analysis of whole leaf samples.

Similar localization of defense gene induction to infection sites have been reported by Roby *et al.* (1990) for a chitinase gene following susceptible infections of tobacco with *Botrytis cinerea, Rhizoctonia solani*, or *Sclerotium rolfsii* and for several defense-related genes following infection of parsley with the nonhost pathogen *Phytophthora megasperma* (Schmelzer *et al.* 1989). However, in other plant-pathogen interactions induction is not always localized to the infection site. For example, the induction of β-1,3-glucanase and chitinase genes in potato leaves during incompatible interactions with *Phytophthora infestans* spreads rapidly throughout the whole infected leaf (Schroder *et al.* 1992). Such observations emphasize the importance of determining the spatial pattern of induction when characterizing defense gene expression.

Temporal and spatial patterns of *gn1:GUS* gene expression in compatible and incompatible interactions.

The patterns of induction mediated by the four *Cf* resistance genes investigated were distinct. Both the number of host cells responding and the speed of the response depended upon the *Cf* gene present. In general, the stronger the inhibition of *C. fulvum* hyphal growth by a particular *Cf-avr* gene combination, the faster the induction. However, the correlation between the number of cells responding and the amount of hyphal growth was less tight. Both *Cf-2* and *Cf-5* confer strong resistance to *C. fulvum*, permitting only very limited hyphal growth, yet the fusion was induced in many more cells not in contact with hyphae in the *Cf-2* genetic background than in the *Cf-5* stock. In both interactions, induction of the fusion was initially observed in the swollen mesophyll cell(s) at the base of the sub-stomatal cavity penetrated by hyphae. The speed of induction relative to the rate of hyphal growth may be another important factor. For example, during incompatible interactions mediated by *Cf-9* and *Cf-4*, the hyphal front was ahead of the area of induction during the early phases of the infection. Often in the *Cf-4* interaction this occurred even as late as 12 days after inoculation. In this interaction the fungus may be "outgrowing" the plant's response, although it should be noted that this level of defense is still sufficient to prevent sporulation.

Induction mediated by *Cf-2* and *Cf-9* was found both throughout the infection lesion and in a border of surrounding cells by the later time points. Thus it is probable that an elicitor of *gn1:GUS* induction (perhaps the fungal AVR product, or a plant-derived signal such as ethylene or salicylic acid) travels ahead of the fungal hyphae. However, some of the GUS staining in the surrounding cells may be due to diffusion of the primary soluble product of the staining reaction prior to oxidative dimerization to form the insoluble indigo dye (Jefferson *et al.* 1987). In contrast, *gn1:GUS* induction in

the *Cf-5-avr5* mediated incompatible interaction was extremely localized even at the later time-points. These data suggest induction may either only occur for a limited period prior to the cessation of hyphal growth or that there was a lack of, or suppression of, secondary signals travelling ahead of the fungal hyphae.

The early and localized induction of the *gn1:GUS* fusion in incompatible interactions is consistent with a role for β-1,3-glucanases in the resistance response of tomato to *C. fulvum*. The precise function of these hydrolases is still unclear. It is possible that their real function is to inhibit the establishment of secondary infections by opportunistic pathogens because the greatest amount of *gn1:GUS* induction was associated with the later phases of the compatible interaction.

In common with studies on other plant-pathogen interactions into the temporal and spatial induction pattern of various defense-related genes (reviewed by Bowles 1990; Lindhorst 1991), the *gn1:GUS* fusion was also transcriptionally activated late in the compatible *C. fulvum* interaction at the onset of sporulation. Initially, *gn1:GUS* induction was not spread evenly throughout the colonized areas but occurred within the infection center where hyphae were in association with vascular tissue. Only later did induction occur throughout the mesophyll layers directly above the sporulating conidiophores. Ethylene biosynthesis increases dramatically with the onset of sporulation (T. Ashfield, unpublished results) and may play a role in the induction of the *gn1:GUS* fusion in the compatible interaction. It is plausible that induction of hydrolases late in compatible interactions represents an attempt by the plant to contain the disease (Pegg and Young 1981; Dong *et al.* 1991).

Although the observations reported are generally in agreement with previous studies on β-1,3-glucanases induction in the *C. fulvum*-tomato interactions (Joosten and de Wit 1989; Van Kan *et al.* 1992; Wubben *et al.* 1992), induction of the fusion was slower than in comparable experiments. This discrepancy is probably due to the slightly slower growth of *C. fulvum* under our environmental regime, the use of different fungal races, and the use of the *Cf* genes in a heterozygous state. Hammond-Kosack and Jones (1994) have demonstrated that *Cf* genes are incompletely dominant in their action (i.e., they permit greater hyphal invasion when present in the heterozygous state) and thus the heterozygosity of these resistance genes may have influenced the kinetics of defense gene induction.

Induction of the *gn1:GUS* fusion by race-specific elicitors.

In this study we also exploited the unique properties of the "gene-for-gene" interaction between tomato and *C. fulvum* in which race-specific elicitors are found in the intercellular washing fluids (IF) isolated from susceptible tomato leaves heavily infected with virulent races of *C. fulvum* (de Wit and Spikman 1982; de Wit *et al.* 1984). These elicitor preparations have the advantage over the living pathogen of representing a more defined challenge that can be applied synchronously. In such circumstances it is easier to measure the kinetics of defense gene induction. No other system provides race-specific elicitors that provoke distinct responses dependent on different resistance genes introgressed into NILs.

Induction of the fusion by IF was *Cf* gene-dependent. The kinetics and magnitude of *gn1:GUS* fusion induction elicited by IF depended on which *Cf* gene was present. *Cf* genes which confer a strong resistance to *C. fulvum* (*Cf-2, Cf-5,* and *Cf-9*) were found to mediate induction of the fusion within 24 hr. In contrast, a relatively weak *Cf* gene (*Cf-4*) did not mediate any detectable induction (at least for 72 hr a.i.). These differences could indicate that the *Cf* genes condition different sensitivity to their respective elicitors because they function via different signal transduction pathways of varying length and/or efficiency. Alternatively, the various race-specific elicitors could occur at different concentrations in the IF, could have different mobilities through the plant cell wall, could bind with varying efficiencies to the *Cf* gene product, or could exhibit different stabilities.

The induction elicited by IF was restricted to the vicinity of the injected panel. This observation demonstrates that the elicitors of *gn1:GUS* induction do not move far from the site of injection and that the fusion is not induced systemically by IF (at least for 72 hr a.i.). These data are consistent with the localized induction characteristic of incompatible interactions with *C. fulvum*.

To further confirm the relevance of these studies to *Cf* gene-dependent induction of effective defense, IF lacking the AVR5 elicitor was prepared from tomato leaves infected with *C. fulvum* race 5. This IF preparation did not induce the fusion in the presence of *Cf-5*, but still induced the fusion in the presence of *Cf-2*. This result supports the hypothesis that the fusion is induced by the race-specific elicitors known to occur in IF. The magnitude of induction of the *gn1:GUS* fusion after IF challenge was dependent on the concentration of the IF injected. This demonstrates that the strength of the defense response activated by the *Cf-2* and *Cf-5* genes is responsive to the concentration of race-specific elicitor applied.

Signal molecules possibly responsible for the induction of the *gn1:GUS* fusion.

Pathogenesis-related (PR) protein accumulation is invariably induced after a biotic agent has caused a necrotic tissue reaction (Linthorst 1991). However, in incompatible *C. fulvum*-tomato interactions conditioned by the *Cf-2, Cf-4, Cf-5,* and *Cf-9* resistance genes, the invoking of a host cell death response is rare until late in the infection time-course and frequently hyphal growth is arrested without its occurrence (Hammond-Kosack and Jones 1994). Also in the IF injection experiments, *gn1:GUS* induction always preceded the necrotic or chlorotic response. Therefore induction of the *gn1* promoter does not require the prior induction of a HR response. Jakobek and Lindgren (1993) have recently shown that the induction of another PR gene, a tobacco chitinase, also occurs in the absence of HR formation. In tobacco leaves infiltrated with a *Pseudomonas syringae* pv. *tabaci* both Hrp$^-$ mutant and wild-type strains induced the accumulation of chitinase transcripts, as well as PAL, CHS, and CHI, even though no HR develops at the Hrp$^-$ mutant infection site.

Known inducers of PR protein genes include ethylene and salicylic acid (SA)(Boller 1991; Enyedi *et al.* 1992). We have evidence that the biosynthesis of both these molecules can be induced by IF in a *Cf* gene-dependent manner at the site of infiltration (T. Ashfield and K. Hammond-Kosack, unpublished results). Although much attention has been focused on

the systemic nature of the SA signal (Enyedi *et al.* 1992), it could also play an important role in localized induction of the plant defense response.

The *Cf* gene-dependent induction of the *gn1:GUS* fusion and the availability of race-specific elicitors should provide a suitable assay system with which to identify the earliest events following *avr* product recognition. To understand the whole chain of events that culminates in *Cf*-gene dependent *gn1:GUS* induction, our attention is now focused on *Cf* gene-dependent events that precede it.

MATERIALS AND METHODS

DNA constructions.

The structure and origin of the *gn1:GUS* fusion have been described elsewhere (Castresana *et al.* 1990). The fusion was subcloned in the binary vector pJE188 (Jones *et al.* 1992) as a *Bam*H1 fragment using standard techniques (Maniatis *et al.* 1982). In the final binary construct (pSLJ1623), the GUS gene of the fusion and the neomycin transferase *(NPT)* transformation marker gene were transcribed in parallel orientation.

Plant transformation.

All transformations were performed with the Cf0 near-isogenic line of *Lycopersicon esculentum* 'Moneymaker.' Binary T-DNA constructs were mobilized into *Agrobacterium tumefaciens* strain LBA4404 (Hoekema *et al.* 1983). Transgenic tomato plants were regenerated as described by Fillatti (1987).

Genetic stocks.

Near-isogenic lines (NILs) of *L. esculentum* 'Moneymaker' homozygous for the *Cf-2, Cf-4, Cf-5,* or *Cf-9* genes (Tigchelaar 1984) were obtained from Richard Oliver (University of East Anglia, Norwich, England). The near-isogenic line lacking all known *Cf* genes (the Cf0 NIL) was also obtained. The nature of the interactions between tomato plants carrying the *Cf-2, Cf-4, Cf-5,* or *Cf-9* genes and the *C. fulvum* races used in this study (races 0, 4, and 5) are presented in Table 1.

The Cf0 NIL was transformed with the *gn1:GUS* fusion. The primary transformants were then crossed to the four *Cf* resistance gene-containing NILs and to untransformed Cf0 individuals. Kanamycin-resistant segregants from such crosses were used as the experimental populations; such plants were heterozygous for both a *Cf* resistance gene (with progeny derived from crosses to the *Cf* gene-containing NILs) and for the T-DNA. Individuals heterozygous for the *Cf-2, Cf-4, Cf-5,* or *Cf-9* resistance genes are referred to as Cf2, Cf4, Cf5, and Cf9 individuals, respectively. Plants lacking a *Cf* gene are referred to as Cf0 individuals. This system of nomenclature is summarized in Table 1.

Two independent Cf0 primary transformants were used in these studies and both appeared to contain a single T-DNA integration site as judged from kanamycin resistance segregation ratios. As the results obtained for both transformants were identical, only data for transformant 1623A is presented.

Growth of *C. fulvum in vitro.*

Cladosporium fulvum (Cooke) (syn. *Fulvia fulva* (Ciferri)) race 0, race 5, and race 4 GUS transformant 9 (Roberts *et al.* 1989) were obtained from Richard Oliver (University of East Anglia, Norwich, England). *C. fulvum* was grown *in vitro* on a quarter strength potato-dextrose agar as described previously (de Wit 1977).

Plant growth and inoculation.

Tomato plants were grown in Levingtons M3 compost under growth room conditions at 23° C with a 16-hr photoperiod. The second or third leaves of 5-wk-old plants were spray inoculated with a suspension of conidia in water (3×10^5 conidia/ml). After spraying the leaves were allowed to dry before the plants were transferred to propagators with the air vents closed. On the fifth day after spraying the vents were half opened to promote stomatal opening.

Isolation and injection of race-specific elicitors.

Intercellular fluids (IF) containing *C. fulvum* race-specific elicitors were isolated from tomato leaves heavily infected with *C. fulvum* as described previously (de Wit and Spikman 1982). The crude intercellular fluid was sterilized by heating for 10 min at 100° C, and the precipitate was removed by centrifugation at 4° C. The supernatant was stored at –20° C prior to use.

IF was injected into the apoplast of the interveinal regions of young, healthy leaves of 5-wk-old tomato plants with a needleless 1-ml disposable syringe as described previously (de Wit and Spikman 1982). In experiments to monitor induction of the *gn1:GUS* fusion only small leaf areas (0.5–2.5 cm in diameter) were flooded. GUS activity was quantified or histochemically localized in leaf disks cut from within the injected panels. In experiments to demonstrate the localization of *gn1:GUS* induction, GUS activity was monitored in leaf disks including both the injected region and surrounding tissues.

β-Glucuronidase assays.

GUS activities were quantified using the fluorometric method described by Jefferson *et al.* (1987), and the GUS values were standardized to protein. Protein concentrations were determined by the method of Bradford (1976) using the protein assay dye concentrate supplied by Bio-Rad Laboratories, and bovine serum albumin as the protein standard. A Titertek Fluoroskan II was used for fluorometric measurements and a Titertek Multiskan Plus for spectrometric ones (ICN, Flow).

The histochemical localization of GUS activity was performed essentially as described by Jefferson *et al.* (1987). To examine the induction of the *gn1:GUS* transgene, leaf disks (1 cm in diameter) were cut with a cork-borer and vacuum infiltrated with a solution containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-Gluc), sodium salt (Biosynthag, CH-9422, Switzerland), 0.05% (v/v) Triton-X100 and 50 mM phosphate buffer, pH 7, and incubated in the dark at 37° C for 16 hr. After staining chlorophyll was removed from the stained samples by incubation in 70% ethanol at room temperature for several days. Samples to be counterstained with acid fuchsin were destained in saturated chloral hydrate (5 g of chloral hydrate to 2 ml of water).

Growth of the GUS expressing *C. fulvum* race 4 was localized as described above, except the X-Gluc concentration was reduced to 0.5 mg/ml and 1 mM potassium ferri/ferro cyanide and 1 mM EDTA were included in the staining solution.

Acid fuchsin staining and microscopic examination.

Staining with acid fuchsin was performed essentially as described by McBryde (1936). Leaf disks were cleared in saturated chloral hydrate for at least 1 wk before being transferred to the staining solution (1 ml 2% [w/v] acid fuchsin in 70% ethanol, 12 ml of saturated chloral hydrate, 8 ml of 95% ethanol) for 1–3 hr. Afterwards, the disks were destained in saturated chloral hydrate until good differentiation was obtained between the host cells and the fungus (2–5 days at room temperature). This often required several changes of chloral hydrate. The destained disks were mounted in chloral hydrate and viewed under phase-contrast optics with a Zeiss Axiophot microscope. Photomicrographs were prepared using Ektachrome 160 Tungsten film (Kodak).

RNA extraction and gel blot hybridization.

RNA was purified from frozen tissue samples by phenol/chloroform/guanidine hydrochloride extraction followed by acetic acid/ethanol precipitation (Logemann *et al.* 1987). Ten-microgram samples of total mRNA were separated by electrophoresis through formaldehyde agarose (1.6%) gels and blotted to nylon membrane (Hybond N, Amersham, UK) as described by Maniatis *et al.* (1982). Ethidium bromide was included in the sample loading buffer at 40 μg/ml, which allowed photography under UV light after electrophoresis to confirm equal sample loading. Hybridizations and washes were essentially according to Feinburg and Vogelstein (1983). ^{32}P-Hexaprime-labeled DNA fragments were prepared using a Pharmacia kit. After hybridization, filters were washed to a final stringency of 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS at 65° C, and exposed to X-ray film at –70° C.

Statistical analysis.

Standard deviations and least significant differences (Q tests) were calculated according to the methods described in Snedecor and Cochran (1980).

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