Incomplete Dominance of Tomato *Cf* Genes for Resistance to *Cladosporium fulvum*

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Different tomato *Cf* resistance genes confer distinct abilities to restrict *Cladosporium fulvum* infections. Measurements of fungal growth revealed that their relative efficiencies decreased in the order *Cf-2*, *Cf-5*, *Cf-9*, *Cf-4*, *Cf-11*, *Cf-3*. Plants homozygous for a given *Cf* gene were more effective in containing infections than when heterozygous. *Cf* homozygotes also responded to a two-fold lower concentration of race-specific elicitor (IF) than heterozygotes. The effectiveness of heterozygotes was even further reduced if produced by crosses to *Lycopersicon pennellii* instead of *L. esculentum*. Incompatibility usually occurred in the mesophyll layers and involved the gradual arrest of hyphal growth, frequent and nondichotomous hyphal branching, and a failure to form straight runner hyphae. Contained hyphae were often swollen and distorted, but those observed at the margin of the larger infections or when hyphae had been arrested within 1–2 days of entry into a substomatal cavity appeared normal. Localized host responses triggered by incompatibility included guard cell death (only in the *Cf-2* containing line), enlargement of lower mesophyll cells, deposition of phenolic extracellular material on cell walls and later some cell death at the center of infections. Compatibility involved rapid colonization of the lower mesophyll apoplast by straight runner hyphae, the accumulation of highly branched mycelium in close proximity to vascular tissue in the lesion center, and finally the death of mesophyll cells directly below the sporulating conidiophores. The implications of the incomplete dominance of *Cf* genes, and the mechanisms by which they restrict fungal growth, are discussed.

Additional keywords: Lycopersicon esculentum, tomato leaf mold.

Cladosporium fulvum Cooke (syn. *Fulvia fulva* (Cooke) Cif.) is a biotrophic imperfect fungus that causes leaf mold disease on tomato (*Lycopersicon esculentum* Mill.). A number of resistance genes (*Cf* genes) have been introgressed to cultivated tomato from various wild *Lycopersicon* species (Stevens and Rick 1988; Dickinson *et al.* 1993), and these are overcome by specific physiological races of *C. fulvum*. *Cf-2*, *Cf-3*, and *Cf-9* originated from *L. pimpinellifolium*; *Cf-1* and *Cf-5* from *L. esculentum* var. *cerasiforme*, *Cf-4* from *L. hirsutum* and *Cf-11* probably from *L. peruvianum*. Earlier reports of macroscopic symptoms following *C. fulvum* infection suggest that these *Cf* genes may arrest hyphal growth at different stages after abaxial leaf penetration. The cytological investigations by Bond (1938) and Lazarovits and Higgins (1976a, 1976b) report the reactions of the *Cf-1* and *Cf*-3 genes to be late, at the onset of conidiophore formation, whereas those of the *Cf-2* and *Cf-4* genes occur earlier, shortly after hyphae penetrate the stomatal cavity (Lazarovits and Higgins 1976a, 1976b; de Wit 1977). No information is available on the cellular phenotypes associated with the *Cf-5*, *Cf-9*, and *Cf-11* resistance genes.

Numerous plant-pathogen interactions have been found to exhibit race-cultivar specificity. Flor (1946) proposed the "gene-for-gene" model to explain this specificity, where incompatibility requires both a dominant resistance gene (*R*) in the host and a dominant avirulence gene (*Avr*) in the pathogen. In the absence of matching resistance and avirulence genes, pathogen recognition does not occur, defense responses are not triggered, and disease ensues. For the *Cladosporium fulvum*-tomato interaction this model is very plausible. It is supported by data obtained from genetic studies on the pathogenicity of different physiological races on a differential series of tomato cultivars (Day 1956) and from an assessment of the plant's response to race-specific elicitor preparations (de Wit and Spikman 1982). The products of the fungal avirulence (*Avr*) genes can be easily isolated in intercellular washing fluids (IFs) obtained from infected tomato leaves supporting a compatible *C. fulvum* interaction. When these IFs are delivered (by injection) into the air spaces of healthy leaves of plants containing *Cf* genes, a necrotic or chlorotic reaction develops in 1–5 days (de Wit and Spikman 1982). Each *Cf* gene conditions a distinct response to IF, and plants lacking any *Cf* genes do not respond to IF. The IF contains a series of small peptides believed to be the products of the fungal avirulence genes (de Wit *et al.* 1985). A 28-amino acid peptide, which has necrosis-inducing ability only when injected into tomato genotypes carrying the *Cf-9* resistance gene, has been purified from IF preparations (Scholtens-Toma and de Wit 1988). The cDNA and genomic clones encoding the peptide have been isolated, and the genomic sequence used to transform a race 9 (normally virulent on *Cf-9* containing plants) to an avirulent one (Van Kan *et al.* 1991; Van den Ackerveken *et al.* 1992). These experiments prove the 28-amino acid peptide is the product of the avirulence (*Avr9*) gene and verify the gene-for-gene hypothesis for the *Cf-9-Avr9* gene combination. Fungal races virulent on *Cf-9* tomato genotypes do not possess the *Avr9* gene (Van den Ackerveken *et al.* 1992).

Recent mapping data on four *Cf* genes (Jones *et al.* 1993) indicate that the *Cf-2* and *Cf-5* are very closely linked to each

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other and are also closely linked to the *Mi* (*Meloidogyne incognita*) resistance gene on chromosome 6. *Cf-4* and *Cf-9* are allelic, or very closely linked, to each other on the short arm of chromosome 1. *Cf-1* also appears to be located on the short arm of chromosome 1 (Kerr and Bailey 1964). In view of the diversity of origin of these *Cf* genes but apparent similarity of their map locations and the incompleteness of previous work, we became interested in carrying out a comprehensive biological characterization of the action of each *Cf* gene at the cytological level. We also wished to know if resistance was equally effective when a plant was homozygous or heterozygous for a given *Cf* gene. The availability of near-isogenic lines of the cultivar Moneymaker carrying either *Cf-2*, *Cf-3*, *Cf-4*, *Cf-5*, or *Cf-9* has enabled the resistance phenotype of these *Cf* genes to be compared in a uniform genetic background. *Cf-11* was also investigated even though it is in a different genetic background.

In the present study we have measured the relative efficiencies of the *Cf-2*, *Cf-3*, *Cf-4*, *Cf-5*, *Cf-9*, and *Cf-11* genes in restricting fungal biomass accumulation *in planta* using a *C. fulvum* race transformed with the β-glucuronidase (*uidA*) reporter gene (Oliver *et al.* 1993) and histological observations.

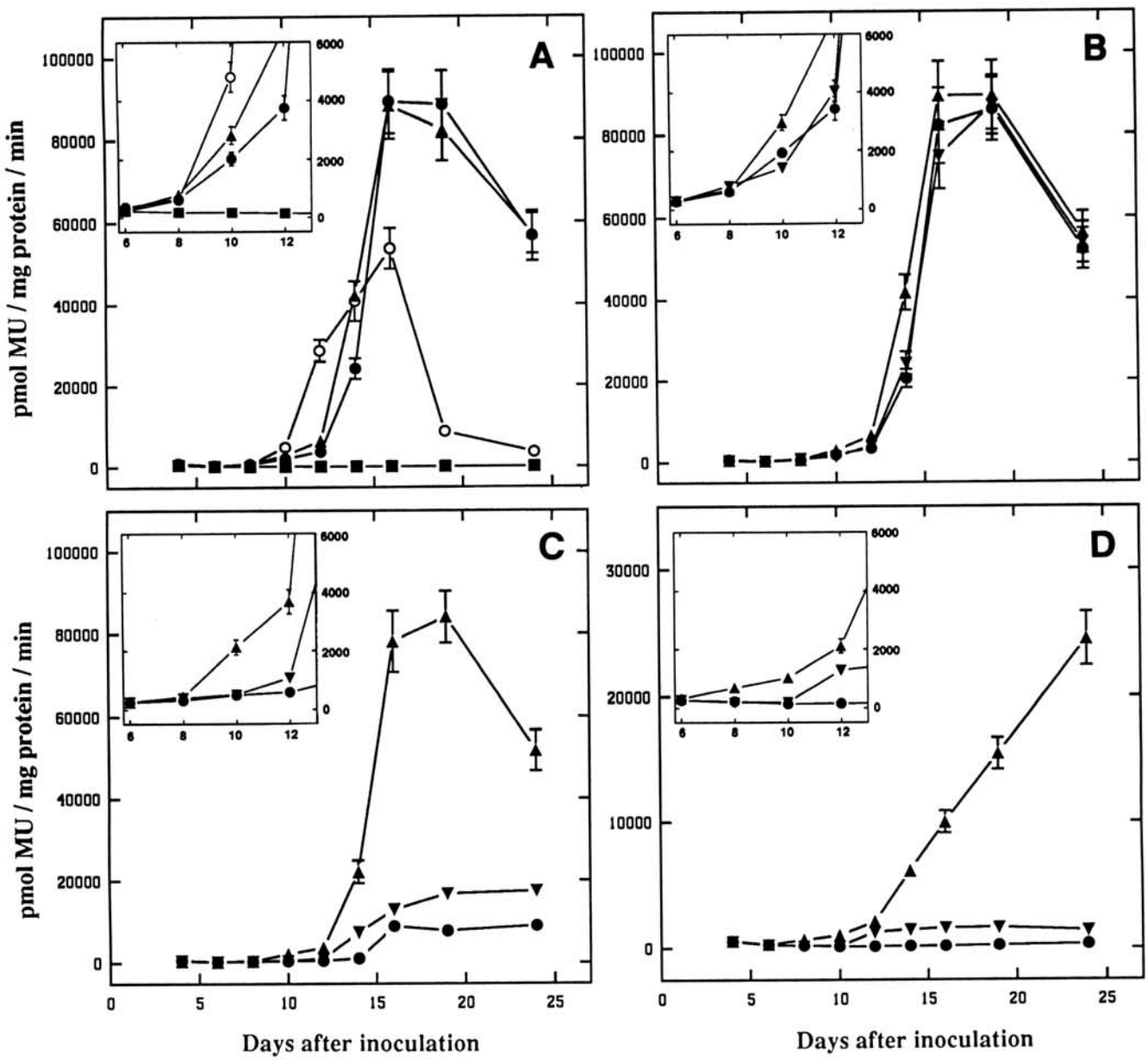

Fig. 1. Comparison of fungal biomass accumulation in various compatible and incompatible interactions differing both in genetic background and the zygosity of the *Cf* resistance gene. A fluorometric β-glucuronidase assay was used to measure the growth of a transformed race 4 of *Cladosporium fulvum* constitutively expressing the *uidA* gene. **A,** Compatible interactions with Cf0 ●, Cf0 × *Lycopersicon pennellii* ▲, *L. pennellii* ○, and incompatible interactions involving *Cf-2*, *Cf-5*, and *Cf-9* homozygotes and heterozygotes in both *L. esculentum* and interspecific hybrid backgrounds ■. **B,** Compatible interactions with *Cf-4* homozygote ●, *Cf-4* heterozygote ▼ and *Cf-4* hybrid heterozygote ▲. **C,** Incompatible interactions with *Cf-3* homozygote ●, *Cf-3* heterozygote ▼, and *Cf-3* hybrid heterozygote ▲. **D,** Incompatible interactions with *Cf*-11 homozygote ●, *Cf-11* heterozygote ∇, and *Cf*-11 hybrid heterozygote Δ . The insert in each panel is an expansion of part of the x-axis and y-axis. Each vertical bar represents the standard error of the sample mean.

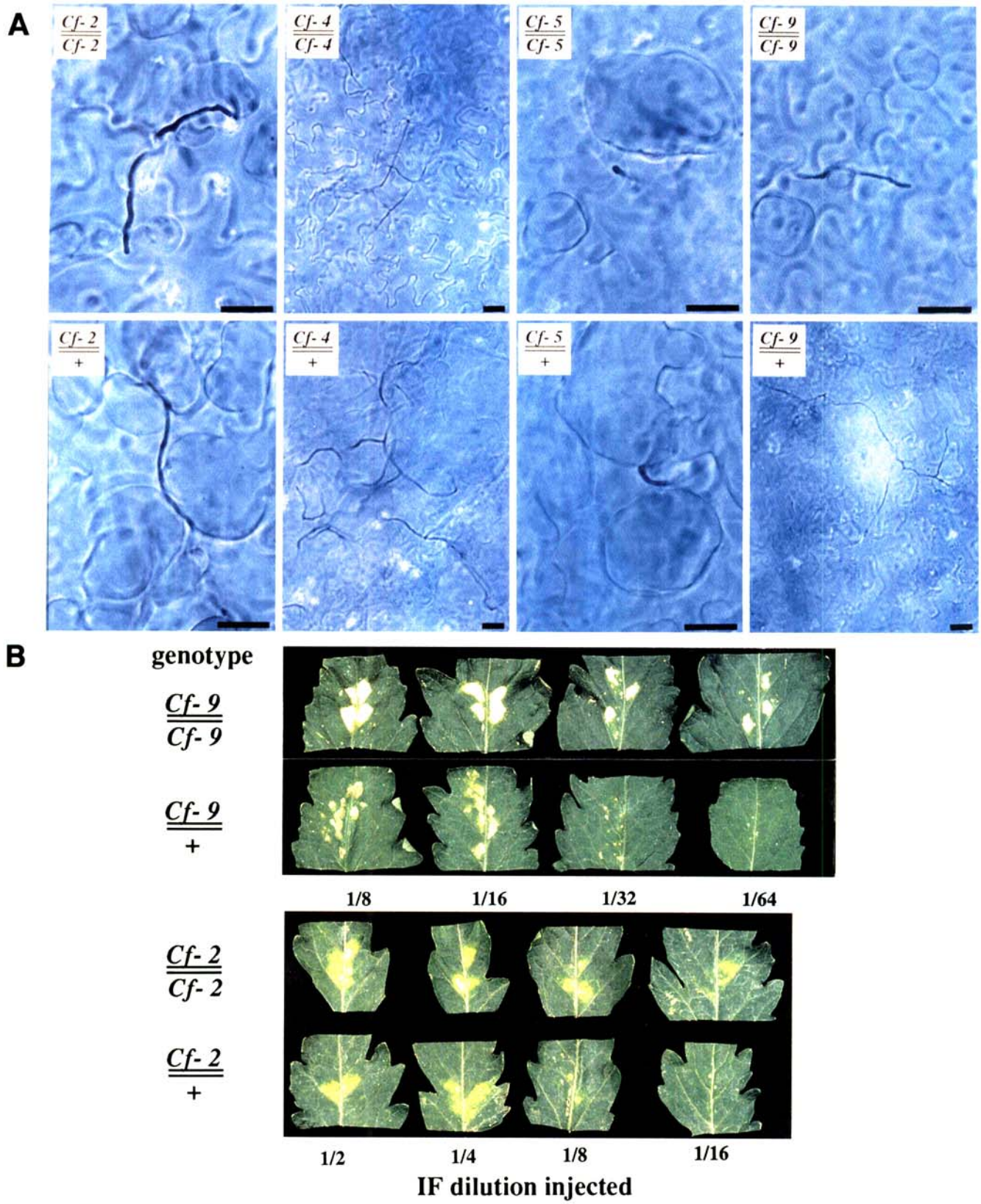

Fig. 2. A, Influence of resistance gene zygosity on *Cladosporium fulvum* growth in interactions involving the *Cf-2*, *Cf-4*, *Cf-5*, and *Cf-9* genes. Photomicrographs of the extent of hyphal growth in the lower mesophyll when each *Cf* gene was present either in a homozygous state (upper panel) or in a heterozygous state (lower panel). The infections shown at the higher magnifications are located directly above the lower epidermis (*Cf-2*/*Cf-2*, *Cf-9*/*Cf-9*), and in mesophyll layers one (*Cf-5*/*Cf-5*), two (*Cf-2*/+), and three (*Cf-5*/+). Leaf samples were taken at 10 days after inoculation with race 0 and stained with lactophenol-trypan blue. The scale bar is equal to 20 μm. **B,** Influence of resistance gene zygosity on the leaf's response to intercellular fluid (IF) injected at different dilutions into the air spaces of healthy leaves. Leaves were photographed after 2 days for *Cf-9* genotypes and after 5 days for *Cf-2* genotypes.

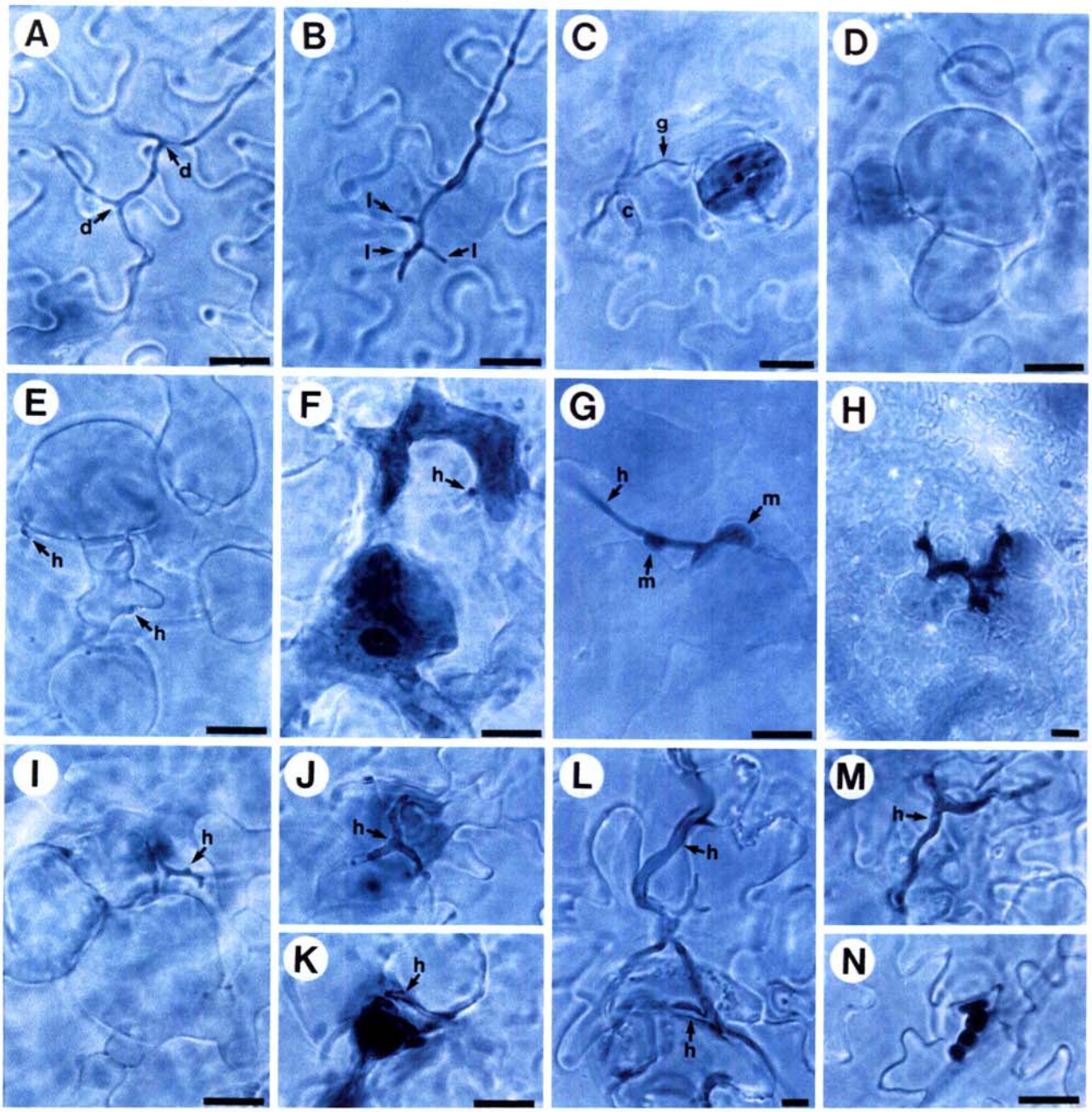

Fig. 3. Appearance of *Cladosporium fulvum* hyphae and tomato cells at selected stages of infection in various interactions involving race 0. Photomicrographs were taken of whole tissue mounts stained with lactophenol-trypan blue and the scale bar is equal to 20 µm. **A,** Hyphae at the edge of an infection in Cf0 exhibiting dichotomous branching [d], day 10; **B,** hyphae at the edge of an infection in a *Cf-3* homozygote exhibiting nondichotomous branching and limited elongation of laterals [l], day 10; **C,** dead guard cells of a penetrated stoma, [c]-conidia, [g]-germ tube, *Cf-2* homozygote, day 10; **D,** enlargement and circularization of lower mesophyll cells at the base of an invaded substomatal cavity [hyphae above the plane of focus], *Cf-2* homozygote, day 10; **E,** several swollen host cells in a deep lower mesophyll layer in the presence of hyphae [h], *Cf-5* homozygote, day 10; **F,** a dead and swollen host mesophyll cell that also contains an enlarged nucleus and a neighboring dead unswollen cell at the site of infection, [h]-hypha, *Cf-5* homozygote, day 10; **G,** deposits of globular material [m] on the outside of a host cell in close proximity to hyphae [h] in the center of an infection, *Cf-4* homozygote, day16; **H,** numerous swollen host cells surrounding a few dead cells at the site of infection, *Cf-2* heterozygote, day 10; *I,* highly branched hyphae [h] surrounded by enlarged host cells, *Cf-5* homozygote, day 16; *J,* swollen and distorted hyphae [h] above enlarged mesophyll cells, *Cf-5* heterozygote, day 16; **K,** extracellular material around the surface of a hypha [h] surrounded by two swollen and one dead mesophyll cell, *Cf-5* heterozygote, day 16; **L,** flattened and distorted hyphae [h] just above the lower epidermis in the center of an infection *Cf-9* heterozygote, day 16; **M,** flattened, distorted, and highly vacuolated hyphae just above the epidermis, *Cf-2* heterozygote, day 16, and, **N,** a swollen and globular but well-stained hyphae in the substomatal cavity, *Cf-2/Cf-5* double heterozygote, day 10.

Table 1. Characteristics of incompatibility conferred by various *Cf* resistance genes when homozygous or heterozygous. The heterozygous *Cf* genes were examined in both *Lycopersicon esculentum* and *L. esculentum* × *L. penneillii* hybrid genetic backgrounds[a]

	Genotype		
	***Cf* homozygote**	***Cf* heterozygote**	***Cf* heterozygote with hybrid background**
Hyphal growth[a]			
Cf-2	LS 0.64 ± 0.021	LS 1.24 ± 0.057	LS 3.76 ± 0.133
	D 0.82 ± 0.024	D 1.47 ± 0.038	D 3.84 ± 0.114
	F 6.41 ± 0.22	F 9.37 ± 0.38	F 11.87 ± 0.32
Cf-3	Sporulation onset	Sporulation onset	Sporulation onset
	16.23 ± 0.41	14.8 ± 0.38	13.8 ± 0.28
Cf-4	LS 12.2 ± 0.42	LS 16.30 ± 0.31	LS 23.32 ± 0.56
	D 4.44 ± 0.11	D 5.14 ± 0.13	D 6.20 ± 0.14
	F 12.87 ± 0.36	F 15.27 ± 0.35	F 21.13 ± 0.43
Cf-5	LS 0.98 ± 0.027	LS 2.85 ± 0.061	LS 3.53 ± 0.122
	D 1.48 ± 0.031	D 2.96 ± 0.068	D 3.47 ± 0.101
	F 8.11 ± 0.27	F 9.82 ± 0.43	F 12.14 ± 0.36
Cf-9	LS 1.41 ± 0.036	LS 3.41 ± 0.117	LS 5.13 ± 0.134
	D 1.48 ± 0.031	D 1.62 ± 0.053	D 3.33 ± 0.097
	F 9.26 ± 0.33	F 11.12 ± 0.46	F 15.83 ± 0.41
Cf-11	LS 0.74 ± 0.023	LS 7.38 ± 0.331	
	D 0.61 ± 0.016	D 2.89 ± 0.081	Sporulation onset
	F 6.76 ± 0.19	F 16.3 ± 0.48	20.2 ± 0.61
No *Cf* gene	Sporulation onset	N/A	Sporulation onset
	11.62 ± 0.35		9.24 ± 0.29
Host response[a]			
Cf-2	gcd 29.7 ± 0.86	S 6.27 ± 0.26	S 9.27 ± 0.41
	S 5.22 ± 0.24	Dm 3.17 ± 0.121	Dm 3.87 ± 0.121
	Dm 1.31 ± 0.043	N 2.43 ± 0.058 (la 1-2)	N 11.43 ± 0.37
	N 2.43 ± 0.061	N 9.11 ± 0.362 (la 3+)	exg ND
	exg ND	exg ND	PS 96.3 ± 0.54
	PS 38.4 ± 1.32	PS 94.4 ± 0.49	
Cf-3	S 13.4 ± 0.46	S 13.8 ± 0.45	S 13.7 ± 0.41
	Dm All	Dm All	Dm All
	exg 14.5 ± 0.38	exg 14.7 ± 0.41	exg 14.38 ± 0.36
	PS 92.4 ± 0.82	PS 89.3 ± 1.12	PS 83.2 ± 1.24
	cd 16.12 ± 0.39	cd 14.62 ± 0.34	cd 13.74 ± 0.47
Cf-4	S 8.37 ± 0.27	S 10.61 ± 0.39	S 10.74 ± 0.41
	Dm 3.78 ± 0.108	Dm 3.84 ± 0.121	Dm 5.83 ± 0.143
	N 18.83 ± 0.71	N 23.23 ± 0.93	N 31.41 ± 0.96
	exg 14.85 ± 0.61	exg 14.35 ± 0.93	exg 14.72 ± 0.39
	PS 93.4 ± 0.73	PS 97.3 ± 0.56	PS 97.4 ± 0.51
Cf-5	S 6.89 ± 0.27	S 8.35 ± 0.33	S 10.61 ± 0.35
	Dm 2.41 ± 0.041	Dm 2.31 ± 0.052	Dm 3.43 ± 0.057
	N 2.83 ± 0.058	N 3.47 ± 0.063	N 3.62 ± 0.067
	exg ND	exg 10.41 ± 0.36	exg 14.84 ± 0.47
	PS 78.2 ± 0.91	PS 93.8 ± 0.53	PS 92.7 ± 0.57
Cf-9	S 8.72 ± 0.28	S 10.41 ± 0.34	S 10.52 ± 0.29
	Dm 1.14 ± 0.032	Dm 1.24 ± 0.037	Dm 1.63 ± 0.034
	N 1.35 ± 0.037	N 2.75 ± 0.083	N 2.81 ± 0.079
	exg ND	exg 14.91 ± 0.46	exg 14.9 ± 0.41
	PS 23.2 ± 0.72	PS 29.2 ± 0.93	PS 58.3 ± 1.2
Cf-11	S 6.34 ± 0.21	S 6.38 ± 0.23	S 10.34 ± 0.29
	Dm 1.18 ± 0.023	Dm 3.41 ± 0.091	Dm 4.33 ± 0.109
	N 6.88 ± 0.28	N 8.23 ± 0.29 (la1-2)	N 26.3 ± 0.97
	exg ND	N 22.0 ± 0.67 (la3+)	exg 14.2 ± 0.42
	PS 96.2 ± 1.3	exg 10.71 ± 0.38	PS 94.3 ± 0.32
		PS 95.4 ± 0.56	
No *Cf* gene	cd 14.82 ± 0.61	N/A	cd 12.4 ± 0.52

[a] Inoculations with race 0 or race 4-*GUS*. For *Cf-4* only race 0 data is presented. LS, lateral hyphal growth measured in epidermal cell length equivalents (1 = 50 μm); D, depth hyphal growth measured in mesophyll cell layer equivalents where 1 denotes the layer directly beneath the epidermis and 6 the layer in the plane of the vascular tissue; F, days after inoculation when no additional hyphal growth was evident; sporulation onset, days after inoculation when conidiophores emerge from stomata; gcd, guard cell death at penetrated stomata (*Cf-2* only). S, days after inoculation when swollen mesophyll cells first observed. Dm, position of responding mesophyll. 1 denotes the layer directly beneath the epidermis and 6 the layer in the plane of the vascular tissue. N, final number of altered host cells (la - denotes specific mesophyll layer). exg, days after inoculation when extracellular globular deposits on host cell walls first observed (ND, not detected). PS, percentage of penetration sites with swollen host cells present. cd, mesophyll cell death.

We have characterized the influence of the zygosity of each *Cf* gene on limiting hyphal growth, and on the plant's responses to IF injections. By examination of the effects of some of the *Cf* genes in paired combinations, their epistatic relationships have also been investigated. Finally, an assessment of the effect of the *L. pennellii* genome on both compatible and incompatible reactions to fungal hyphae and IF is reported. *L. pennellii* × *L. esculentum* F_2 populations are frequently used to map genes on the tomato RFLP map because of the considerable genetic diversity in this interspecific cross (Tanksley *et al.* 1993).

RESULTS

Comparison of the effectiveness of different *Cf* genes in restricting fungal hyphae.

The transgenic race 4 of *C. fulvum* that expressed the β-glucuronidase gene, permitted a comparison to be made between the effectiveness of the *Cf-2, 3, 5, 9,* and *11* resistance genes in restricting fungal biomass. In Figure 1 the time courses of β-glucuronidase activity accumulation in these different incompatible interactions are shown. The incompatible interactions are compared to two compatible interactions; one in which a *Cf* resistance gene is absent (Cf0) (Fig. 1A) and a second in which use is made of the *Cf-4* resistance gene (overcome by the *C. fulvum* race 4) (Fig. 1B). Compatible *C. fulvum* conidia germinated and their germ tubes grew extensively over the leaf surface for the first 3 days before penetrating stoma. Hyphae then thickened upon entry into the leaf and sequentially colonized the lower and upper mesophyll layers by growing between the host cells to form a dense net of mycelium containing numerous straight runner hyphae. After 10–12 days, conidiophores emerged from stoma at the center of each infection to form an expanding sporulating lesion on the lower leaf surface, which was visible macroscopically 12–14 days after inoculation. By day 16 the entire lower leaf surface was covered with a matt of white conidiophores upon which mature pale brown conidia were evident. This pattern of mycelial growth was reflected in the rapid rise in β-glucuronidase activity in both compatible interactions from day 8 onwards. In *Cf-2*, *Cf-5*, *Cf-9,* and *Cf-11* interactions, GUS activity remained very low throughout the time-course, reflecting limited hyphal growth. Fungal sporulation was never observed on these lines. A moderate rise in GUS activity was obtained for *Cf-3* up to day 16, which peaked at a level 10-fold lower than found in the two compatible interactions. Very sparse sporulation was evident on this line from day 16 onwards when leaves were examined under a dissecting microscope.

From the histological observations shown in Figure 2A, upper panel, and Table 1, clear distinctions are apparent in the relative effectivenesses with which the *Cf-2, 4, 5,* and *9* genes retard fungal growth, which were either not revealed in their respective GUS activities or could not be assessed (*Cf-4*). On Cf2 and the Ontario 7716 line containing *Cf-11*, hyphal ingress was restricted to the substomatal cavity or within one cell of it and further advances of hyphae were rarely observed after day 6. In Cf5 the penetrating hyphae immediately entered into lower mesophyll layers but rarely progressed more than two cell layers. These hyphae exhibited limited lateral branching and by day 10 all growth was arrested. With Cf9, hyphae grew up to one to two epidermal cell lengths from the penetrated stoma, but these were often distorted and had limited lateral branches, and the infections were mainly confined to the intercellular space immediately adjacent to the lower epidermis. No further growth was evident after day 10. Greater hyphal ingress occurred with *Cf-4*, by day 8, numerous highly branched hyphae with occasional straight runner hyphae were present in the entire lower mesophyll and a few hyphae had penetrated deeper into the upper mesophyll layers. However, by day 14 no further increases in the extent of the infection were apparent. *Cf-3* allowed more mycelial colonization than *Cf-4*, but from day 6 onwards the pattern of growth was again distinct from that occurring in compatible interactions. The tips of the hyphae often failed to branch in a dichotomous manner and the side branches developed poorly (cf. Fig. 3A and B). By day 16, straight runner hyphae became evident at the margin of the mycelium but in its center extremely thick and highly vacuolated hyphae were often present. Conidiophore formation was severely delayed and reduced and conidia were rarely formed. Lazarovits and Higgins (1976a) had previously noted that the *Cf-3* gene had its effect on *C. fulvum* development around the onset of conidiophore formation. Our data reveal some earlier effects of *Cf-3*.

Tomato host cells involved in incompatible interactions with *C. fulvum* hyphae exhibited a range of responses, the phenotype and timing of which differed with each *Cf* gene (Table 1). In Cf2 at approximately 30% of the infection sites, the guard cells accumulated trypan blue (Fig. 3C) and were presumed dead (Keogh *et al.* 1980). In the other incompatible interactions this response was only observed beneath large clumps of germinating spores. No other macroscopic responses of the L1 (epidermal layer) were ever observed in any interaction; all visible host reactions were confined to the L2 (mesophyll) and L3 (vascular) layers of the leaf. In Cf2, Cf5, and Cf11 lines a consistent feature was the swollen and circular appearance of mesophyll cells directly beneath the penetrated substomatal cavity. This was apparent within 1 or 2 days of germ tube entry on Cf2 (Fig. 3D) but was delayed until 3 days postpenetration on Cf5 and Cf11. In Cf5, hyphae were

Table 2. Necrosis-inducing activity of intercellular fluid from a compatible Cf0-race 0 interaction on different tomato genotypes; the value[a] given is the dilution endpoint of necrosis-inducing activity of the intercellular fluid

	Genotype		
	***Cf* homozygote**	***Cf* heterozygote**	***Cf* heterozygote with hybrid background[b]**
No Cf gene[c]	...[d]	...	...
Cf-2[e]	16	8	8
Cf-3[e]	2	...	...
Cf-4[e]	8	4	4
Cf-5[e]	16	8	8
Cf-9[f]	64	32	4–8[g]
Cf-11[e]	2	...	...

[a] The response of the eight plants tested was the same.
[b] *Lycopersicon esculentum* × *L. pennellii* F_1 hybrid.
[c] Chlorosis and necrosis induction were assessed 7 days after injection.
[d] No necrosis was visible after injection of the undiluted intercellular fluid.
[e] Chlorosis and necrosis were assessed 4 days after injection.
[f] Gray necrosis was visible 8 hr after injection, but assessments were done 1 day after injection.
[g] In this experiment, 16 plants were tested, eight plants responded to the 1/4 IF dilution, and eight plants responded to the 1/8 IF dilution.

often seen in advance of the initially reacting host cells, and a second round of host cell enlargement occurred in deeper mesophyll layers immediately in advance of the hyphae, 6–9 days postpenetration (Fig. 3E). At these later time points host cells distal to hyphae in the deeper mesophyll layers also enlarged in Cf2 and Cf11, and this response extended two and four cells, respectively, from the arrested hyphae. With Cf9 and Cf4 some host cell enlargement occurred later (from day 8 onwards) and was confined to the infection center where hyphae had attempted to colonize the deeper mesophyll layers. At later time-points in all these interactions some swollen cells in immediate contact with hyphae accumulated trypan blue and their nuclei appeared considerably enlarged (Fig. 3F). From day 14 onwards in Cf4 and Cf3, globular extracellular material (predominantly consisting of lignin on the basis of staining with phloroglucinol-hydrochloric acid and autofluorescence under UV light) was found on the surfaces of mesophyll cells in close association with hyphae in the lesion center (Fig. 3G). In addition, a proportion of both swollen and unswollen mesophyll cells in the center of these infections accumulated trypan blue. In Cf9 and Cf4 a host response was never evident at the hyphal front directly above the epidermis even when growth was finally arrested. With the Cf3 line, the extensive collapse of cells in the center of the lesion occurred at the onset of conidiophore formation and was characterized by the retention of either the GUS or trypan blue histochemical stain. In compatible interactions, no obvious host cell response to hyphae occurred during the first 10 days of infection, except for a few infection sites in which fungal ingress was rapidly arrested (described in detail below).

Influence of *Cf* gene zygosity on the efficiency with which fungal hyphae are restricted.

Homozygous *Cf* lines were crossed to Cf0 to generate F_1 plants heterozygous for the *Cf* genes. MUG activities obtained from these plants after inoculation with the *GUS* race of *C. fulvum* were significantly higher in the heterozygotes for *Cf-3* and *Cf-11* than in homozygotes. Their final GUS activities were two- and four-fold higher, respectively (Fig. 1C and D). No significant differences in activity were observed between heterozygotes and homozygotes for *Cf-2*, *Cf-5*, and *Cf-9*. Their GUS activities remained minimal throughout the course of the experiment (Fig. 1A). However, the detailed histological observations revealed that these *Cf* genes were in fact significantly weaker when present in a heterozygous state (Fig. 2A, cf. upper and lower panels; Table 1). In *Cf-2*, heterozygotes hyphae were evident up to two mesophyll cell lengths from the substomatal cavity at approximately 30% of penetration sites, compared to ≤ one cell length in homozygotes. In *Cf*-5 heterozygotes, hyphae extended two to four cell lengths and often penetrated into the deeper mesophyll layers, but rarely progressed more than two cell layers in homozygotes. *Cf-9* heterozygotes permitted hyphae to penetrate two to six cell lengths, but as in homozygotes, hyphae were mainly restricted to the mesophyll layer immediately adjacent to the epidermis and most did not advance further after day 12. A few thickened hyphae in the center of lesions, <3 per leaf disk, penetrated deeper into the second and third mesophyll layers above the epidermis (layers 2 and 3), and these continued to grow until day 16. In *Cf-11* heterozygotes, hyphae extended four to 10 cell lengths by day 16 and also penetrated the deeper mesophyll layers. The mycelial growth permitted by *Cf-4* heterozygotes was considerable by day 8, extending up to 20 cell lengths, but it was low in overall density and predominantly devoid of straight runner hyphae. Greatest mycelial growth was supported in *Cf-3* heterozygotes although it was distinguishable throughout the time course from compatible infections because of the low abundance of straight runner hyphae. Sparse conidiophore development occurred from day 14 onwards.

In *Cf*-gene heterozygotes, reactions to *C. fulvum* were delayed but otherwise had phenotypes similar to those seen on the corresponding homozygotes (Table 1). A greater number of host cells eventually responded; this appeared to be due primarily to the greater fungal biomass present in each incompatible interaction. However, with *Cf-2* and *Cf-11* heterozygotes, more host cells in the deeper tissue layers appeared to respond per unit fungal hyphae (Fig. 3H). The visible response extended four to six and eight to 10 cells from the contained hyphae with *Cf-2* and *Cf-11*, respectively. In the

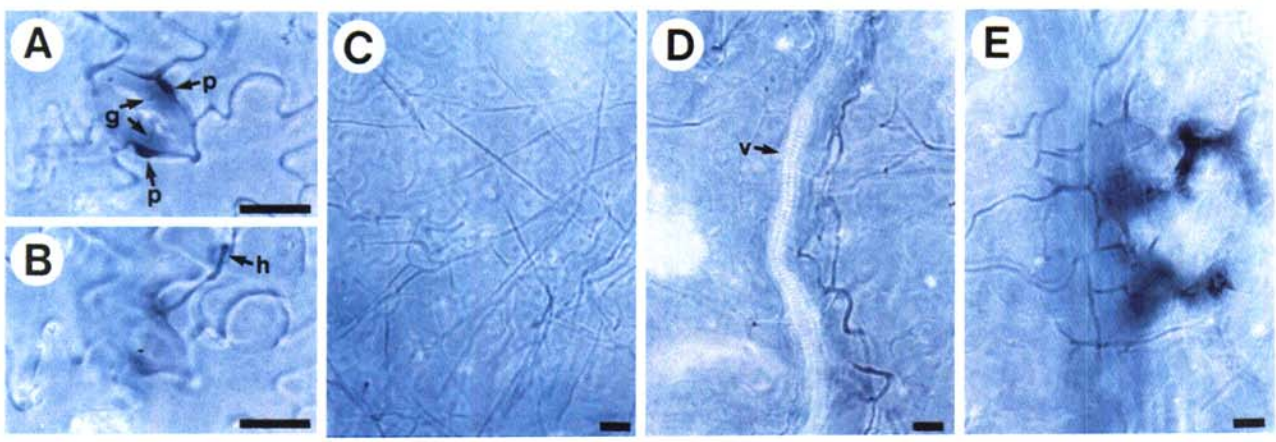

Fig. 4. Features of compatible *Cladosporium fulvum* interactions in Cf0 involving race 0. Photomicrographs were taken of whole tissue mounts stained with lactophenol-trypan blue, and the scale bar is equal to 20 μm. **A,** Unsuccessful germ tube [g] penetrations of a stoma causing the localized deposition of material in guard cell wall's to form papillae [p]; **B** the same as A but photographed in the leaf plane above, one of the hyphae [h] extends < 1 cell length into the lower mesophyll, day 10; **C,** successful infections with abundant straight runner hyphae present in the lower mesophyll, day 10; **D,** accumulation of highly branched and slightly thickened hyphae around the vascular tissue [v], day 10 and, **E,** dead mesophyll cells adjoining veins surrounded by branched hyphae in an area of the infection where sporulating conidiophores were present on the lower leaf surface, day 16.

Cf-5 heterozygote, the column of responding host cells surrounding the deeper penetrating hyphae remained relatively constant in width. In these three interactions no further increase in the total number of responding cells was evident after day 16, although for *Cf-11* heterozygotes the hyphae at the lesion margin were not surrounded by swollen cells. In the *Cf-9* and *Cf-4* heterozygotes hyphae present immediately above the epidermis failed to evoke any visible host reaction. Only when hyphae penetrated into cell layers 2 and 3 did host cell swelling occur. At the margins of these infections which continued to expand, albeit slowly, a host response was never evident. From day 14 onwards, numerous globular extracellular deposits (as described above) were observed in the center of lesions in *Cf-3*, *Cf-4,* and *Cf-9* heterozygotes and also to a limited extent in the *Cf-11* and *Cf-5* heterozygotes from day 10 onwards.

The fates of fungal hyphae in the various incompatible interactions conditioned by *Cf* genes in heterozygotes were similar to those in homozygotes. It appeared that the timing of final containment and hence the overall size of the infection was dependent on the zygosity of the *Cf* gene. A consistent feature of incompatibility was the frequent abortive attempts to initiate lateral branches. Hyphae became distorted in appearance because of the high density of the small lateral initials (Fig. 3I) or because the tip of the sole hypha in some infections on *Cf-2*, *Cf-5,* and *Cf-11* homozygotes had itself become slightly swollen (examples in Fig. 1). Up to 7 days postpenetration, all hyphae stained well with trypan blue. In most infections that had extended < two cell lengths and had remained just above the lower epidermis, no further alterations in hyphal morphology were evident throughout the time course. From day 10 onwards an increasing number of swollen hyphae which stained poorly were evident in the various interactions (Fig. 3J–M). These hyphae either lacked cytoplasmic contents or contained abundant vacuoles, were flattened, and had extracellular material deposited around their surface. These alterations to hyphae were evident when the deeper mesophyll layers had been penetrated and particularly where hyphae were surrounded by swollen host cells. A similar fate befell hyphae in the center of larger infections, where the hyphae had remained just above the lower epidermis (Fig. 3L). This type of infection was particularly evident in *Cf-9* heterozygotes and all lines involving *Cf-4* and *Cf-3*. In these infections mycelium at the margins appeared normal, and when growth was finally arrested there were no obvious alterations to hyphae, which remained well stained and of normal dimensions for the remainder of the time course.

In some inoculation experiments conducted in the glasshouse greater *C. fulvum* growth has been observed in incompatible interactions than is reported above. However, the relative efficiencies of the various *Cf* genes to restrict hyphae was never altered, and the types of host cell responses triggered were similar but involved additional mesophyll cells.

Influence of *Cf* gene zygosity on host responsiveness to race-specific elicitors.

From compatible *C. fulvum* infections intercellular fluid (IF) can be prepared that contains the active products of all the avirulence genes possessed by the infecting race. Thus IF, like race 4 *GUS* used above, can be used as a reagent to quantify the biological effectiveness of the *Cf* genes in their homozygous and heterozygous states. By using an IF prepared from a compatible Cf 0–race 0 interaction and serially diluting these preparations to 1/64th of their original concentration, the lowest titer at which a chlorotic or necrotic response was induced on lines containing the *Cf* genes was determined. The results are summarized in Table 2. They reveal that homozygotes for each *Cf* gene can respond to a two-fold lower titer of IF than heterozygotes. The responses for the *Cf-3* and *Cf-11* genes were so weak that no macroscopic reactions to IF were seen on heterozygotes. Interestingly, neither the nature of the reaction nor the timing of its appearance were altered at the IF titers giving positive reactions. *Cf-9* conditioned a gray necrotic reaction within 24 hr of IF injection, *Cf-2* a strong chlorotic and necrotic reaction by day 4, *Cf-5* a strong chlorotic reaction again by day 4, *Cf-4* a moderate chlorotic reaction by day 7, and *Cf-3* and *Cf-11* a weak chlorotic reaction by day 7 in homozygotes. The types of responses observed on the different homozygous *Cf* lines to race-specific elicitor (IF) challenge and the timing of their appearance were identical to those originally reported by de Wit and Spikman (1982) and Higgins and de Wit (1985). Examples of the influence of *Cf* zygosity on macroscopic responses to IF are given for the *Cf-9* and *Cf-2* genes in Figure 2B.

Influence of the *L. pennellii* genome on *Cf* gene action.

Homozygous *Cf* lines were crossed to *L. pennellii* to generate F_1 plants heterozygous for the *Cf* genes with a 50% *L. pennellii* 50% *L. esculentum* background. Significantly higher levels of fungal biomass were found in all heterozygotes with this hybrid background compared to a *L. esculentum* background (Fig. 1 and Table 1). Using race 4 *GUS*, the only detectable changes to GUS enzyme activity were discernible in the *Cf-3* and *Cf-11* heterozygotes in a hybrid background, the final values were 5- and 30-fold greater than the respective homozygotes. On the *Cf-3* heterozygotes in a hybrid background sporulation was first visible on day 16, but by day 19 was comparable to that on the Cf0 × *L. pennellii* hybrid at this time point. On the *Cf-11* heterozygote in a hybrid background small pustules (2–4 mm in diameter) of fungal sporulation were observed from day 21 onwards, but they never attained normal size (7–10 mm in diameter). For the *Cf-2*, *Cf-5*, and *Cf-9* heterozygotes in a hybrid background the GUS activity remained low throughout the time course (Fig. 1A).

Microscopic observations (summarized in Table 1) revealed that although the relative efficiency of hyphal containment by *Cf* genes remained unaltered, greater *C. fulvum* hyphal growth was permitted in all the *Cf* heterozygotes with hybrid background than *L. esculentum* background. Hyphae extended two to six cell lengths in the *Cf*-2 and *Cf*-5 heterozygotes and often reached the mesophyll layer in the plane of the veins. In the *Cf-9* heterozygote lateral hyphal growth extended two to eight cell lengths and in the lesion center hyphae penetrated to mesophyll layers 3 and 4. Greater than 20 cell lengths were colonized in the *Cf-4* heterozygote, and hyphae extended into the upper mesophyll. Although straight runner hyphae were quite abundant, there was minimal accumulation of branched hyphae around the veins and conidiophores were absent. In the *Cf-2*, *Cf-5,* and *Cf-9* heterozygotes further increases in the total extent of colonization were rarely evident after day 16, although hyphal thickening and further branching occurred within its boundary.

In the various incompatible interactions on the *L. pennellii* hybrid heterozygotes, visible host cell responses were delayed until hyphae penetrated deeper into the mesophyll in the infection center (5–7 days postpenetration). The phenotypes of the responding cells were identical to those observed on the *L. esculentum* lines. However, throughout the time course on the *Cf-9*, *Cf-4,* and *Cf-11* hybrid heterozygotes a greater number of nonresponding host cells separated the hyphal front from the responding host cells than in the corresponding *L. esculentum* lines. Deposition of globular extracellular materials on host cell walls was also particularly evident in the center of lesions on all lines, except *Cf-2* heterozygotes, from day 14 onwards. A host response specific to *L. pennellii* and the *Cf* × *L. pennellii* lines was the increased number of highly granulated cells as infections proceeded. This novel cell type is formed in a range of plant species when exposed to stress conditions and appears to be the site of calcium oxalate accumulation. In the *L. pennellii* lines these granulated cells were not clustered around the infection sites but were evenly distributed in the mesophyll cell layer in the plane of the vascular tissue and were found in both compatible and incompatible interactions.

The necrotic and chlorotic responses to IF challenge in the heterozygotes with hybrid background were identical to those observed on the *L. esculentum* heterozygotes with the exception of *Cf-9* (Table 1). The characteristic gray necrotic response conditioned by *Cf-9* was only apparent at an IF titer 8- or 16-fold lower than for the homozygous line but the timing of its appearance was unaltered. By day 4 a weak chlorotic reaction was evident at the first IF dilution titer that did not give a gray necrotic reaction.

_Cf_ gene epistasis.

To explore the combined effects of two different *Cf* genes on *C. fulvum*, double heterozygotes containing *Cf-2*/*Cf-4*, *Cf-2*/*Cf-5*, *Cf-4*/*Cf-5,* and *Cf-4*/*Cf-9* were generated. Microscopic observations following *C. fulvum* inoculation revealed some alterations to the infection phenotypes in two of these lines compared to their corresponding *Cf* heterozygote line. On *Cf-2*/*Cf-5*, infections were more confined than on the *Cf-2* heterozygote, *Cf-5* homozygote or *Cf-5* heterozygote but were equivalent to those observed on the *Cf*-2 homozygote. Within 1 day of germ tube entry, one or two host cells at the base of the substomatal cavity swelled, and the infections rarely proceeded further. No additional visible host responses were evoked but often by day 10 the hyphae had an extremely distorted appearance (Fig. 3N). This unusual alteration to hyphal appearance was never observed in the other interactions examined. With the *Cf-4*/*Cf-5* double heterozygote although the degree of hyphal containment was equivalent to that conditioned by the *Cf-5* heterozygote, the column of responding host cells surrounding the contained hyphae was increased in width by three to five cells. Double heterozygotes *Cf-2*/*Cf-4* and *Cf-4*/*Cf-9* gave infection phenotypes comparable to those observed on *Cf-2* and *Cf-9* heterozygotes, respectively. When these four lines were challenged with IF, the necrotic and chlorotic responses invoked were similar in appearance to that conditioned by the heterozygote *Cf* line containing the *Cf* gene conditioning the stronger reaction, i.e., double heterozygotes *Cf-2*/*Cf-4* and *Cf-2*/*Cf-5* were both equivalent to the *Cf-2* heterozygote, and double heterozygotes *Cf-4*/*Cf-5* and *Cf-4*/*Cf-9* were equivalent to *Cf-5* and *Cf-9* heterozygotes, respectively. Thus, the resistance phenotype controlled by *Cf-2* is epistatic over that conditioned by either *Cf-5* or *Cf-9*, and the *Cf-5* and *Cf-9* genes are epistatic in action to *Cf-4*.

Additional features of compatible interactions.

As germ tubes entered stomata, a small proportion (< 5%) evoked the localized deposition of cell wall material to form a callose containing papillae beneath the wall on the lower surface of the guard cells. Hyphal growth either proceeded no further or a nonthickened hyphae extended up to 1 epidermal cell length (Fig. 4A,B). This type of infection was invariably observed where conidia had germinated in the center of an interveinal area of the leaf, and was evident in all interactions at about the same frequency. At the rest of compatible infection sites, the subsequent period of host tissue colonization was characterized by radial enlargement of the mycelial colony. Predominantly long straight runner hyphae grew in the lower mesophyll layers (Fig. 4C), while more branched hyphae subsequently colonized the palisade mesophyll layer. Although there was no obvious directional growth of hyphae, from day 8 onwards a progressively increasing amount of mycelium in the lesion center was associated with vascular tissue (Fig. 4D). The minor and major leaf veins appeared to present a physical barrier to hyphae advancing in this plane of the leaf because hyphae in neighboring leaf planes crossed the veins and continued to grow away from the lesion center. Hyphae associated with the vascular tissue branched frequently, and at subsequent time-points considerable increases in hyphal diameter were evident. This latter feature was particularly striking in the lines derived from *L. pennellii.* A shift to increased susceptibility was evident in all compatible interactions involving the *L. pennellii* lines. Sporulation was visible first on *L. pennellii* at day 9, occurred next on Cf0 × *L. pennellii* at day 10 and last on Cf0 plants at day 12. However, the total biomass of *C. fulvum* finally supported on *L. pennellii* was significantly lower than in the other compatible interactions (Fig. 1A).

On *L. pennellii* extensive and simultaneous host cell death was evident from day 8 onwards in mesophyll layers directly above the emerging conidiophores. These areas expanded rapidly and tissue browning and chlorosis were evident from day 14 onwards. The reduced GUS activity evident on *L. pennellii* from day 19 onwards was presumably due to host cell death eliminating enzyme activity. On the Cf0 × *L. pennellii* and Cf0 lines, host cell death was delayed until 1 or 2 days after sporulation and then extended more gradually. Isolated host cells immediately adjacent to the vascular tissue and directly above sporulating conidiophores died first (Fig. 4E). Their cytoplasm also became very granular in appearance. Subsequently, adjacent host cells either associated with vascular tissue or in the surrounding mesophyll died. As the sporlating lesions expanded, extensive areas of dead mesophyll cells were evident within their boundaries and the associated vascular tissues became filled with brown pigmented materal. Death of epidermal cells was never apparent in any of the comatible interactions.

In this study, the ability to quantify fungal biomass by using the *GUS* race of *C. fulvum* revealed no residual resistance activity of the *Cf-4* gene towards race 4 in either the *L. esulentum* or hybrid genome backgrounds.

DISCUSSION

This study shows that the invasion of *C. fulvum* hyphae in tomato leaves can be contained at almost any stage during the infection of the mesophyll cell layers and if sporulation cannot be prevented, conidiophore development can be delayed and their final numbers reduced. Collectively, fungal biomass quantification and microscopic observations show that the efficiency with which hyphal ingress was contained in heterozygotes decreased in the *Cf* gene order 2, 5, 9, 4, (11), 3. Because of the influence of both the plant *Cf* gene and the fungal *Avr* gene on the interaction outcome, the relative strengths of the *Avr* genes can likewise be ascribed as decreasing in an identical gene order. The ranking of the Cf-11 gene has been placed in brackets because of the disproportionate increase in fungal growth in the *Cf-11* heterozygotes compared to the other *Cf* lines. Possibly, the Ontario 7716 background contributes to the extreme resistance phenotype of the homozygous line or the extreme suseptibility of the heterozygote. In addition, the data reveal each *Cf* gene when present in a homozygous state to be more effecive in containing infections than when present in a heterozygous state, and also that each *Cf* gene was less effective in the *Cf* × *L. pennellii* F_1 hybrid lines. The considerable amount of fungal growth permitted by the *Cf-4* gene in these experiments was unexpected. de Wit (1977) had previously reported hyphae to be contained within four cell lengths of the stoma entered. We are unable to explain this discrepancy which has been consistently observed for the past 3 yr in our laboratory under a range of greenhouse and growth room regimes and with different fungal races incompatible with *Cf-4*.

In general, the timing and severity of the leaf's macroscopic responses to IF were in agreement with the above gene order with the exception of the *Cf-9-Avr9* combination, which now ranked first. Possibly, a greater rate of *Avr9* gene transcription or greater stability of the AVR9 peptide compared to other *Avr* genes and their products, accounts for the abundance of AVR9 in IF collected from leaves supporting *C. fulvum* sporulation. At the hyphal-plant cell interface in incompatible interactions, the amount of AVR9 perceived by individual host cells is probably considerably less than from an IF injection. At infection sites, the effectiveness of the gene combination *Cf-2-Avr2* and *Cf-5-Avr5* in the triggering of successful defense responses were greater than for *Cf-9-Avr9*. The relative contribution of each gene product in a specific *Cf-Avr* combination to the final outcome of incompatibility is unknown.

Interestingly, a correlation appeared to exist between the resistance phenotype each *Cf-Avr* gene combination conferred and the genetic relationship of the *Cf* genes to one another (where known). The *Cf-2* and *Cf-5* genes, which may be allelic (Jones *et al.* 1993), are both strong resistance genes. They permitted very limited hyphal growth and this was frequently accompanied by a pronounced enlargement of the surrounding host cells. By contrast, the *Cf-9* and *Cf-4* genes, which may also be allelic genes but at another locus (Jones *et al.* 1993), confer moderate and weak resistance, respectively. Although differing considerably in their relative strengths, the modes of action of the *Cf-9* and *Cf-4* genes exhibit striking similarities. Both initially restricted hyphal colonization to just above the lower epidermis and only later, as hyphae in the center of the lesion attempted to penetrate into deeper mesophyll layers, did host cells enlarge. The apparent differences in relative strengths of the *Cf-9* and *Cf-4* genes may be due to different relative concentrations of active *Avr4* and *Avr9* gene products at the infection sites and not to intrinsic differences in the action of each *Cf* gene. The weak resistance gene *Cf-1* (Bond 1938) may also be allelic to *Cf-4* and *Cf-9* (Jones *et al.* 1993). These findings suggest qualitatively distinct resistance mechanisms are initiated by different *Cf-Avr* gene combinations and that these might be related to the genetic location of the *Cf* gene. By mapping the corresponding *Avr* genes and additional *Cf* genes this hypothesis can be tested. Collectively, our data do not reveal an obvious correlation between *Cf* resistance phenotype and the wild *Lycopersicon* species from which the genes were introgressed.

Containment of *C. fulvum* hyphae was frequently observed without a rapid host cell death, "hypersensitive" response. When evident, host cell death was evoked between 3 and 8 days after *C. fulvum* infection. Classically, hypersensitivity is defined as the death of host cells within a few hours of pathogen contact (Stakman 1915). Therefore, the delayed response of the tomato cells precludes use of this term, except for interactions involving *Cf-2*. In other studies (de Wit 1977; Lazarovits and Higgins 1976a), rapid host cell death was recognized to be associated with the action of the *Cf-2* and *Cf-4* resistance genes. The extracellular mode of colonization of *C. fulvum* hyphae could account for why this biotrophic fungus is contained without the need for host cell sacrifice; host defense responses could be targeted to the outside of the cell where they may inflict less damage to the host itself. Alternatively, the burden of defense can be shared by all cells neighboring the invading hyphae and thereby not require an individual cell's death. Other obligate biotrophic fungal species, e.g., powdery and downy mildews and the rusts, penetrate host cell walls and form a specialized feeding organ, the haustorium, that is surrounded by the plasma membrane of the host cell. In an incompatible interaction the defense response is likely to be limited initially to the invaded cell and always appears to involve its death.

The frequent observation of enlarged and circular mesophyll cells either in contact with hyphae or slightly in front of the advancing hyphae was interesting. This feature of incompatibility was also noted by Lazarovits and Higgins (1976a) when exploring the *Cf-2* and *Cf-4* genes in combination in the cv. Purdue and the *Cf-3* gene in the cv. V121. Similar alterations to host cell appearance are evident on Cf9 plants after IF injection into interveinal leaf panels (K.H-K, unpublished). These altered cells were able to accumulate the vital stain fluorescein diacetate and to be plasmolyzed by osmotically active solutions and so are considered to be alive. Similarly in this study the swollen cells did not accumulate trypan blue. These changes to host cell morphology suggest induction of cell wall loosening reactions, increased synthesis of plasma membrane and water uptake. The contribution of these events to resistance is not known, but similar events were never observed in the various compatible interactions examined.

Hyphal growth was gradually arrested in each incompatible interaction, except in Cf2. Depending on the *Cf* gene involved, termination of hyphal advance was not evident until 10 days or later. The results of the detailed examination of the fate of hyphae are consistent with the presence of defense responses that are particularly effective against hyphal tips and lateral

branch initials. Such a role in defense may be played by plant hydrolytic enzymes, e.g., chitinases and β-1,3 glucanases (Boller 1987; Mauch *et al.* 1988). These proteins have been shown to accumulate earlier in incompatible interactions, from day 6 onwards (Joosten and de Wit 1989; Joosten *et al.* 1990). However, in the *Cf-2* and *Cf-5* interactions the inhibition of hyphal growth often occurred earlier and was not accompanied by any obvious morphological distortions of the hyphae. Similarly, in the *Cf-9*, *Cf-4*, and some of the *Cf-11* interactions, hyphae at the lesion margin were unaltered in appearance when their growth was arrested. Thus, other defense mechanisms may also be operating on *C. fulvum* hyphae in incompatible interactions. Two other candidate defense reactions that could inhibit hyphal growth are the generation of active oxygen species, i.e., superoxide and hydroxyl radicals and hydrogen peroxide, and the action of lipoxygenase enzymes to produce toxic lipid degradation products. Both are known to be induced when tomato cells are challenged with *C. fulvum* race-specific elicitors (Peever and Higgins 1989; Vera-Estrella *et al.* 1992). This study also indicates that when *C. fulvum* infections are rapidly arrested, there is considerable uniformity in the final size of the infection and that the timing of the host's reaction and the spatial location of the responding cells is relatively constant. However, in infections that proceed further, increased variation was observed between individual penetration sites in the total mycelial growth present and the host cell layers entered, and the spatial and temporal patterns of the responding host cells became less synchronous.

In compatible interactions, the formation of enlarged hyphae in association with the vascular tissue may be indicative of the better nutritional environment in this region of the leaf. These apparently fortuitous encounters with vascular tissue, lead hyphae to accumulate both in close proximity with host cells involved in phloem loading and unloading and in an apoplastic zone where water from the xylem vessels first enters the leaf via transpiration. However, the contribution of these hyphae to the feeding of the entire infection is not known. As host cell death occurred first in the regions adjoining vascular tissue, at the time of sporulation, this suggests these host cells were heavily supporting *C. fulvum* parasitism and were eventually unable to cope with this burden. The increased rate of *C. fulvum* growth on *L. pennellii* and the Cf0 × *L. pennellii* F_1 hybrid indicates that either the available nutrients provided by this species were better than those provided by *L. esculentum* or deleterious chemicals, enzymes, and inducible reactions were reduced.

The weakening effects of the *L. pennellii* genome on each resistance phenotype were striking and reveal that other loci are crucial for resistance besides the *Cf* genes. The reduced gray necrotic response of the Cf9 × *L. pennellii* line to IF challenge was not unexpected. Rapid necrosis on Cf9 is dependent on the supra-optimal opening of stoma and can be abolished if plants are maintained under high humidity conditions or sprayed with the abscisic acid (K.H-K, unpublished). *L. pennellii* is known to be a drought tolerant species (Rick 1988) and presumably can tightly regulate water loss through stoma. The data also indicate that the fine mapping of *Cf* genes which confer only moderate resistance in a *L. esculentum* background may be problematic using the standard *L. pennellii* × *L. esculentum* F_2 population. Even with *Cf-4*, reliable scoring of disease responses has proven difficult in this mapping population (P. Balint-Kurti, personal communication).

The increased *C. fulvum* invasion of host tissue and the higher titer of IF required to elicit a necrotic or chlorotic response in lines where a *Cf* gene was present in a heterozygous state indicates the Cf genes are incompletely dominant. Presumably two copies of the gene result in higher amounts of active *Cf* protein and this accounts for the discernible effect. By inference, the lower concentrations of *Cf* protein in heterozygotes must have limited the activation of the plant's defense responses and suggest *Cf* proteins are naturally in low abundance. Several other examples of the incomplete or partial dominant of genes for resistance to various viral, bacterial, and obligate biotrophic fungal pathogens are known. These include the L^1 and L^3 genes of *Capsicum chinense* which are effective against the virus TMV (Boukema 1980), the *Dm6* gene of lettuce effective against *Bremia lactuca* (Crute and Norwood 1986), the *Pto* gene of tomato effective against *Pseudomonas syringae* pv. *tomato* (Carland and Staskawicz 1993), several alleles of the *Mla* locus of barley effective against *Erisyphe graminis* f. sp. *hordei* (Jahoor *et al.* 1993) and the *Arabidopsis thaliana* gene *RPP5* effective against *Peronospora parasitica* (Parker *et al.* 1993). It is highly probable that other resistance genes would be reclassified as incompletely dominant in action if examined in detail under controlled environmental conditions and in uniform genetic backgrounds. However, some resistance genes are completely dominant (Crute 1985; Flor 1971; Jahoor *et al.* 1993).

As no resistance genes to biotrophic pathogens have been isolated, one can only speculate as to how increased concentrations of resistance gene protein in the *Cf* homozygous lines might result in more effective containment of *C. fulvum* hyphae. If resistance genes encode a protein with a receptor/surveillance function, a theory currently in vogue, then the perception of pathogen invasion could be heightened by increasing receptor concentrations. If the resistance gene product and the *Avr* gene product are both present at low concentrations, the rate of the reaction that is the outcome of their interaction (i.e., a signal to activate defense) will be proportional to the concentration of both participants, as in a second-order chemical reaction. The differences in the extent of hyphal growth and the timing of containment between *Cf* gene homozygotes and heterozygotes would support this notion. By cloning corresponding resistance and avirulence genes, the relative concentrations of the respective protein product can then be manipulated and this hypothesis rigorously tested.

MATERIALS AND METHODS

Strains and media.

C. fulvum (Cooke) race 0 and a race 4 transformed with the *uidA* reporter gene (Roberts *et al.* 1989) were both obtained from R. Oliver (University of East Anglia, Norwich, UK). The latter race is designated as race 4 *GUS*. *C. fulvum* was propagated on a quarter-strength potato-dextrose agar as described by Harling *et al.* (1988). For long-term storage, conidiospore suspensions were prepared at a density of 10^6 spores ml^{-1} in 60% glycerol and kept at –70° C. Spore sus-

pensions for plant inoculations were derived from culture plates which had been subcultured at maximum four times. Five *Cladosporium*-resistant near-isogenic lines of the *Cladosporium*-susceptible cultivar Moneymaker, homozygous for resistance genes *Cf-2*, *Cf-3*, *Cf-4*, *Cf-5*, and *Cf-9*, respectively (Tigchelaar 1984), and a line homozygous for *Cf-11* in the nonisogenic Ontario 7716 background were obtained from R. Oliver. The cultivar Moneymaker is here designated Cf0 (because of its lack of detectable resistance genes) and the other lines Cf2, Cf3, Cf4, Cf5, Cf9, and Cf11 (consistent with the *Cf* resistance genes they carry), respectively. Seeds of *L. pennellii* LA 716 were obtained from C. Rick (University of California, Davis). To obtain the *Cf* heterozygotes in either a 100% *L. esculentum* or a 50% *L. esculentum* 50% *L. pennellii* background, the appropriate crosses were made using the *Cf* gene containing lines as the female parent. These lines are designated *Cf* heterozygote and *Cf* hybrid heterozygote, respectively. Crosses between two different *Cf*-gene-containing lines, to produce the *Cf* double heterozygotes, were done reciprocally.

Growth and inoculation of plants.

L. esculentum plants were grown singly in plastic 12-cm^2 pots (Plantpak, Maldon, Essex) in Levingtons compost (F2) in a growth room at 24° C during the 16-hr day and 16° C at night. Light was supplied by Osram Power Star (HQI-T) 400W lamps to give a photon flux density of 650 μE m^2 s^{-1} at the plant surface. Conidial suspensions of *C. fulvum* containing 2 × 10^5 spores ml^{-1} were prepared in sterile, distilled water. When plants had four fully expanded leaves the lower surfaces of leaves 2, 3, and 4 were inoculated; spore suspensions were sprayed onto the leaves with an aerosol gun (Humbrol) almost to the point of droplet coalescence. Inoculated plants were kept for 3 days in a polythene chamber to maintain humidity near saturation, then the relative humidity was reduced to approximately 80% by opening the vents at the top of the chamber. The plants were staged on a bed of capillary matting and this was watered twice daily.

All inoculation experiments were performed three times using a minimum of five plants per genotype per *C. fulvum* isolate.

Plant infection studies.

Leaf samples were taken at 4, 6, 8, 10, 12, 14, 16, 19, and 24 days after inoculation. Five leaves were harvested for each time point per host genotype per fungal isolate combination. The sampling time was always 5 hr into the daylight period. Two types of histochemical staining of plant tissue were performed. β-Glucuronidase (GUS) activity was visualized *in planta* by vacuum infiltrating 8-mm-diameter tissue disks, cut with a cork borer, with 0.5 mg ml^{-1} X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide cycloammonium salt, Biosynth AG, Zurich) in a 50 mM sodium phosphate buffer pH 7.0 containing 1 mM EDTA, 1 mM potassium ferricyanide/ferrocyanide and 0.05% (v/v) Triton X-100 (Jefferson 1987). The X-Gluc solid was first dissolved in dimethyl formamide (20 mg ml^{-1}) before adding to the buffer. Tissue pieces were incubated overnight at 37° C in the dark. Plant tissue clearing was achieved by successive washes in 70% ethanol. Conventional histochemical staining of fungal hyphae was performed using lactophenol-trypan blue and destaining with chloral hydrate (Keogh *et al.* 1980). Microscopical observations were made on a Zeiss "Axioskop" instrument under phase contrast and photomicrographs were prepared with Kodak Ektachrome 160 Tungsten film. The results presented are based on a minimum of 150 observations of penetration sites per interaction per time point for each experimental replica.

Fungal biomass within plant tissue was quantified in inoculations with the race 4 *GUS* transformant of *C. fulvum*. Fluorometric GUS assays utilizing the substrate MUG (4-methylumbelliferyl β-D-glucuronide) were performed essentially as described by Jefferson (1986; 1987), except reactions were scaled down to allow all manipulations to be carried out in 96-well microtiter plates. Protein levels in samples were estimated by the method of Bradford (1976) using a kit supplied by Bio-Rad Laboratories and bovine serum albumin as the standard. Samples were prepared from the same leaves from which the two tissue disks had been cut for the histochemical analyses. Additional leaf samples were taken to identify callose and lignin polymers synthesized by the host cells using the histological methods of Lazarovits and Higgins (1976a) and Bradbury (1973), respectively.

Stastistical analyses were conducted according to Snedecor and Cochran (1980) and graphs were produced using the program SigmaPlot.

Isolation of elicitors and assay of their biological activity.

Tomato leaves on which *C. fulvum* was sporulating over the entire lower leaf surface (16–19 days after inoculation) were harvested for the isolation of race-specific elicitors. Leaves were vacuum infiltrated with distilled water and the intercellular washing fluid (IF) containing the elicitors was recovered by a low-speed centrifugation as originally described by de Wit and Spikman (1982). The IF was sterilized by heating for 10 min at 100° C, then centrifuged at 1,500 *g* for 10 min, and the supernatant stored at –20° C.

Six- to seven-week-old plants were used for injection with intercellular fluid. Injections were done with a 1-ml disposable syringe fitted with a 21 gauge, 6% luer tipped needle (Terumo, Leuven, Belgium). Two interveinal panels per leaflet were completely flooded with IF of a single dilution, requiring about a 25-μl volume per injection site. The third, fourth, and fifth fully expanded compound leaves were injected on each plant with a serial dilution of IF. Injected plants were in a greenhouse at temperatures between 22 and 27° C in the light and 12–16° C in the dark where 16 hr of light was supplied at a photon flux density of 300–650 μE m^2 s^{-1} at the plant surface and the relative humidity was about 80%. The necrosis- or chlorosis-inducing activity of elicitor on the different tomato genotypes was expressed as the reciprocal of the dilution endpoint which still induced necrosis. All assessments were based on a minimum of eight plants for each genotype.

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