Plant Physiol. (1996) 110: 1381–1394

Race-Specific Elicitors of *Cladosporium fulvum* Induce Changes in Cell Morphology and the Synthesis of Ethylene and Salicylic Acid in Tomato Plants Carrying the Corresponding *Cf* Disease Resistance Gene[1]

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Defense responses mediated by the genetically unlinked *Cf-9* and *Cf-2* genes were compared with those involving no *Cf* gene (Cf0). Compatible tomato (*Lycopersicon esculentum*)-*Cladosporium fulvum* intercellular washing fluids were injected into tomato cotyledons, and the kinetics of responses was monitored under conditions of 70 and 98% relative humidity. The latter conditions suppressed the normal macroscopic responses. For the *Cf-9-Avr9* interaction, stomatal opening was induced within 3 to 4 h and after 9 h mesophyll cell death commenced. A burst of ethylene production occurred between 9 and 12.5 h and remained elevated. Free salicylic acid levels increased after 12 h, peaked at 24 h, and thereafter declined. For the *Cf-2-Avr2* interaction, stomata became plugged after 8 h, and salicylic acid and ethylene levels increased by 12 and 18 h, respectively, and thereafter declined. Host cell death commenced around vascular tissue by 24 h. Cell death in both incompatible interactions was frequently preceded by cell enlargement. For Cf0-injected plants, no significant responses were detected. High humidity delayed and reduced the *Cf-Avr*-gene-dependent cell death and ethylene synthesis, whereas induced salicylic acid levels were unaffected for *Cf-2-Avr2* and reduced in magnitude only for *Cf-9-Avr9*.

An array of highly localized plant defenses are induced rapidly and coordinately at the site of genetically incompatible plant-pathogen interactions. Subsequently, the growth of the invading virus, bacteria, fungus, or nematode is retarded, and eventually the microbe either dies or remains quiescent. Activation of localized plant defenses is dependent on mutual recognition between plant and microbe. This recognition is hypothesized to result from the interaction of the product of a dominant or semidominant plant *R* gene with the direct or indirect product from the corresponding dominant pathogen *Avr* gene (Flor, 1971; Keen, 1990; Dangl, 1995; Staskawicz et al., 1995). Subsequent signal transduction events coordinate the resistance phenotype that includes the generation of active oxygen species, ion fluxes across membranes, changes in protein phosphorylation, transcriptional activation of plant defense-related genes, and the rapid death of host cells around the penetration site (the HR) (Klement, 1982; Bowles, 1990; Dixon et al., 1994; Godiard et al., 1994; Staskawicz et al., 1995). Since the plant defense responses induced by diverse microbes are similar, it has been suggested that only a limited number of signaling pathways initiate defense via *R*-gene action (Lamb, 1994; Dangl, 1995). In the absence of pathogen recognition, plant defense responses are not activated and disease occurs (Keen, 1990).

Several plant disease-resistance genes have recently been isolated that confer incompatibility in distinct host-microbe interactions, and various classes of *R*-gene products are now recognized (reviewed by Dangl, 1995; Staskawicz et al., 1995). How do the *R*-gene products function? The initial and probably diverse signal cascades, emanating from the action of each different class of *R*-gene product, may rapidly converge into a few common signaling pathways that activate a general plant disease-resistance response. Alternatively, different classes of *R* genes could each engage different signaling pathways that coordinate a specific resistance phenotype tailored to each pathogen type. Detailed characterization of the mode of action of different classes of *R* genes by combined genetic, biochemical, cell biology, and plant physiology approaches is necessary to distinguish these possibilities.

We are interested in the resistance phenotype conferred by tomato (*Lycopersicon esculentum*) *Cf* genes against distinct physiological races of the extracellular biotrophic fungal pathogen *Cladosporium fulvum* that cause leaf mold disease (de Wit, 1992; Hammond-Kosack and Jones, 1995). We also wish to discover whether the gene products encoded by genetically unlinked *Cf* genes activate similar or

[1] This work was supported by grants from the U.S. Department of Agriculture/Competitive Research Grants Office (grant No. 90–37261–5659), Division of Energy Biosciences of U.S. Department of Energy grant No. DE-FG05–91ER20049, New Jersey Commission for Science and Technology grant No. 93–240380–1, and the New Jersey Agricultural Experimental Station to P.S. and I.R. and a grant from the Gatsby Charitable Foundation to K.E.H.-K. and J.D.G.J.

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Abbreviations: *Avr* gene, avirulence gene; Avr, avirulence protein; h a.i., hours after injection; HR, hypersensitive cell death response; IF, intercellular fluid; *R* gene, resistance gene; SA, salicylic acid; TMV, tobacco mosaic virus.

dissimilar resistance mechanisms. The *Cf-9* gene on chromosome 1 has been isolated (Jones et al., 1993, 1994). The DNA sequence of *Cf-9* predicts a transmembrane glycoprotein with a putative signal peptide, 28 imperfect Leu-rich repeats of 24 amino acids each, flanked by two domains with homology to polygalacturonase inhibitor proteins and a C-terminal membrane anchor. The *Cf-2* gene on chromosome 6 (Dickinson et al., 1993) also encodes a protein with Leu-rich repeats and with structural domains similar to those of Cf-9 (Dixon et al., 1996). Thus, although both Cf-9 and Cf-2 proteins appear to lack an apparent intracellular signaling domain, they could each act through the same signaling pathway.

In this paper and in May et al. (1996), we have explored the action of two tomato disease-resistance genes, *Cf-9* and *Cf-2* (Jones et al., 1994; Dixon et al., 1996). Exploration of *R*-gene action is simplified for this host-pathogen interaction because of the ability to isolate *Avr*-gene products from IFs obtained from the air spaces of tomato leaves supporting heavy fungal sporulation (de Wit and Spikman, 1982). When IF carrying the complementary race-specific elicitor is injected into the air spaces of healthy cotyledons or leaves of *Cf*-carrying stocks, a characteristic macroscopic chlorotic or necrotic response develops after 1 to 5 d of ambient humidity, the exact phenotype of which is dependent on the *Cf-Avr* gene combination involved (de Wit and Spikman, 1982; Hammond-Kosack and Jones, 1994).

SA serves as a signal molecule in localized plant defense responses as well as in the development of systemic acquired resistance to infection by other pathogens (Malamy et al., 1990; Metraux et al., 1990; Raskin, 1992; Ryals et al., 1994). In tobacco, SA levels increase more than 40-fold in the vicinity of necrotic lesions that form around the sites of incompatibility conferred by *N*-gene action against TMV (Enyedi et al., 1992). Delaney et al. (1994) used transgenic tobacco and *Arabidopsis thaliana* plants that express the bacterial enzyme salicylate hydroxylase (*nahG*), and so do not accumulate SA, to reveal that the absence of SA significantly weakens the local resistance responses conferred by *N* against TMV, *RPS2* against *P.s.* pv *tomato*, and *RPP* genes against *Peronospora parasitica*. Thus, for a few *R* genes that are effective against very different microbial pathogens, an indispensable role for SA in localized resistance has been established. Treatment of plants with SA is known to induce the accumulation of numerous pathogenesis-related proteins that are found at incompatible infection sites (Raskin, 1992; Ryals et al., 1994). Many of these pathogenesis-related genes have an SA-responsive site, the AS-1 element, within the promoter (Goldsborough et al., 1993; Horvath and Chua, 1994).

Ethylene, a gaseous plant hormone, is also produced in tissues undergoing an HR (De Laat et al., 1981) and in response to treatment of plant cells with pathogen-derived elicitors (Boller, 1991). Therefore, it has been proposed that ethylene acts as a signal molecule during incompatible plant-microbe interactions. In support of this hypothesis, exogenous application of ethylene or the ethylene-releasing compound ethephon has been shown to induce the expression of many defense-related genes (Raz and Fluhr, 1993; Lawton et al., 1994). However, by using ethylene-insensitive *ein1* and *ein2* mutants of Arabidopsis that expressed the resistance genes *RPS2* and *RPM1*, Bent et al. (1992) showed these plants still to be resistant to avirulent strains of *P.s.* pv *tomato* that expressed the corresponding *Avr* genes *avr-Rpt2* or *avrRpm1/avrB*, respectively. These data suggest that ethylene is not required for active defense against avirulent bacteria. Similarly, tobacco *N*-mediated resistance to TMV also appears not to involve ethylene. When tobacco tissue was treated with an inhibitor of ethylene action, 2,5-norbornadiene, no significant changes to HR development toward TMV were evident (Silverman et al., 1993). Possibly the role of ethylene biosynthesis at sites of genetic incompatibility is to activate plant responses that will prevent the ingress of secondary opportunistic pathogens.

To determine whether tomato *Cf*-gene-mediated resistance activates localized plant defenses by mechanisms similar to those thought to be crucial for the action of other *R*-gene products, the tomato plant's response to *C. fulvum* Avr elicitors has been investigated. In this paper we describe *Cf-2-Avr2-* and *Cf-9-Avr9*-dependent changes in cell morphology, induction of ethylene and SA biosynthesis, and changes to cell viability. In the accompanying paper (May et al., 1996), we characterized changes in superoxide formation, electrolyte leakage, lipid peroxidation, glutathione levels, and catalase, superoxide dismutase, and lipoxygenase activity. The kinetics and magnitude of each response were documented by injecting IF into entire cotyledons, thereby providing a synchronous challenge. Near-isogenic tomato stocks carrying the resistance genes *Cf-9* and *Cf-2* or no known *Cf* genes were used.

MATERIALS AND METHODS

Plant Material, IF Preparation, and Experimental Regimes

All experiments were performed exactly as described by May et al. (1996) on 14- to 16-d-old Cf0, Cf2, and Cf9 tomato (*Lycopersicon esculentum* [Mill]) seedlings, using a partially purified IF preparation (Cf0-race 0 compatible interaction) and under the identical environment conditions. For experiments at 70% humidity and 24 h of darkness, a second growth cabinet was used in which the temperature was maintained at 24°C for 16 h and 18°C for 8 h. Plants were placed in this cabinet for 24 h prior to IF injection, and seed sowings were staggered for each time analyzed. IF injections of seedlings from the dark regime were conducted in the presence of an appropriate safety lamp, as was the harvest of samples.

Cell Viability

Viability of cotyledon cells after treatment with IF was determined by the accumulation of fluorescein diacetate in the cells (Widholm, 1972) and by their ability to exclude trypan blue stain, as described previously (Keogh et al., 1980; Hammond-Kosack and Jones, 1994). Microscopic observations were made on a Zeiss Axioskop instrument under either epifluorescence illumination or phase contrast. Photomicrographs were prepared using Kodak Tri-Pan-X film.

ABA Treatment

A 20 mM stock solution of ABA (Sigma) was freshly prepared in 90% methanol and then diluted to 20 μM in sterile deionized water. ABA was sprayed onto the upper and lower surfaces of tomato cotyledons with an aerosol gun (Humbrol, Hull, UK) almost to the point of droplet coalescence at various times after IF injection.

Scanning Electron Microscopic Analysis of Tomato Cotyledon Surfaces

Specimen casts were prepared by the procedure of Green and Linstead (1990) and examined in a CamScan MR4 scanning electron microscope (Cambridge Scanning, Cambridge, UK). Cotyledon surface replicas were prepared at 1-h a.i. intervals for the first 8 h and then at 24, 48, and 72 h a.i. Because there was a high likelihood of introducing artifacts while preparing samples from the high-humidity regime and from the dark regime, since the room where the safety lamp was located did not have humidity control, the effects of these two environmental regimes on L1 morphology were not examined.

Determination of Ethylene Production

At 0.5, 3, 6, 9, 12, 15, 18, 21, and 24 h after injection of IF, individual cotyledons were excised and placed separately into 9-mL glass bottles, sealed with a gas-proof septum, and left in the growth cabinet. Three and one-half hours later, 1-mL aliquots of gas were removed from each bottle using a gas-tight syringe (Hamilton, Dundee, UK) and injected into a gas chromatograph. For each *Cf* genotype a minimum of eight cotyledons were examined for each time.

Extraction and Quantitation of SA

Levels of free and total (free plus conjugated) SA and β-*O*-D-glucosylsalicylic acid in tomato cotyledons (approximately 250 mg of tissue) were determined by online UV absorption and fluorescence detectors after separation on a C_{18} reverse-phase HPLC column (Enyedi and Raskin, 1993). All data were corrected for SA recovery, which ranged from 42 to 66%. The identity of the SA molecule isolated from tomato cotyledons was confirmed by MS.

Experimental Design and Statistical Analysis

All experiments were performed with a minimum of four tissue sample replicates per treatment per time. Each experiment was carried out at least twice with similar results. Data from each experiment are expressed as the means ± SE (unless otherwise stated) and either paired *t* test or an analysis of variance was performed to determine significant differences between treatments at the $P < 0.05$ level (Snedecor and Cochran, 1980).

RESULTS

Macroscopic Responses of Cf0, Cf2, and Cf9 Plants to Race-Specific Elicitors under Three Different Environmental Regimes

The *Cf*-gene-dependent induction of chlorosis (tissue yellowing) and necrosis (tissue appearing gray or brown) following *C. fulvum* IF challenge was influenced by both RH and light. At 70% RH under a 16-h light/8-h dark regime, IF injection into cotyledons or leaves resulted in the *Cf*-gene-dependent formation of macroscopically visible necrosis or chlorosis. No response was observed in Cf0 plants that lacked a *Cf* gene. The type of symptom induced was dependent on the *Cf* stock challenged. Plants homozygous for the *Cf-9* gene developed gray necrosis within 12 to 16 h of IF injection, whereas plants homozygous for the *Cf-2* gene developed yellow chlorosis with some brown necrosis by 3 to 4 d a.i., as shown in Table I. These data are in agreement with those originally reported by de Wit and Spikman (1982).

At 70% RH and continuous darkness, the *Cf-9*-dependent gray necrosis still occurred but was delayed in appearance until 48 h a.i. (Table I). If Cf9 plants kept for 24 h in the dark after IF injection were returned to the light, gray necrotic lesions formed within 2 to 3 h (data not shown). This latter observation confirms the original report by Peevers and Higgins (1989). For Cf2 plants kept under continuous darkness, the macroscopic responses to IF occurred earlier and became more severe. By 48 h a.i., Cf2 cotyledons had collapsed entirely and exhibited severe chlorosis and brown necrosis. No macroscopic response of Cf0 plants to IF occurred in the dark.

When the 16-h light/8-h dark regime was maintained but the RH was elevated to 98%, the development of macroscopic symptoms was almost entirely abolished on both Cf9 and Cf2 plants (Table I). Sometimes pale chlorosis was visible 4 d after injection on both genotypes. If Cf9 plants kept for 24 h at high humidity after IF injection were returned to 70% humidity, gray necrosis formed within 3 to 6 h (data not shown). No macroscopic response to IF occurred at high humidity in Cf0 plants.

Macroscopic Responses of Cf2 Plants to Higher IF Concentrations

To investigate whether the *Cf-2*-dependent chlorosis response could be converted into a *Cf-9*-like reaction resulting in gray necrosis, concentrated IF was injected into cotyledons or leaves. At 2, 4, 8, 16, 32, and 64 times the titer of the IF originally isolated from *C. fulvum*-infected tomato leaves, these IF injections did not result in gray necrosis (data not shown). At the higher IF concentrations (8-fold and above), severe chlorosis with some brown necrosis was visible by 48 h a.i. (data not shown). Cf0 plants gave no macroscopic responses following a challenge with any of the higher concentrations of IF (data not shown). Thus, the *Cf-2-Avr2*-dependent response is qualitatively distinct from the *Cf-9-Avr9*-dependent response.

Loss of Cell Viability in Response to IF

Two different assays were used to assess cell viability in the IF-challenged cotyledons. The fluorescein diacetate stain relies on the ability of living cells to accumulate and retain the fluorescent product (Widholm, 1972). In contrast, the trypan blue stain is based on the capacity of living cells to exclude the dye; only dead cells can accumulate it (Keogh et al., 1980). At crucial times at approximately the onset of viability loss, the ability of host cells to be plasmolyzed by 2 M Suc was also determined. The percentages of palisade mesophyll cells still viable following IF injection into Cf0, Cf2, and Cf9 plants under the three experimental regimes are shown in Figure 1. For Cf0 plants, cell viability remained unchanged (97 ± 3%) throughout the entire time course.

For Cf2 plants no significant loss of cell viability was detectable by 24 h a.i. in the 16-h light/8-h dark regime at either 70 or 98% RH (Fig. 1A). Most cell viability loss occurred during the period 36 to 48 h a.i. and reached a maximum of 50%. Death occurred initially in mesophyll cells associated with vascular tissue as shown in Figure 2. Even by 24 h a.i. cells in the immediate vicinity of the vascular tissue exhibited a slightly enhanced staining with trypan blue (Fig. 2B). Under continuous darkness the decline in cell viability occurred more rapidly, with all cells becoming nonviable by 48 h a.i.

For Cf9 plants maintained at 70% RH in either the 16-h light/8-h dark or continuous dark regimes, a significant reduction in cell viability commenced 9 h a.i., and by 24 h a.i. all mesophyll cells were nonviable (Fig. 1B). The loss of viability occurred in patches throughout the cotyledon's lower and palisade mesophyll layers. These areas expanded, coalesced, and eventually encompassed the entire cotyledon. At 98% RH the decline in cell viability commenced later and even by 72 h a.i approximately 50% of plant cells remained viable. However, most viable cells by 48 h a.i. appeared to fluoresce to a lesser extent than cells in unchallenged cotyledons of a comparable age.

The microscopic analyses revealed two additional features common to both the *Cf-9-Avr9* and *Cf-2-Avr2* incompatible interactions. First, all epidermal cells retained viability until at

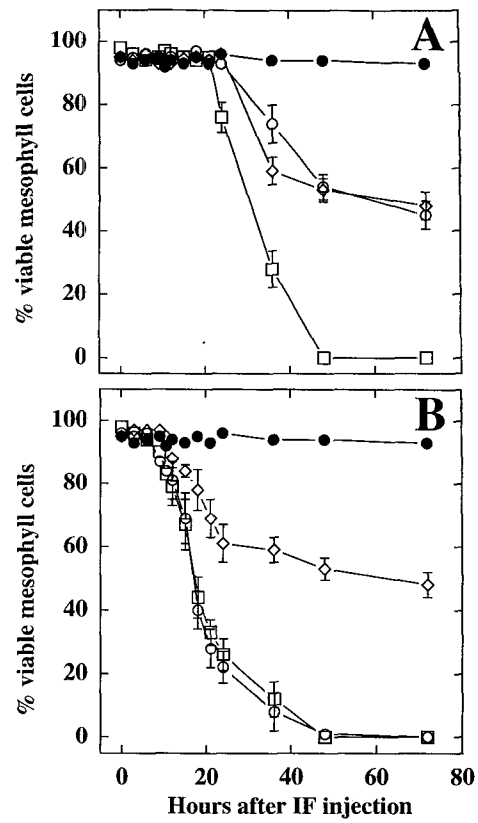

Figure 1. Effect of *C. fulvum* race-specific elicitors on mesophyll cell viability in tomato cotyledons during incompatible interactions mediated by the gene combinations *Cf-2 Avr2* (A) and *Cf-9-Avr9* (B) and in a compatible interaction involving a near-isogenic line lacking known *Cf* genes (●). After IF injection (1 in 2 dilution titer) the plants were maintained under three different environmental regimes: 70% RH in a 16-h photoperiod (circles), 70% RH in continuous darkness (squares), or 98% RH in a 16-h photoperiod (diamonds). Cell viability was determined using two vital stains, fluorescein diacetate and trypan blue. Sample times were 0, 3, 6, 9, 12, 15, 18, 21, 24, 36, and 48 h a.i. Each data point is the mean (±SE) of eight replicate cotyledons and the experiment was performed three times.

Table I. *The effect of environmental conditions on the chlorosis and necrosis-inducing activity of C. fulvum race-specific elicitors (IF[a]) on tomato near-isogenic lines containing either no Cf gene or the Cf-2 or the Cf-9 gene*

Experimental Regime	*Cf* Gene	Days after IF Injection				
		1	2	3	4	5
70% RH, 16-h light/8-h dark	None	–	–	–	–	–
	Cf-2	–	–	++[b]	+++	++++
	Cf-9	G	G	A	A	A
70% RH, continuous darkness	None	–	–	–	–	–
	Cf-2	–	++++	A	A	A
	Cf-9	–	G	A	A	A
98% RH, 16-h light/8-h dark	None	–	–	–	–	–
	Cf-2	–	–	–	±	±
	Cf-9	–	–	–	±	±

[a] IF from a compatible Cf0–race 0 *C. fulvum* interaction. [b] Chlorosis and necrosis induction were assessed by an arbitrary rating (–, ±, ++, +++, ++++, G) indicating no symptoms, little chlorosis, much chlorosis, chlorosis with brown necrosis, much chlorosis and brown necrosis, and gray necrosis. Chlorosis is defined as the loss of Chl causing the tissue to appear yellow. Necrosis is defined as cell death resulting in the tissue becoming brown (Cf2) or gray and then brown (Cf9). A, Denotes abscission of the injected cotyledon. Each experiment was repeated at least 12 times using a minimum of eight plants per genotype.

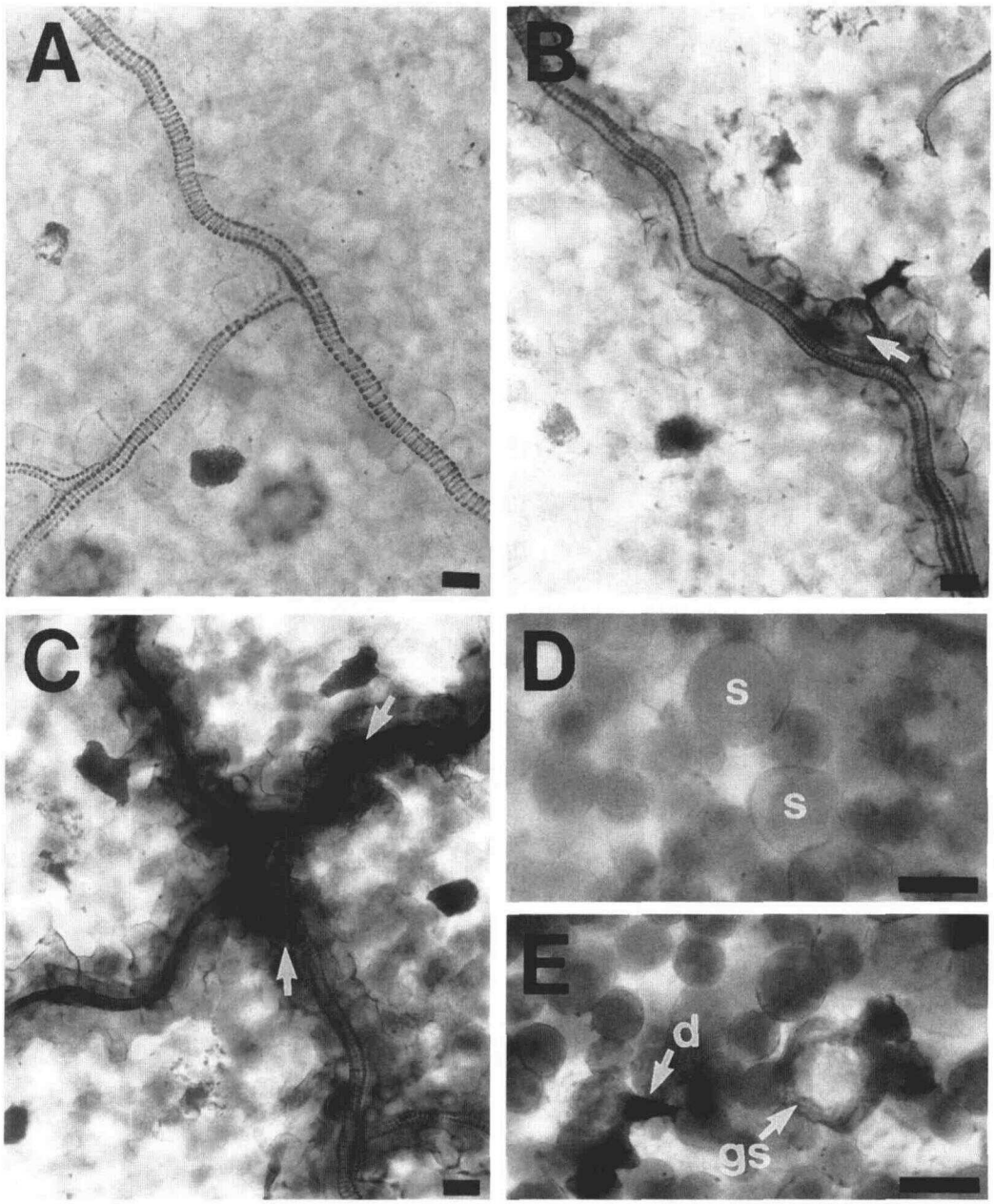

Figure 2. Photomicrographs of the appearance of Cf0, Cf2, and Cf9 tomato cotyledon cells at different times after injection of *C. fulvum* race-specific elicitors at a 1 in 2 dilution titer. The whole-tissue mounts have been stained with trypan blue-lactophenol. Nonviable cells accumulate the stain. A, B, and C, Photographed in the plane of the L3 layer. A, Control, Cf0 at 24 h a.i.; B, Cf2 at 24 h a.i.; C, Cf2 at 48 h a.i. The arrows in B and C indicate the responding cells beside the vascular tissue that by 48 h have died and accumulated the trypan blue stain. D and E, Photographed in the plane of the lower part of the palisade mesophyll at 12 h a.i. into Cf9 tissue. In D two highly swollen mesophyll cells are indicated (s) and in E a highly granulated swollen cell (gs) and several collapsed nonviable cells that have accumulated the trypan blue stain (d) are shown. Bar = 30 μm.

least 72 h a.i. under each of the environmental conditions explored. Second, when the concentration of the IF injected was reduced to 1 in 8 or 1 in 64 of the original titer, the enlargement and circularization of viable mesophyll cells were frequently observed. An example of this phenomenon for the *Cf-9-Avr9* interaction is shown in Figure 2, D and E. These enlarged cells later collapsed and died.

Overall, a good correlation existed between the rate and magnitude of mesophyll cell viability loss and the timing and severity of the macroscopically visible symptoms. The only exception to this was at 98% RH, at which, although the cotyledons remained green for at least 72 h a.i., 50% of cells were dead by 24 h a.i. for Cf9 plants and by 48 h a.i. for Cf2 plants.

Modifications to L1 Layer Cells in Response to IF

To explore the changes in appearance of plant cells belonging to the L1 (epidermal) layer (Satina and Blakeslee, 1941) in response to IF challenge, specimen casts were prepared of the upper and lower cotyledon surfaces by the procedure of Green and Linstead (1990) and examined in a scanning electron microscope. Only samples from the 70% RH, 16-h light/8-h dark environmental regime were analyzed.

In response to IF infiltration, gross changes to L1 cells were apparent by 3 h a.i. on Cf9 cotyledons and by 6 h a.i. on Cf2 cotyledons, whereas no alterations to Cf0 cells occurred (Fig. 3). For Cf9 modifications to both epidermal cells and stomatal guard cells were observed, whereas for Cf2 changes were predominantly restricted to the epidermal cells. By 3 to 4 h a.i., isolated patches of supraoptimally open stomata, accompanied by the collapse of intervening epidermal cells, were found on both the upper and lower surfaces of the the Cf9 cotyledons, as shown in Figure 3B. During the subsequent 6 h, the areas with supraoptimally open stomata expanded and all epidermal cells within these areas collapsed. The kinetics of *Cf-9-Avr9*-dependent supraoptimal opening of stomata is shown in Figure 4. For Cf2 small patches where the stomatal pores were occluded with amorphous material were evident by 6 h a.i. on both lower and upper cotyledon surfaces, and after 8 h almost all stomatal pores exhibited this phenotype, but the guard cells still retained turgor (Fig. 3D). By 24 h a.i. all Cf2 epidermal cells were collapsed and distorted and the guard cells had also collapsed (Fig. 3E).

In response to the toxin fusicoccin, produced by the fungal pathogen *Fusicoccum amygdali*, the supraoptimal

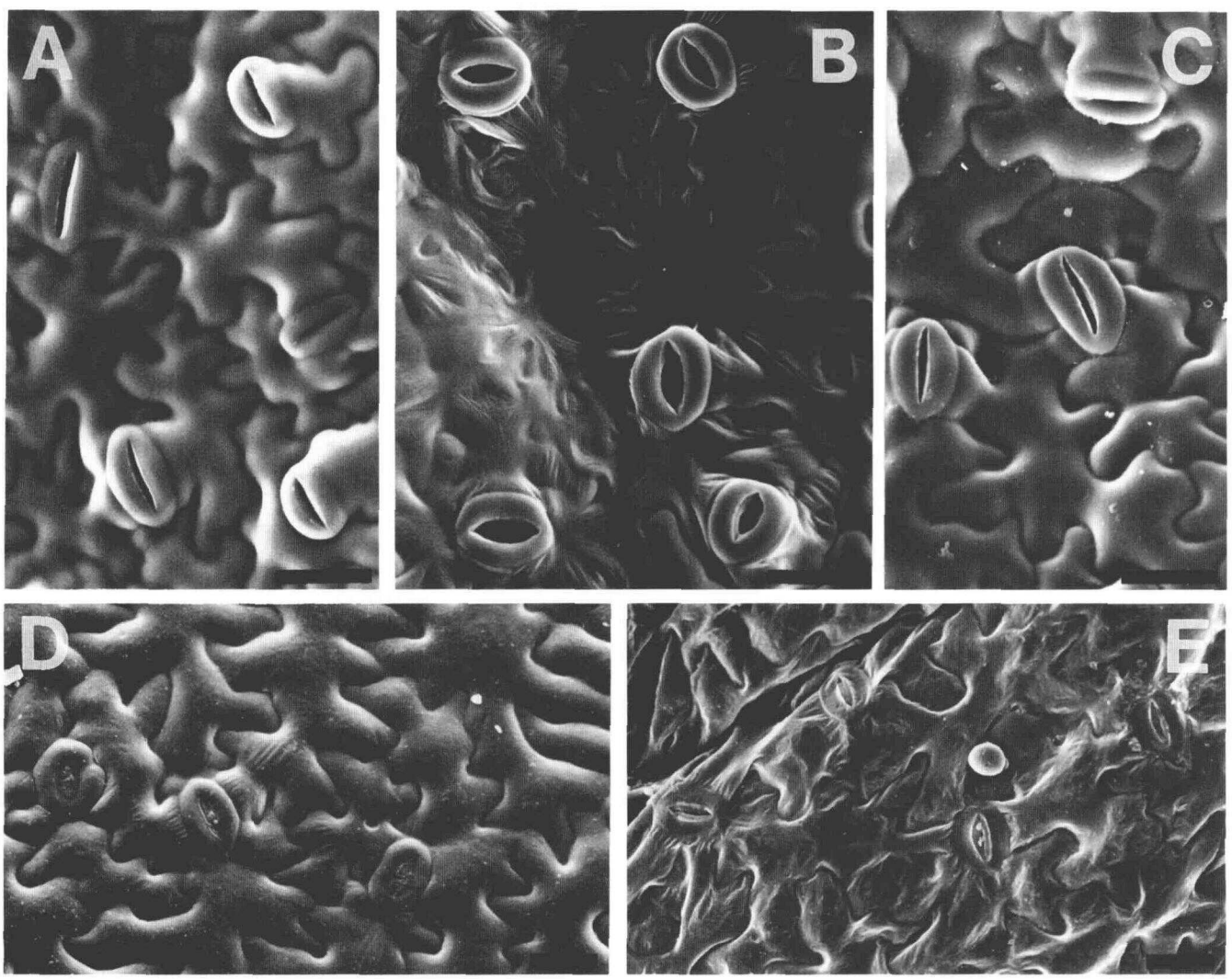

Figure 3. Scanning electron micrographs of lower surface replicas taken from Cf0, Cf2, and Cf9 cotyledons at various times after IF injection at a 1 in 2 dilution. A, Cf0, 6 h control; B, Cf9, 6 h with supraoptimally open stomata and collapsed epidermal cells; C, Cf9, 6 h having also received a 20 μM ABA spray application at 1 h, with normal stomata and epidermal cells; D, Cf2, 8 h with plugged stomata and normal epidermal cells; and E, Cf2, 24 h with closed and collapsed stomata and severely distorted and collapsed epidermal cells. Bar = 30 μm.

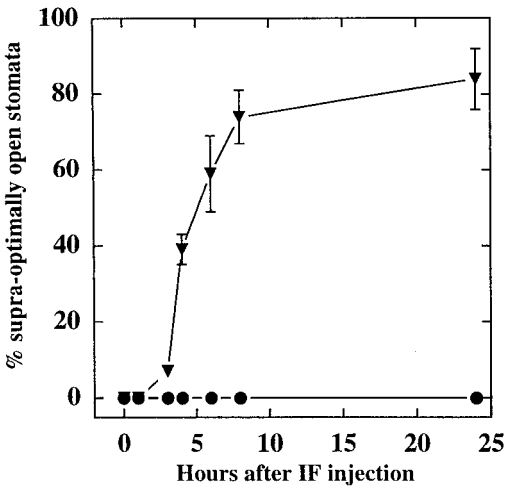

Figure 4. Influence of *C. fulvum* race-specific elicitors on stomatal opening in incompatible interactions mediated by the gene combinations *Cf-9-Avr9* (▼) and *Cf-2-Avr2* (●) and in a compatible interaction involving a near-isogenic line lacking known *Cf* genes (also ●). The proportion of stoma with pores wider than 30 μm was assessed from SEM replicas of the lower cotyledon surface prepared at 0, 1, 3, 4, 6, 8, and 24 h a.i. of a 1 in 2 dilution titer of IF. The plants were maintained at 70% RH in a 16-h photoperiod. Each data point is the mean (±SE) of six replicate cotyledons. The experiment was performed three times.

opening of stomata has been observed in many plant species (Marre, 1979). These modified guard cells cannot be induced to close the stomatal pore by known stimuli, for example, darkness or treatment with ABA. To test whether the supraoptimally open stomata in Cf9 plants challenged with IF could be prevented from opening or were capable of closing once supraoptimally opened, cotyledons were sprayed with 20 μM ABA at either 1 or 7 h a.i. or at both 1 and 7 h a.i. In samples prepared during the period 4 to 9 h a.i., all stomata were found to be closed and the epidermal cells appeared normal if the cotyledons had received an ABA spray at 1 h a.i. (Fig. 3C). When ABA treatment was delayed until 7 h a.i., the stomata were closed within 1 h but approximately 50% of the intervening epidermal cells remained collapsed at the 9-h sampling point.

When IF-challenged Cf9 plants were sprayed at 6-h intervals with 20 μM ABA, no *Cf*-dependent macroscopic response developed (data not shown). Because the prolonged ABA treatment induced premature cotyledon senescence in both challenged Cf9 and Cf0 cotyledons after 5 d, it was not possible to determine whether a delayed *Cf-9*-dependent chlorotic response developed (data not shown). The single ABA spray at 1 h a.i. blocked the formation of gray necrosis by 24 h, but this phenotype was evident by 48 h. The ABA spray at 7 h a.i. permitted the formation of several discrete patches of gray necrosis approximately 2 to 3 mm in diameter on each cotyledon by 24 h, followed by confluent gray necrosis by 48 h. Cf2 plants sprayed regularly with 20 μM ABA developed the typical IF-dependent macroscopic chlorotic response commencing on d 3 to 4. Control Cf0-injected plants sprayed with ABA did not become chlorotic during the period examined (0–5 d).

Loss of Cotyledon Fresh Weight in Response to IF

For Cf9 plants kept at 70% RH within a 16-h light/8-h dark regime, a significant reduction in cotyledon fresh weight was detected 24 h a.i. (Fig. 5A). In contrast, the injected Cf2 cotyledons continued to gain fresh weight at the same rate as the injected Cf0 cotyledons until 48 h a.i. but thereafter declined. At 70% RH and continuous darkness (Fig. 5B), the *Cf-9-Avr9*-dependent loss in fresh weight was slower, but by 48 h a.i. an 80% reduction in fresh weight was again evident. Under continuous darkness the injected Cf2 cotyledons declined in fresh weight by 75% during the period 24 to 48 h a.i. A reduction in injected Cf0 cotyledon fresh weight was evident from 48 h onward. This was presumably due to the absence of photosynthesis for a total of 72 h. Increasing the RH to 98% while maintaining a 16-h light/8-h dark regime (Fig. 5C) abolished the dramatic loss in fresh weight in the Cf9-injected cotyledons. However, in both the Cf2 and Cf9 cotyledons, the rate of fresh weight increase was less than for Cf0 cotyledons from 24 h a.i. By 72 h a.i. the injected Cf2 and Cf9 cotyledons were only 80% of their original fresh weight.

Overall, the timing and magnitude of the *Cf-Avr*-dependent alterations to cotyledon fresh weight correlated well with the changes to cell viability and effects on stomatal opening observed within the different environmental regimes.

Ethylene Production in Response to IF

At 70% RH and with a 16-h light/8-h dark regime, significant increases in ethylene production were detected during the sampling period 9 to 12.5 h a.i. for Cf9 plants as shown in Figure 6A. Coincident with this, an epinastic response of the injected cotyledons was visible by 9.5 to 10 h a.i. (data not shown). Ethylene levels then decreased by 50% but remained elevated for the rest of the time course (last sample period examined 24–27.5 h). For Cf2 plants a significant increase in ethylene production occurred later, during the period 15 to 18.5 h a.i., and thereafter declined. By 18 h a.i. the rate of ethylene production from both Cf9 and Cf2 cotyledons was similar and both were significantly higher than for the Cf0-injected cotyledons.

At 98% RH an epinastic response of Cf9 cotyledons was again evident by 9.5 to 10 h a.i. and a similar increase in ethylene production was detected during the sampling period 9 to 12.5 h a.i. (Fig. 6B). Ethylene production decreased during the sampling period 12 to 15.5 h a.i. and then remained relatively constant. For Cf2 plants significant increases in ethylene production occurred during the period 18 to 21.5 h a.i. and thereafter declined. An epinastic response was visible by 24 h a.i. At the 21 h point the rates of ethylene production for both Cf9 and Cf2 IF-injected cotyledons were similar and both were 11-fold higher than for the Cf0 injected coty-

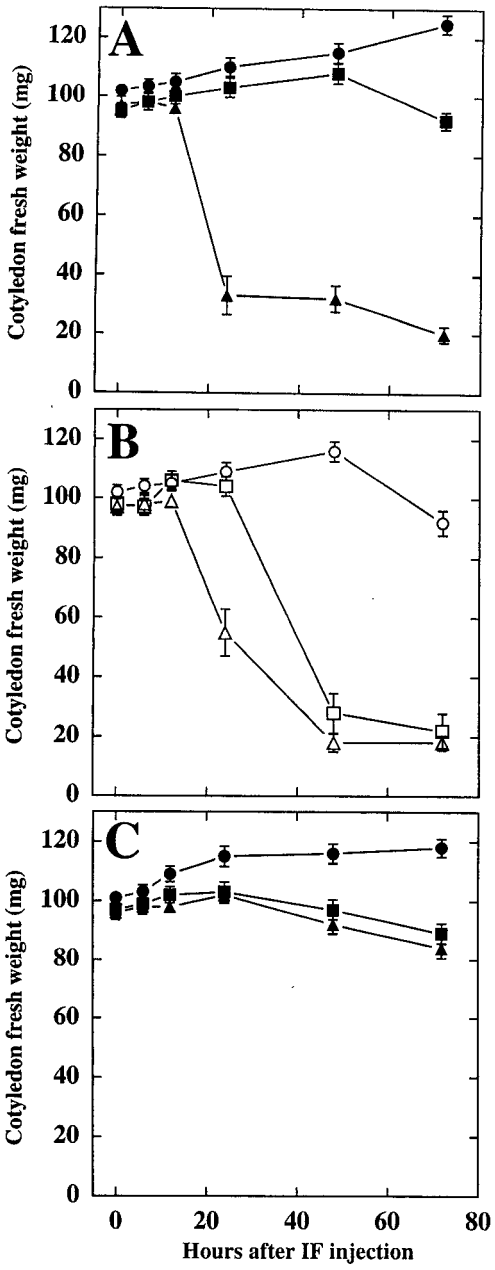

Figure 5. Changes in tomato cotyledon fresh weight following IF injection at a 1 in 2 dilution titer in incompatible interactions mediated by the gene combinations *Cf-9-Avr9* (triangles) and *Cf-2-Avr2* (squares) and in a compatible interaction involving a near-isogenic line lacking known *Cf* genes (circles). After injection the plants were maintained at 70% RH in a 16-h photoperiod (A), at 70% RH in continuous darkness (B), or at 98% RH in a 16-h photoperiod (C). Each data point is the mean (±SE) of eight replicate cotyledons.

ledons. Ethylene production in the continuous darkness regime was not determined.

SA Accumulation in Response to IF

Significant and *Cf*-gene-dependent increases in the levels of free SA in response to IF occurred in each of the three environmental regimes examined. However, the timing and magnitude of the SA increase was distinct in each regime and for each *Cf* gene. Because tomato tissue had not been analyzed previously for SA, MS was used to confirm the authenticity of the peak that was presumed to be SA in the HPLC elution profile (data not shown).

For both Cf9 and Cf2 plants kept at 70% RH and a 16-h light/8-h dark regime, a significant 4-fold increase in free SA levels above basal levels was detectable by 12 h a.i. as shown in Figure 7A. Subsequently, free SA levels increased in the Cf9 cotyledons to a peak value of 18 μg/g fresh weight, 55-fold above the basal level, by 24 h a.i. and then declined. For Cf2 cotyledons free SA levels gradually declined during the period 12 to 48 h a.i. but were always significantly higher than in the Cf0 challenged cotyledons. The basal levels of free SA in IF-challenged Cf0 cotyledons (approximately 0.45 ± 0.02 μg/g fresh weight) were not significantly different from

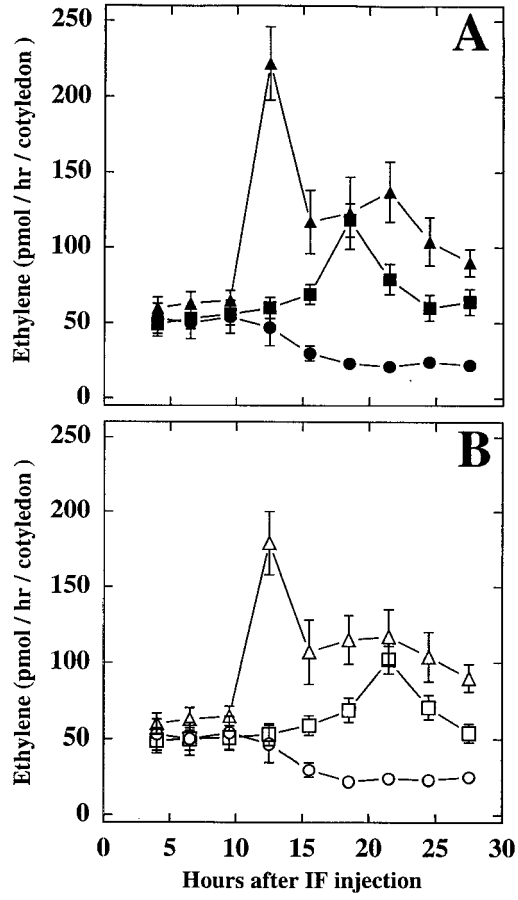

Figure 6. Levels of ethylene produced from tomato cotyledons following IF injection at a 1 in 2 dilution titer in incompatible interactions mediated by the gene combinations *Cf-9-Avr9* (triangles) and *Cf-2-Avr2* (squares) and in a compatible interaction involving a near-isogenic line lacking known *Cf* genes (circles). After injection the plants were maintained at 70% RH in a 16-h photoperiod (A) or at 98% RH in a 16-h photoperiod (B). Cotyledons were detached at various times after IF injection and the total ethylene produced per cotyledon during the subsequent 3.5 h was determined. Each data point represents the mean (±SE) ethylene levels produced by eight replica cotyledons. The experiment was repeated twice.

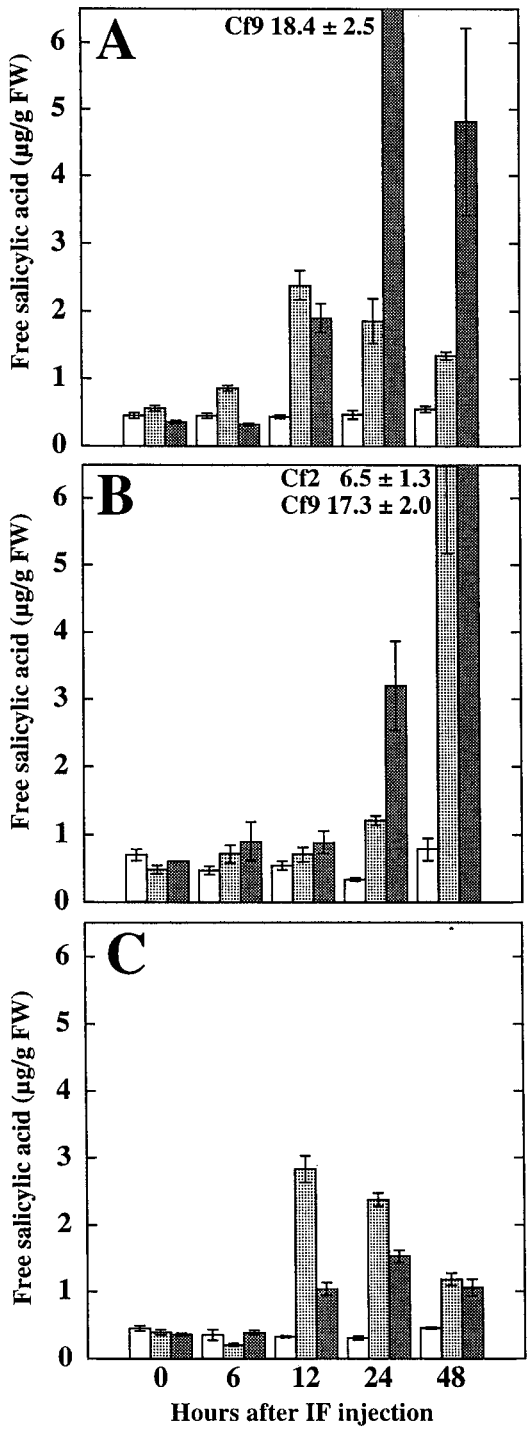

Figure 7. Levels of free SA in tomato cotyledons following injection with *C. fulvum* race-specific elicitors (1 in 2 dilution titer). Incompatible interactions mediated by the gene combination *Cf-9-Avr9* (solid bars) or *Cf-2-Avr2* (hatched bars) and in a compatible interaction involving a near-isogenic line lacking known *Cf* genes (open bars). After injection the plants were maintained at 70% RH in a 16-h photoperiod (A), at 70% RH in continuous darkness (B), and at 98% RH in a 16-h photoperiod (C). Each bar is the mean (±SE) of four replicate cotyledon extracts. The experiment was repeated twice. FW, Fresh weight.

the levels found in unchallenged cotyledons for any genotypes (data not shown).

At 70% RH under continuous darkness, increases in free SA levels were delayed until 24 h a.i., when a 5-fold increase was evident for Cf9 plants and a 2-fold increase was evident for Cf2 plants (Fig. 7B). By 48 h a.i. further increases in free SA levels occurred for both genotypes but were more pronounced for the Cf9 plants. At this time the SA level for Cf9 was comparable to that recovered at 24 h a.i. in Cf9 plants maintained at 70% RH in the 16-h light/8-h dark regime. For Cf2, SA levels at 48 h a.i. were more than 5-fold higher than in unchallenged cotyledons at the same time point.

At 98% RH and the 16-h light/8-h dark regime, increases in free SA levels were again evident by 12 h a.i. (Fig. 7C). Under these conditions SA levels increased in the Cf2-challenged plants to a peak of 3 μg/g fresh weight, which was 3-fold greater than for Cf9-injected plants. At subsequent times, the free SA levels declined for Cf2 to 1 μg/g fresh weight at 48 h. For Cf9-injected plants SA levels increased slightly at the 24-h point to 1.5 μg/g fresh weight and then declined.

Overall, the kinetics and magnitude of SA induction for Cf2 plants was not significantly modified by changing the RH conditions after IF injection. For Cf9 plants the onset of SA induction also did not appear to be delayed by increasing RH, but the overall magnitude of the response was reduced. Keeping plants in continuous darkness caused only a delay in the *Cf-9-Avr9-* and *Cf-2-Avr2*-dependent elevation in SA levels but not a reduction in the magnitude of the response. Indeed, for Cf2 plants 5-fold more SA was eventually synthesized in the dark than in the light. These data also reveal that the increases in SA levels in Cf2 plants always preceded the onset of cell viability loss by a minimum of 12 h (cf. Fig. 1 and Fig. 7).

Dilute titers of IF also caused *Cf*-gene-dependent increases in free SA levels. Only plants maintained in the 70% RH and a 16-h light/8-h dark regime were assessed. When a 1 in 8 dilution titer of IF was used, although there was a delay in the onset of SA synthesis in Cf2 plants until 24 h a.i., the subsequent kinetics and the overall magnitude of the response were not altered (data not shown). In Cf9 plants, even though both the onset and initial increase in SA levels by 12 h a.i. were identical with that observed with the 1 in 2 titer of IF, the peak in SA levels was reduced more than 6-fold to 3 μg/g fresh weight by 24 h and thereafter remained at this level (data not shown). When a 1 in 64 dilution titer of IF was injected into Cf9 plants, a peak of free SA of 2.18 ± 0.18 μg/g fresh weight was evident by 24 h a.i., and thereafter the SA levels declined (data not shown). Because a 70% loss in fresh weight occurs by 24 h a.i., these data reveal that at least a doubling of free SA levels was induced following the injection of the lowest IF titer into Cf9 cotyledons.

Detection of SA Conjugates and Total SA Levels

Total levels of SA released from Cf9 and Cf2 cotyledons injected with IF following base and acid hydrolysis (Enyedi et al., 1992) yielded significantly more SA from most tissue

samples. Significantly more SA was also recovered when samples were analyzed following enzymatic hydrolysis with a β-glucosidase that releases Glc-conjugated SA. However, there was considerable variation between experimental replicas in the timing and magnitude of *Cf*-gene-dependent increases in total SA levels derived from both treatments (data not shown). For this reason, free SA was seen to be a more meaningful measure of *Cf-Avr*-gene-dependent induction of SA biosynthesis.

DISCUSSION

In this paper we demonstrate four early *Cf*- and *Avr*-gene-dependent events. These are (a) changes in cell morphology within the L1 layer, (b) the accumulation of free SA, (c) ethylene biosynthesis, and (d) host cell death. The kinetics of each response was monitored by injecting a partially purified *C. fulvum* IF preparation into tomato cotyledons, thus providing a synchronous challenge to a population of plant cells. To determine whether the responses conferred by unlinked *Cf* genes exhibited qualitative differences, the *Cf-9-Avr9*- and *Cf-2-Avr2*-incompatible interactions were compared with each other and with a compatible interaction involving a near-isogenic line (Cf0) lacking known *Cf* genes. By identifying environmental conditions that suppressed the development of macroscopic *Cf*-dependent necrotic and chlorotic reactions and altered the kinetics of host cell death, we were able to determine which of the induced responses arose directly as a consequence of the *Cf-Avr* recognition event and which arose indirectly.

Stomatal Opening

The rapid induction of stomatal opening, accompanied by the collapse of the intervening epidermal cells in the *Cf-9-Avr9* interaction, was the only response not evident in the *Cf-2-Avr2* interaction. The reasons underlying this divergence are at present not known. A discrepancy in the Avr9 and Avr2 titer injected is unlikely to be the cause because stomatal opening was not induced when concentrated IFs were used. The *Cf-9-Avr9* induction of supraoptimally open stomata has also been observed in cotyledons of F_1 seedlings that carried both *Cf-9* and a *35S:SP:Avr9* transgene (Hammond-Kosack et al., 1994).

Plasma membrane H^+-ATPases are the effectors of stomatal opening (Kearns and Assmann, 1993). Thus, the detection of this response in the *Cf-9-Avr9* interaction indicates that one consequence of Cf-9 activation is the stimulation of signaling pathways that activate proton pumps in guard cells. This signaling apparently differs from that induced by either the *F. amygdali* toxin, fusicoccin (Marre, 1979), or the *Rhynchosporium secalis* necrosis-inducing peptides to stimulate plasmalemma localized H^+-ATPases activity (Wevelsiep et al., 1993), because in these examples the opened stomata cannot be closed by subsequent ABA treatment. Probable intermediate components of stomatal signal transduction include carbon metabolism, G proteins, Ca^{2+}, inositol-1,4,5-triphosphate, and phosphatases (Kearns and Assmann, 1993).

Cf-Avr-gene-dependent activation of the proton pump in other cell types is also likely because highly swollen mesophyll cells were found in injected Cf2 and Cf9 cotyledons. In several incompatible *C. fulvum* interactions, highly enlarged lower mesophyll cells form around fungal hyphae (Lazarovits and Higgins, 1976; Ashfield et al., 1994; Hammond-Kosack and Jones, 1994) and indicate that cell-wall-loosening reactions, mediated in part by proton extrusion, are occurring at sites of fungal incompatibility. Finally, when a *C. fulvum* IF preparation containing the *Avr5* gene product was added to a Cf5 tomato cell-suspension culture, a rapid increase in H^+-ATPase activity, coincident with the acidification of the extracellular medium, was detected (Vera-Estrella et al., 1994). Thus, several lines of evidence indicate that the interaction of various *Cf*- and *Avr*-gene products activates a signal transduction pathway that stimulates H^+-ATPase activity.

SA

In the *Cf-9-Avr9* interaction increases in the levels of free SA occurred simultaneously under both low- and high-humidity regimes and the onset of this increase also coincided with the initial decline in host cell viability 9 to 12 h a.i. Thus, SA biosynthesis could have provoked cell death, as suggested by Levine et al. (1994), or vice versa. In other incompatible plant-microbe interactions involving tobacco and cucumber plants, free SA levels typically reach approximately 5 μg/g fresh weight coincident with tissue collapse and visible lesion formation (Malamy et al., 1990; Raskin, 1992; Silverman et al., 1993; Ryals et al., 1994). The levels of SA attained under low-humidity conditions (18 μg/g fresh weight) were considerably higher than reported anywhere else. Possibly the rate of conversion of free SA to various conjugated forms (Enyedi et al., 1992) is slower in tomato than other plant species. However, since a 70% reduction in fresh weight had occurred by 24 h a.i., presumably through water loss from the supraoptimally open stomata, this may be the main reason why on a fresh weight basis such high free SA levels were measured.

In the *Cf-2-Avr2* interaction the increase in free SA levels preceded the onset of host cell death by 8 to 12 h in both 16-h daylength regimes. These data indicate that the signaling pathway(s) involved in inducing SA arose from the initial *Cf-Avr*-mediated recognition event and not indirectly via host cell death. However, under continuous darkness SA levels only increased slightly by the onset of cell death. Thus, mechanisms other than elevated levels of SA can promote host cell death after 48 h in the dark (see below).

The increases in free SA levels in both incompatible interactions were transient. SA decreases may be due to SA translocation out of these injected tissues or due to metabolic turnover including the formation of a β-*O*-D-glucosylsalicylic acid. A preliminary comparison of total and free SA levels in Cf9 and Cf2 cotyledons after IF challenge indicated that various conjugated forms of SA were formed including the β-*O*-D-glucoside (P. Silverman and K.E. Hammond-Kosack, unpublished data).

Signaling Interrelationships

In the accompanying paper by May et al. (1996) we have demonstrated that both Cf-2-Avr2 and Cf-9-Avr9 mediate the activation of an oxidative burst and increased enzyme lipoxygenase activity prior to any of the responses detailed here. A compilation of the data presented in both papers is presented in Figure 8. Therefore, activation of these intermediate responses may be initiated by the molecular species generated in the oxidative burst, indirectly following the formation of secondary signals, i.e. lipid breakdown products or low reduced glutathione levels, or because of the generally perturbed state of plant cell metabolism. Alternatively, some or all of these delayed responses may arise directly from Avr elicitor recognition via other direct or indirect signaling pathways.

H^+-ATPase activation in response to Avr elicitor challenge could be an indirect consequence of the earlier oxidative burst. In the macrophage oxidative burst induction of the NADPH oxidase is coupled to the opening of a proton channel to prevent intracellular acidification (Henderson et al., 1987). It is, however, unclear why the oxidative burst of tomato cells mediated by *Cf-9-Avr9* leads to activation of a proton pump in guard cells, whereas the responses mediated by *Cf-2-Avr2* and *Cf-5-Avr5* (K.E. Hammond-Kosack, unpublished data) do not. Differences in the tissue-specific expression and/or relative concentration of the Cf2 and Cf9 proteins are a possible explanation.

One possible function of an extracellular decrease in pH around the activated host cells would be to increase the rate of production of more reactive oxygen radicals. The half-life of superoxide anions ($O_2^{\cdot -}$) at pH 8.5 is 50 s, whereas at pH 6.5 it is 0.5 s (Sutherland, 1991). The swelling of plant cells is also known to stimulate an oxidative burst (Low and Dwyer, 1995).

Increases in SA levels may also be a consequence of the oxidative burst. Both UV light and ozone elicit a burst of active oxygen species production in plant tissue (Imbrie and Murphy, 1984; Dixon and Lamb, 1990) and also cause free SA levels to increase in the absence of necrotic lesion formation (Yalpani et al., 1994). Leon et al. (1995) demonstrated that H_2O_2 injection into tobacco leaf apoplast stimulates SA biosynthesis. In the TMV-*N*-gene incompatible interaction in tobacco a similar temporal relationship between the generation of superoxide anions and SA synthesis appears to exist (Doke and Ohashi, 1988; Leon et al., 1993). However, a direct *Cf-Avr*-dependent induction of the phenylpropanoid pathway leading to SA synthesis is also possible and worthy of further investigation.

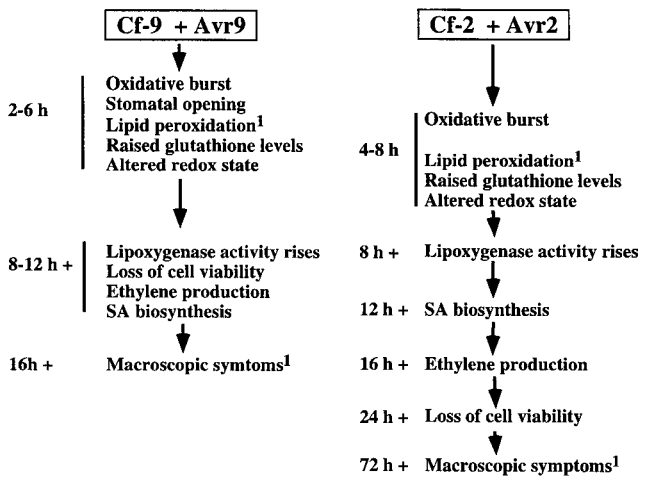

Figure 8. Temporal order of activated plant responses in incompatible interactions mediated by Cf-9-Avr9 and Cf-2-Avr2 when the tomato plants injected with *C. fulvum* race-specific elicitors were maintained at either 70 or 98% RH under a 16-h photoperiod. [1]At 98% RH neither lipid peroxidation nor macroscopic symptoms was detected.

Ethylene has been proposed to be a part of the signaling pathway leading to SA induction (Van Loon, 1977). However, the time course presented for the levels of these two molecules in the *Cf-2-Avr2*-mediated defense responses makes this unlikely. Silverman et al. (1993) also found that an inhibitor of ethylene action, 2,5-norbornadiene, did not significantly alter SA accumulation in incompatible TMV infections on tobacco leaves of plants carrying the *N*-resistance gene.

High SA levels could also heighten the level of cell stress imposed by stimulating the continuation of the oxidative burst. SA can bind to catalase, an enzyme that catalyzes the dismutation of H_2O_2 (Chen et al., 1993). Inhibition of catalase activity by SA would lead to a localized elevation in H_2O_2, possibly to levels toxic to the plant cell, especially if the Fenton reaction leading to $OH^{\cdot}$ production is occurring. Sanchez-Casas and Klessig (1994) demonstrated that the activity of tomato catalases is inhibitable by SA.

Elevated levels of SA could provoke cell stress and lead to cell death. SA is known to inhibit stomatal closure (Rai et al., 1986) and so elevated SA levels may establish a positive feedback loop that drives the desiccation process in the *Cf-9-Avr9*-mediated interaction. SA is also known to be toxic to both plant and microbial cells in the micromolar range (Malamy and Klessig, 1992; Raskin, 1992). Therefore, the magnitude of the initial oxidative burst and the potential for stress-induced cell death may be increased, by a combination of elevated SA levels and catalase enzyme inhibition, from 12 h onward in both *Cf-2-Avr2* and *Cf-9-Avr9* incompatible interactions.

The function of ethylene and SA in incompatible interactions may be to amplify defense responses both locally and systemically. An array of defense-related genes can be induced by the exogenous application of ethylene (Raz and Fluhr, 1993; Lawton et al., 1994) or SA (reviewed by Horvath and Chua, 1994). Ashfield et al. (1994) demonstrated that at the site of incompatible *C. fulvum* infections, and in response to *C. fulvum* IF injection into *Cf*-containing tomato lines, a β-1,3-glucanase promoter:GUS reporter construct is rapidly induced in a highly localized manner. The promoter element of this construct contains an SA-responsive element (Goldsborough et al., 1993). Wubben et al. (1996) and van Kan et al. (1995) also showed that several of the pathogenesis-related proteins inducible by the exogenous application of SA are induced earlier in incompatible than in compatible *C. fulvum* infections. An increase in SA levels may provoke an increase in H_2O_2 levels and the induction

of additional signaling pathways (Levine et al., 1994; Chen et al., 1995).

What Stops *C. fulvum* Growth?

Several possible explanations can be proposed to explain the containment of *C. fulvum* hyphae during incompatible interactions. Growth inhibition could be due to induced polyacetylenic phytoalexins, induced chitinase, and β-1–3-glucanase defense proteins or the rapid host cell death, HR. Each of these factors acting alone is unlikely to be able to arrest *C. fulvum* because equivalent phytoalexin levels were produced during both compatible and incompatible interactions (de Wit and Flach, 1979; de Wit and Kodde, 1981), no antifungal activity toward *C. fulvum* hyphae was demonstrated for PR proteins (Joosten et al., 1995), and the HR response (Lazarovits and Higgins, 1976; de Wit, 1977) can be absent in situations in which *C. fulvum* arrest occurs (Hammond-Kosack and Jones, 1994). Thus, other plant defense responses must contribute to resistance.

Several reactive oxygen intermediates may be directly toxic to *C. fulvum* hyphae. Also, the apparent localized decline in extracellular pH would lead to increased spontaneous $O_2^{\cdot -}$ dismutation to the more reactive and membrane-permeable oxygen species, H_2O_2, $HO_2^{\cdot}$, and $OH^{\cdot}$ and therefore heighten the potency of the oxidative burst toward hyphae. A similar strategy occurs in the vacuoles of mammalian macrophages where an oxidative burst under low-pH conditions kills the engulfed microbe (Baggiolini and Wymann, 1990; Segal and Abo, 1993). H_2O_2 is toxic to microbes at concentrations known to be produced in planta (Peng and Kuć, 1992; Svalheim and Røbertson, 1993). Alternatively, or in addition, the elevated levels of reactive oxygen intermediates could drive the rapid oxidative cross-linking of cell-wall structural proteins (Bradley et al., 1992; Brisson et al., 1994) and elevate the production of lignin polymer precursors via increased peroxidase activity (Gross et al., 1977). Changes to host cell-wall permeability would significantly decrease the nutrient and water supply to the *C. fulvum* hyphae in the extracellular spaces and thus compromise hyphal fitness. Generated $HO_2^{\cdot}$ and $OH^{\cdot}$ radicals would also result in a direct attack on fatty acids within the membranes of both plant and *C. fulvum* cells and lead to self-perpetuating lipid peroxidation. Many of the fatty acid breakdown products arising from lipoxygenase activity are antimicrobial (Croft et al., 1993). Finally, alterations to lipid membrane composition would cause an increase in electrolyte leakage and eventually plant cell death. This is frequently observed as a late response at the center of various *Cf-Avr*-mediated incompatible infections (Lazarovits and Higgins, 1976; Hammond-Kosack and Jones, 1994), particularly at lower humidity. Cell leakage would release both induced and preformed cytoplasmic and vacuolar contents into the apoplast, which could be fungitoxic. For example, SA is inhibitory to the growth of many fungal species (reviewed by Malamy and Klessig, 1992), whereas the alkaloid saponin tomatine, constitutively present in tomato vacuoles, lyses *C. fulvum* hypha (P. Bowyer, unpublished data). Thus, synchronous and coordinate activation of numerous plant defense responses both in advance of and within *C. fulvum* infections could enhance the chances of effective microbe containment while minimizing plant tissue damage.

ACKNOWLEDGMENTS

The authors wish to thank Sara Perkins for her excellent horticultural assistance and Graham Hills for assistance with the scanning electron microscope analysis.

Received July 5, 1995; accepted December 30, 1995.

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