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Involvement of Reactive Oxygen Species, Glutathione Metabolism, and Lipid Peroxidation in the *Cf*-Gene-Dependent Defense Response of Tomato Cotyledons Induced by Race-Specific Elicitors of *Cladosporium fulvum*[1]

Mike J. May[2,3], Kim E. Hammond-Kosack[2], and Jonathan D. G. Jones*

The Sainsbury Laboratory, John Innes Centre, Colney Lane, Norwich, NR4 7UH, United Kingdom (K.E.H.-K., J.D.G.J.); and Department of Plant Sciences, University of Oxford, South Parks Road, Oxford, OX1 3RB, United Kingdom (M.J.M.)

The chronological order of responses to *Cladosporium fulvum* (Cooke) (*Cf*) race-specific elicitors was assessed in cotyledons of three near-isogenic tomato (*Lycopersicon esculentum* Mill.) lines carrying either *Cf-9* or *Cf-2* or no *Cf* gene. The responses observed were dependent on the presence of a *Cf* gene, *Avr*-gene product dose injected, and the relative humidity (RH) of the growth chamber. At ambient RH, superoxide formation and lipid peroxidation occurred after 2 h (Cf9) and 4 h (Cf2). At elevated RH (98%) and at lower avirulence elicitor dose, *Cf-Avr*-dependent lipid peroxidation was considerably attenuated. Significant electrolyte leakage occurred by 18 h but only at the lower RH. Total glutathione levels began to increase 2 to 4 h and 4 to 8 h after challenge of Cf9 and Cf2 cells, respectively, and by 48 h reached 665 and 570% of initial levels. A large proportion of this accumulation (87%) was as oxidized glutathione. When the RH was increased to 98%, increases in glutathione levels were strongly attenuated. Increased lipoxygenase enzyme activity was detected 8 h postchallenge in either incompatible interaction. These results indicate that the activation of the *Cf-Avr*-mediated defense response results in severe oxidative stress.

Early events during the interaction between a specialized pathogen and its host ultimately determine whether the attempted infection succeeds or fails. Plant-pathogen interactions often exhibit race-cultivar specificity. To explain this, the "gene-for-gene" hypothesis has been proposed (Flor, 1946), wherein incompatibility requires the presence of both a dominant plant resistance (*R*) gene and a complementary dominant pathogen avirulence (*Avr*) gene (Gabriel and Rolfe, 1990; Keen, 1990).

The interaction between the fungal pathogen *Cladosporium fulvum* (syn. *Fulva fulva* [Cooke] Cif.) and tomato (*Lycopersicon esculentum* Mill.) provides an excellent experimental system with which to elucidate the mechanism of the gene-for-gene model. Progress in determining the genetic, cytological, biochemical, and molecular details of this interaction has been rapid, in large part because of specific features of the interaction that make it highly amenable to experimental manipulation and analysis (reviewed by de Wit, 1992; Hammond-Kosack and Jones, 1995). NILs are available, each carrying a different resistance (*Cf*) gene (Stevens and Rick, 1988; Dickinson et al., 1993), as are an array of *C. fulvum* races that produce the corresponding functional *Avr*-gene products either singly or in combination. Thus, it has been possible to assess the resistance phenotype conferred by each *Cf-Avr*-gene combination within a uniform genetic background (Hammond-Kosack and Jones, 1994). Also, because of the exclusively intercellular growth habit of *C. fulvum* hyphae (Bond, 1938), the *Avr*-gene products are easily purified from IFs obtained from tomato leaves supporting heavy fungal sporulation (de Wit and Spikman, 1982). Stocks that carry a *Cf* gene, but not ones that lack a *Cf* gene, respond when IF containing the complementary *Avr*-gene product is injected into the air spaces of healthy cotyledons or leaves of *Cf*-carrying stocks. Each *Cf-Avr*-gene combination confers a characteristic macroscopic chlorotic or necrotic response 1 to 5 d after IF challenge at ambient humidity (de Wit and Spikman, 1982; Hammond-Kosack and Jones, 1994).

Whereas the isolation of the *Cf-9* gene has obvious implications for plant biotechnology (Jones et al., 1994), it is essential to have a detailed understanding of the biochemical consequences of *Cf*-gene function for the rational design of strategies to manipulate effective disease resistance. Incompatibility in numerous plant-pathogen interactions is associated with the synthesis of molecules that debilitate or injure the pathogen, including ROIs ($O_2^{\cdot-}$, H_2O_2, and $OH^{\cdot}$), phytoalexins, and a number of pathogenesis-related pro-

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[2] These two authors contributed equally to the work in this paper and should be considered joint first authors.

[3] Present address: Laboratorium voor Genetika, Universiteit Gent, KL Ledeganckstraat 35, B-9000 Gent, Belgium.

* Corresponding author; e-mail jonesj@bbsrc.ac.uk; fax 44–1603–250024.

Abbreviations: Avr, avirulence; CAT, catalase; h a.i., hours after injection; IF, intercellular washing fluid; LOX, lipoxygenase; NBT, nitroblue tetrazolium (2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-[3–3′-dimethoxy-4,4′-diphenylene]-ditetrazolium chloride); NIL, near-isogenic line; ROI, reactive oxygen intermediate; SA, salicylic acid; SOD, superoxide dismutase; TBA, thiobarbituric acid; TBARS, TBA-reactive species.

teins, including hydrolytic enzymes, glucanases, and chitinases (Lamb, et al., 1989; Dixon and Lamb, 1990; Sutherland, 1991; Mehdy, 1994). These events are accompanied by the establishment of processes that limit pathogen spread, such as callose and lignin deposition, and the reinforcement of plant cell walls by the cross-linking of proteins; rapid host cell death (the hypersensitive response), which is thought to deprive biotrophic pathogens of a food base; the activation of defense signaling through the synthesis of ethylene and SA; or the production of lipid peroxides via enhanced LOX enzyme activity (Klement, 1982; Bowles, 1990; Bradley et al., 1992; Koch et al., 1992, Raskin, 1992; Croft et al., 1993; Brisson et al., 1994). However, the causal involvement of only one induced response, namely SA accumulation, in localized disease resistance in three host-pathogen interactions has been proven (Delaney et al., 1994).

To unravel the complexity of the *Cf-Avr*-mediated defense responses and to allow subsequent analysis of how the expression and activity of individual components of the response are coordinated, we have undertaken a detailed biochemical analysis of the chronology of events initiated immediately following a synchronous activation of the resistance response. The analysis of two different *Cf-Avr*-mediated incompatible interactions, namely *Cf-9-Avr9* and *Cf-2-Avr2*, was selected to determine whether the resistance response conferred by genetically unlinked *Cf* genes was similar or dissimilar (Jones et al., 1993). In this paper we describe several *Cf-Avr*-gene-dependent events indicative of oxidative stress. In the accompanying paper (Hammond-Kosack et al., 1996), we characterize the subsequent changes in cell viability, macroscopic alterations to cell morphology, and ethylene and SA formation.

MATERIALS AND METHODS

Plant Material

All experiments were performed on the cotyledons of 14- to 16-d-old tomato (*Lycopersicon esculentum* [Mill]) seedlings. The three tomato genotypes investigated were NILs of the cv Moneymaker (Tigchelaar, 1984). They contained the *C. fulvum* resistance gene *Cf-9* or *Cf-2* in a homozygous state or carried no known *Cf* gene. The three plant lines are designated Cf9, Cf2, and Cf0, respectively.

IF Preparation

Cladosporium fulvum (Cooke) race-specific elicitors were isolated in IF from Cf0 tomato leaves on which race 0 was sporulating over the entire leaf surface, as originally described by de Wit and Spikman (1982). The *C. fulvum* race 0 used for IF preparation causes an incompatible interaction when inoculated on NILs expressing either the resistance gene *Cf-9* or *Cf-2* (Hammond-Kosack and Jones, 1994). Thus, race 0 possesses functional copies of the Avr genes *Avr9* and *Avr2*. The proteins present in the IF were precipitated overnight in 40% (v/v) acetone at −20°C. After the sample was centrifuged for 15 min at 3000*g*, proteins in the supernatant were precipitated overnight in 80% (v/v) acetone at −20°C. The pellet obtained after centrifugation (15 min at 3000*g*) was freeze dried, resuspended in distilled water to give the original volume, and stored at −20°C. The biological activity of the IF preparation was assessed as described previously (Hammond-Kosack and Jones, 1994). An identical IF preparation was used for all experiments. This gave a gray necrotic response on Cf9 plants in which IF was injected within 24 h down to a 1 in 64 titer and a chlorotic response on Cf2 plants within 4 d down to a 1 in 8 titer (Hammond-Kosack and Jones, 1994). The relative intensity of Coomassie blue staining of the Avr9 peptide and the pathogenesis-related protein P14 after electrophoretic separation in the IF preparation used throughout this study was reported previously (Hammond-Kosack et al., 1994a, fig. 2B).

Experimental Regimes

Seedlings were grown in a growth cabinet maintained at 24°C during the 16-h light period and 18°C during the 8-h dark period. Light was supplied by 400-W lamps (Power Star [HQI-T]; Osram Ltd., Middlesex, UK) to give a photon flux density of 600 μE m^{-2} s^{-1}, and the RH was maintained at 70%. For experiments at 98% RH 30 min after IF injection, the seedlings were placed in plastic propagators with closed vents and 3-mm-deep water in the lower trays. The plant pots were held above the water surface on plastic trays. Each propagator contained the plants for analysis at a single time. For experiments at 70% RH the seedlings were covered with plastic propagator lids with the vents fully open.

All IF injections were done 3 h after the onset of the 24°C regime. A 1 in 2 dilution of IF was injected into both cotyledons of each seedling using a 1-mL disposable syringe fitted with a 21-gauge 6% Luer-tipped needle (Terumo, Leuven, Belgium). The apoplastic domain of each cotyledon required about 50 μL to be entirely flooded.

NBT Staining

Histochemical staining for $O_2^{\cdot-}$ production in whole tissue was based on the ability of cells to reduce NBT, as described by Doke and Ohashi (1988). Whole cotyledons were vacuum infiltrated with 10 mM potassium phosphate buffer (pH 7.8) containing 0.5% (w/v) NBT, 10 μM NADPH, and 10 μM EDTA. After 15 min of staining at 25°C in the light, the cotyledons were placed in a chloral hydrate solution (2.5 g/mL) to remove Chl and preserve tissue integrity.

LOX Activity

Extracts for the measurement of LOX activity were prepared according to the method of Koch et al. (1992). Following injection and incubation, cotyledons were frozen in liquid nitrogen and stored at −80°C until use. Approximately 0.2 to 0.3 g of tissue was ground in a mortar and pestle in 0.8 mL of ice-cold 0.1 M potassium phosphate buffer (pH 7, 1% [w/v] PVP, 0.1% [v/v] Triton X-100, and 0.04% [w/v] sodium metabisulfite). The homogenate was centrifuged at 16,000*g* for 10 min at 4°C and the clear

supernatant (hereafter termed cotyledon extract) was assayed immediately.

LOX activity in the cotyledon extracts was measured using the polarographic method of Christopher et al. (1970). A 10 mM stock of the LOX substrate, the sodium salt of linoleic acid (*cis*-9,*cis*-12 octadecadienoic acid) was prepared for the assay as described by Koch et al. (1992). A 1.4-mL aliquot of 0.1 M potassium phosphate buffer, pH 7, was equilibrated at 25°C for 2 min in an oxygen electrode (Hansatech, Norfolk, UK) attached to a strip chart recorder. Fifty microliters of cotyledon extract were added and the rate of oxygen consumption was measured. When the trace was stable, 50 μL of linoleic acid were added with a syringe and the rate of oxygen uptake was recorded. LOX activity was expressed as μmol O_2 min^{-1} mg^{-1} protein using the standard oxygen content of air-saturated water at 25°C (0.258 μmol mL^{-1} water).

Total Glutathione and GSSG Determinations

Cotyledons were ground in 1 mL of distilled water and 300 μL were taken for analysis. Protein and cell debris were precipitated by addition of 50 μL of 5% (w/v) sulfosalicylic acid (made up in 0.1 M potassium phosphate buffer, pH 7.6, containing 5 mM EDTA) and centrifugation at 15,000*g* for 10 min. Two samples of 100 μL of the supernatant were neutralized by addition of 300 μL of 0.5 M potassium phosphate buffer, pH 7.6; one sample was used for the determination of total glutathione and the other was used for the determination of GSSG. Glutathione concentrations in the neutralized extracts were determined as described by Smith (1985). GSH was determined as the difference between total glutathione and GSSG and was expressed as nmol mg^{-1} protein. Recovery experiments were performed in which a known concentration of GSH or GSSG was added prior to grinding. Recovery was 95 ± 2%.

Lipid Peroxidation Analysis

Samples of 375 μL of the cotyledon homogenate prepared as described for glutathione determination were assayed for the products of lipid peroxidation by the TBA method as described by Oteiza and Bechara (1993) with the following modifications. To the homogenate was added 125 μL of 3% (w/v) SDS, 250 μL of 3% TBA in 50 mM NaOH, and 250 μL of 25% (v/v) HCl with thorough mixing in between each addition. The mixture was heated at 80°C in a water bath for 20 min and snap-cooled on ice. TBARS were extracted with 600 μL of butan-1-ol and the specific A_{532} of the organic phase was measured and the nonspecific A_{600} was subtracted. Measurements were expressed as A_{532} mg^{-1} protein.

Electrolyte Leakage

Conductivity measurements were made on 8-mm cotyledon discs, prepared and analyzed as described by Peevers and Higgins (1989).

Protein Determination

Protein determinations were carried out using the method of Lowry (1951) as modified by Peterson (1977).

Experimental Design and Statistical Analysis

All experiments were performed with a minimum of three tissue sample replicates per treatment per time point. Each experiment was done three times. Data from each experiment are expressed as the means ± SE (unless otherwise stated).

RESULTS

The effects of a *C. fulvum* race-specific elicitor preparation IF, containing the products of the fungal Avr genes *Avr9* and *Avr2* on Cf0, Cf2, and Cf9 NILs of tomato, were assessed under two different humidity regimes. The 70% RH was selected so that the data obtained could be directly compared with a previous investigation of *Cf-9-Avr9*-dependent induced responses on leaves (Peevers and Higgins, 1989). The 98% RH was chosen to mimic the conditions required for successful *C. fulvum* pathogenesis. The high humidity also eliminates the macroscopic *Cf*-gene-dependent necrotic and chlorotic responses to IF challenge (Hammond-Kosack et al., 1996). Biochemical parameters indicative of oxidative stress, such as changes in the cellular GSH status, increases in lipid peroxidation, and NBT staining, were analyzed. The physiological consequences of oxidative damage to membranes after IF treatment were determined by analyzing changes in solute leakage from the treated cotyledons. In parallel we investigated the effect of IF on the activity of SOD, CAT, and LOX. Thus, the chronological sequence of oxidative events after race-specific elicitation of defined genotypes of tomato was determined and their physiological consequences were assessed.

Effect of IF on NBT Staining

NBT staining is a good indicator of elevated levels of ROIs, in particular superoxide anions ($O_2^{\cdot-}$), in tissues: NBT turns from a pale yellow solution to an insoluble blue/purple formazan product in the presence of ROI (Doke and Ohashi, 1988). Following the delivery of a 1 in 2 dilution of IF into Cf0, Cf2, and Cf9 cotyledons maintained at 70% RH, *Cf-Avr*-gene-dependent formazan formation was observed, as shown in Figure 1. Patches of NBT-positive staining were evident 2 h a.i. on Cf9 plants and by 4 h a.i. on Cf2 plants. The intensity and extent of NBT staining increased over the subsequent 10 h on Cf9 plants and thereafter declined, whereas on Cf2 plants increased NBT staining was sustained until 24 h. At later times in the Cf2-injected cotyledons (24–72 h a.i.), the intensity of NBT staining gradually declined (data not shown). The earlier decline in staining in Cf9 cotyledons probably reflected the onset of host cell death and tissue necrosis induced by the IF. Similar results were obtained when a 1 in 8 or 1 in 64 dilution was injected into Cf9 cotyledons or a 1 in 8 dilution into Cf2 cotyledons. No positive NBT staining in Cf2 was obtained when a 1 in 64 dilution of IF was used. No

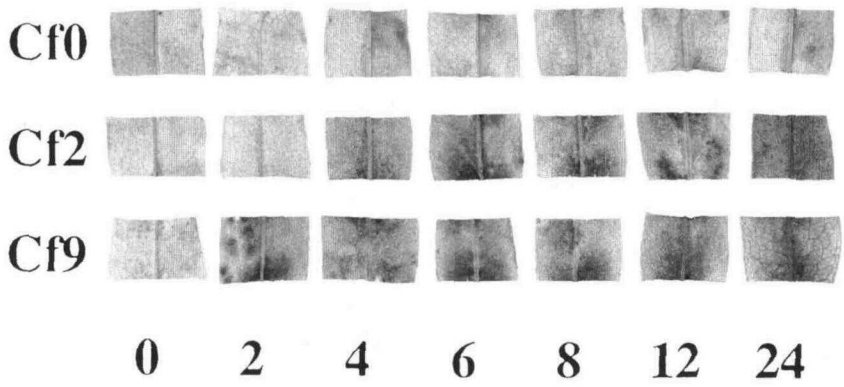

Figure 1. Time course of NBT staining in Cf0, Cf2, and Cf9 tomato cotyledons induced by IF from race 0. The cotyledons were excised at various hours after a 1 in 2 titer of elicitor was injected into the intercellular air spaces. Incompatible interactions involved Cf2-Avr2 and Cf9-Avr9 and the compatible interaction involved Cf0. The black staining indicates the presence of reactive oxygen species. The weaker staining in the Cf9 sample at 24 h was due to IF-induced tissue necrosis.

increased NBT staining was apparent in Cf0 cotyledons after IF injection. For all three genotypes, a limited and variable amount of positive NBT staining was evident, specifically around the wounded tissue at the injection site by the 2-h point (data not shown).

When the above experiment was undertaken at elevated humidity (98% RH), NBT staining was detectable in all injected tomato genotypes at the 2-h time. The intensity of the staining increased over the subsequent 24 h (data not shown). An identical result was obtained when IF at a 1 in 8 or 1 in 64 dilution titer was injected. Noninjected cotyledons of the three plant genotypes maintained at high humidity did not exhibit increased NBT staining (time course examined: 2–48 h after placement of seedlings at 98% RH). We conclude from these data that the wounding and/or the temporary anoxia caused by IF injection induces at least one additional stress response under high-humidity conditions, namely, enhanced ROI generation.

Effect of IF on Lipid Peroxidation

The *Cf*-gene-dependent induction of lipid peroxidation after *C. fulvum* IF infiltration was measured by determining the accumulation of TBARS at various times after IF injection into Cf0, Cf2, and Cf9 cotyledons. Since malonaldehyde is not the only molecule to react with TBA (Gutteridge and Halliwell, 1990), measurements were expressed as A_{532} mg^{-1} protein rather than as the concentration of malonaldehyde estimated from the absorbance of the sample after TBA reaction. A 2-fold dilution of IF induced rapid and substantial accumulation of TBARS when injected into the cotyledons of Cf9 and Cf2 plants maintained at 70% RH (Fig. 2A). A negligible change was measured in Cf0 plants at either 70 or 98% RH, even 48 h a.i. (Fig. 2A). For Cf9 accumulation of TBARS was measurable 2 h post-IF treatment, whereas for Cf2 they were not measurable until 4 h. By 24 h post-IF injection, the magnitude of lipid peroxidation detectable was 179% of the initial level for Cf2 plants and 260% of the initial level for Cf9 plants.

Considerable attenuation of the *Cf*-dependent accumulation of TBARS was observed when IF-treated Cf0, Cf2, and Cf9 plants were maintained under the same light regime but the RH was elevated to 98% (Fig. 2B). Both *Cf*-2- and *Cf*-9-dependent accumulation of TBARS was almost completely abolished. Under these environmental conditions the macroscopic response to IF injection is abolished (Hammond-Kosack et al., 1996).

Effects of IF on Electrolyte Leakage from Cells

Previously, it was demonstrated that when *Cf*-9-containing tomato leaves were challenged with IF increased electrolyte leakage from plant cells was detectable by 3 to 6 h in an *Avr*9-dependent manner (Peevers and Higgins, 1989). To compare the overall kinetics of the *Cf*- and *Avr*-dependent responses in cotyledons with those reported earlier in leaf tissue, cotyledon tissue discs were cut at various times after IF injection and conductivity measurements were made. In Figure 3 the levels of net electrolyte leakage in the first 3 h after cutting the tissue discs are presented for each *Cf-Avr* interaction. In Cf9 and Cf2 plants maintained at 70% RH, following elicitor injection at a 1 in 2 dilution, increased leakage commenced at 9 to 12 h and 15 to 18 h, respectively, and continued to increase thereafter. The final magnitude of the increase by 48 h was 9-fold in Cf9 and 4-fold in Cf2 plants (Fig. 3A). When the *Cf*-containing plants were maintained in high-humidity conditions after IF injection, only a 2-fold increase in electrolyte leakage was detectable by 48 h (Fig. 3B). In Cf0 plants the IF injection caused no increase in electrolyte leakage during the 0- to 48-h period in either humidity regime.

Effect of IF on the Level of Total Glutathione and the GSH:GSSG Ratio

It has previously been demonstrated that glutathione accumulates in plants exposed to oxidative stimuli (Smith, 1985; May and Leaver, 1993). *Cf*- and *Avr*-dependent ROI production occurs rapidly upon IF injection; therefore, we measured the levels of total glutathione and also the level of GSSG to discover whether this oxidative stress induced cell protection mechanisms that involved glutathione.

At 70% RH, injection of IF into the cotyledons of Cf2 and Cf9 resulted in considerable accumulation of total glutathione during the 0- to 48-h sampling period (Fig. 4A) relative to Cf0. In both cases levels of GSSG were significantly higher than the controls after 2 to 4 h (Fig. 4C), amounting to about 50% of total glutathione at 4 h (compare insets in Fig. 4, A and C). The kinetics of these two responses follow

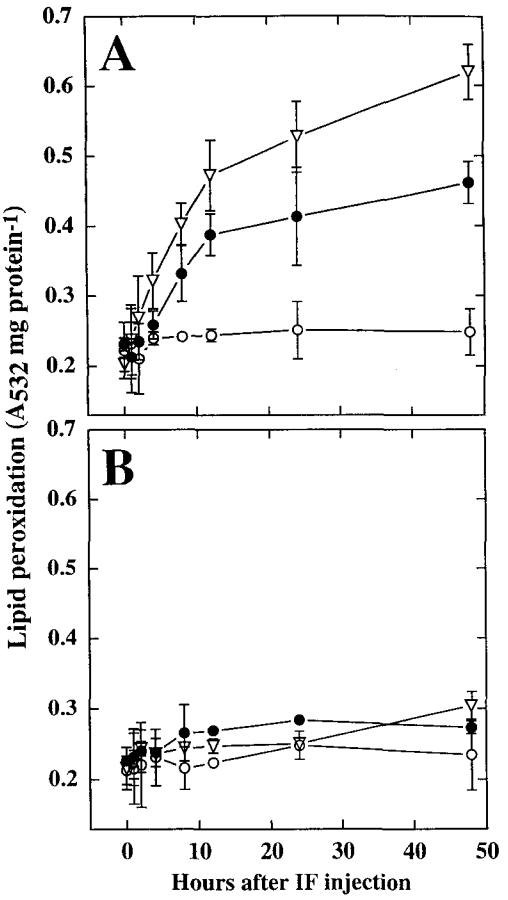

Figure 2. Degree of lipid peroxidation, as measured by the accumulation of TBARS, induced in Cf0, Cf2, and Cf9 tomato cotyledons in response to IF from race 0. The plants were maintained at 70% RH (A) or 98% RH (B) after injection of a 1 in 2 titer of IF. Macroscopic chlorotic and necrotic symptoms induced in the incompatible interactions, Cf-2-Avr2 (●) and Cf-9-Avr9 (▽), that developed at 70% RH within 24 or 72 h, respectively, are absent at 98% RH. In the compatible interaction involving Cf0 (○) no macroscopic symptoms to IF developed in either humidity regime. Vertical bars represent the SES.

closely the kinetics of changes in the level of lipid peroxidation, both in the speed and magnitude of expression. In Cf0, which lacks any *Cf* genes, a small increase in the level of total glutathione was measured and up until 24 h; <25% of this was GSSG (Fig. 4, A and C). Such marked increases in the level of GSSG are strong evidence that IF challenge results in severe oxidative stress in a *Cf-Avr*-gene-dependent manner. The accumulation of total glutathione continued in Cf2 and Cf9 until the end of the sampling period at 48 h (Fig. 4A), at which point 87% was GSSG in both lines (Fig. 4C). Total glutathione accumulated in Cf2 to levels 20% less than in Cf9, 538 and 665% higher than the initial levels, respectively.

Increasing the RH to 98% while keeping the same light and temperature regime markedly delayed and attenuated GSH and GSSG accumulation in response to IF challenge (Fig. 4, B and D). Importantly, increases in the level of total glutathione were only detected 8 to 12 h a.i. in Cf9 and 12 to 24 h a.i. in Cf2. At the end of the sampling period increases in total glutathione were lower (39 and 45% of levels in Cf2 and Cf9, respectively, at 70% RH) and the relative proportion of GSSG was also lower. At 48 h after IF challenge, GSSG levels as a proportion of total glutathione in Cf2 and Cf9 were 40 and 34%, respectively, compared to 87% at 70% RH. Thus, the kinetics and magnitude of total glutathione accumulation and an increase in the ratio of GSSG:GSH are strongly *Cf-Avr*-gene dependent. Increases in the ratio of GSSG:GSH followed the measured *Cf-Avr*-dependent increases in the level of lipid peroxidation and together with *Cf-Avr*-dependent NBT staining and solute leakage provide strong evidence for *Cf-Avr*-gene-dependent oxidative stress in response to race-specific IF challenge. The marked reduction in the magnitude and timing of changes in the development of these four parameters clearly indicates that *Cf-Avr*-dependent oxidative stress in response to IF challenge is strongly influenced by the RH.

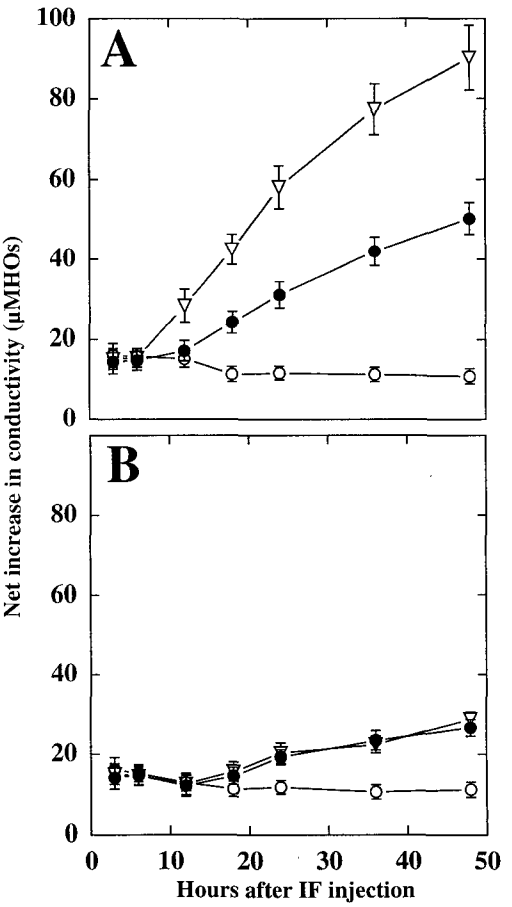

Figure 3. Effects of IF on electrolyte leakage from Cf0, Cf2, and Cf9 tomato cotyledon discs. IFs at a 1 in 2 titer were injected into attached cotyledons and the plants were maintained at either 70% RH (A) or 98% RH (B). At various times after elicitor injection a single tissue disc was cut from each cotyledon and floated on water (4 discs/2.5 mL). Values are the mean conductivity readings taken 3 h after the discs were cut. A compatible interaction involved Cf0 (○), and incompatible interactions involved Cf2-Avr2 (●) and Cf9-Avr9 (▽). Vertical bars represent SES.

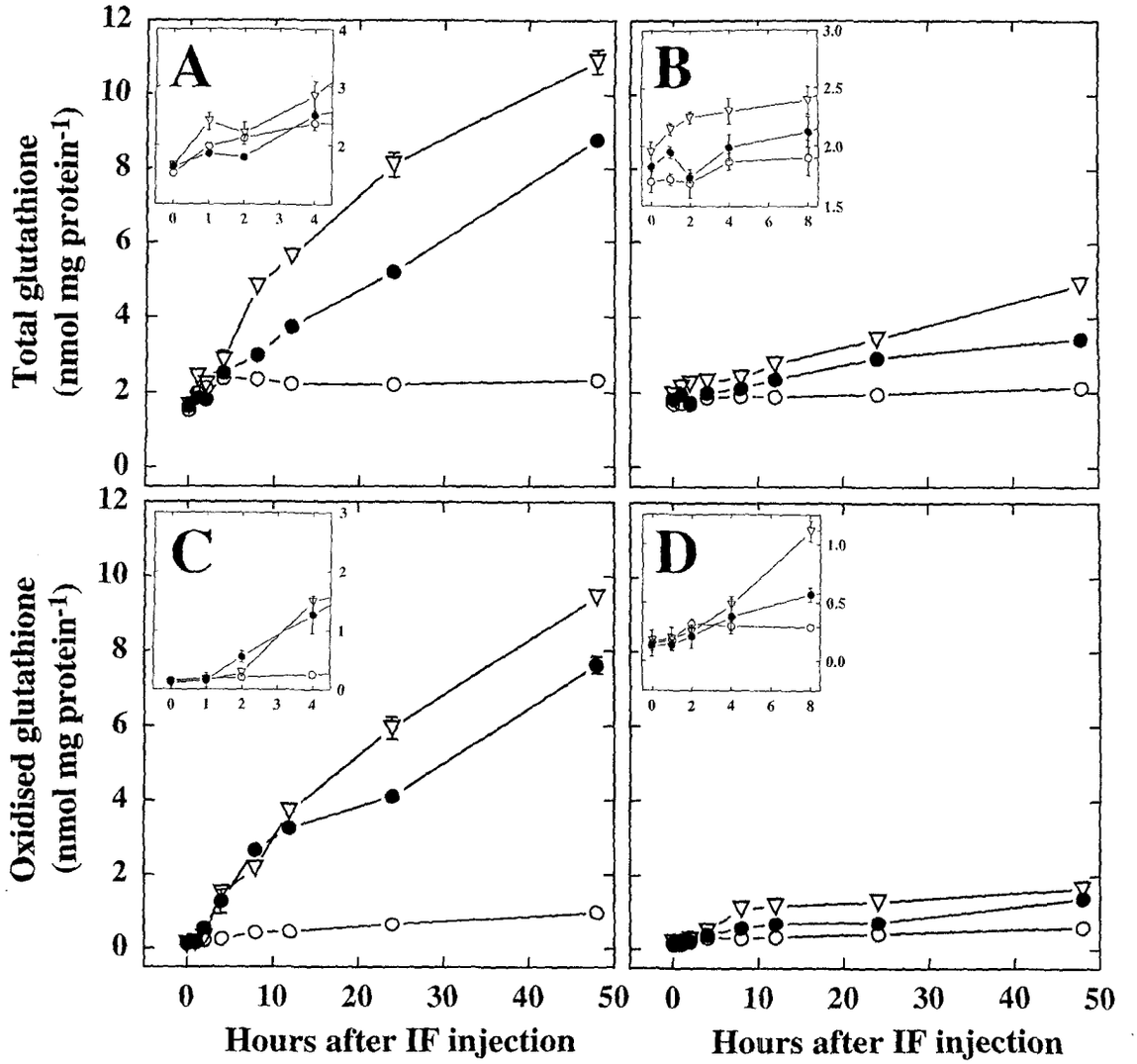

Figure 4. A comparison of the induction of total glutathione (A and B) and GSSG (C and D) in Cf0, Cf2, and Cf9 tomato cotyledons after the injection of a 1 in 2 titer of IF from race 0. A compatible interaction involved Cf0 (○), and incompatible interactions involved Cf2-Avr2 (●) and Cf9-Avr9 (▽). The plants were maintained at 70% RH after injection (A and C) or at 98% after injection (B and D). The inset in each graph is an expansion of the *y* axis over the earlier times and the vertical bars represent the SES.

The Effects of IF Dilution on *Cf-Avr*-Dependent Lipid Peroxidation and Glutathione Levels

The effects of IF dilution on lipid peroxidation and glutathione levels was investigated to determine the dose dependency and lower threshold level of IF challenge. Dilutions of 1 in 8 and 1 in 64 of the original IF extract were prepared and injected into the cotyledons of Cf0, Cf2, and Cf9 maintained at 70% RH. The increase in the level of TBARS and accumulation of total glutathione and GSSG were determined at 2, 4, 8 and 24 h (Figs. 5 and 6). Reducing the titer of IF to 1 in 8 had a dramatic effect on the appearance of TBARS (Fig. 5). In Cf2 TBARS were only measurable at 24 h and were reduced to 76% of the level resulting from injection of an IF titer of 1 in 2 (Fig. 5A). The reduction in the increase of TBARS in response to challenge with an IF titer of 1 in 8 in Cf9 was less marked and at 24 h 90% of the level obtained with IF at a 1 in 2 titer was evident (Fig. 5B). However, although TBARS levels were significantly increased above control levels in Cf9 plants at 4 to 8 h after challenge with IF at a dilution of 1 in 8 (110–133% of control levels), this is 2 to 4 h later than at a 1 in 2 titer. When the IF was diluted to 1 in 64 and injected into Cf2 cotyledons, IF-dependent TBARS accumulation was almost completely abolished. In contrast, significant increases in the level of TBARS in cotyledons of Cf9 plants challenged with the same dilution could still be measured, even at 8 h a.i., and at 24 h the increases were 57% higher than initial levels, only 33% lower than levels measured in response to challenge with a titer of 1 in 2.

The pronounced *Cf-Avr*-gene-dependent effects on lipid peroxidation as a function of IF titer were not reflected in

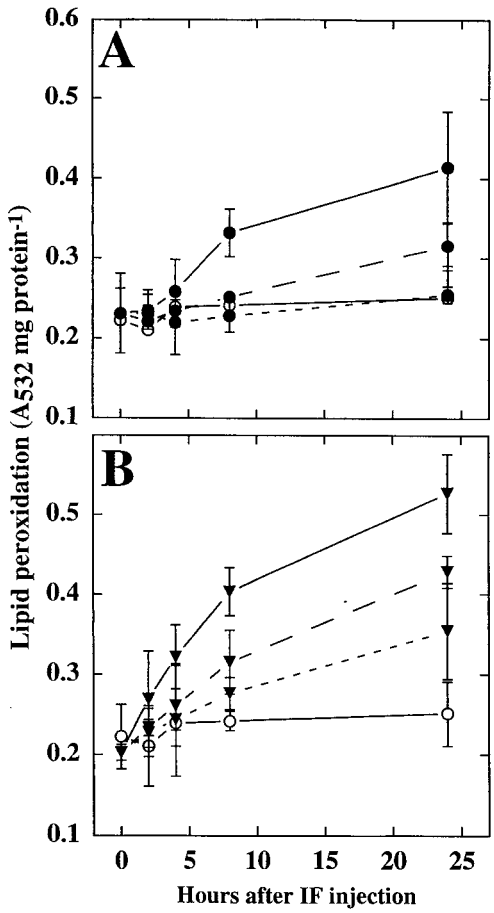

Figure 5. Effect of reducing the titer of the IF on the degree of lipid peroxidation induced in Cf2 (A) and Cf9 (B) tomato cotyledons. The IF titers injected were 1 in 2 (solid lines), 1 in 8 (long dashed lines), and 1 in 64 (short dashed lines). O, Lipid peroxidation levels induced in Cf0 cotyledons after challenge with IF at a 1 in 2 titer. The plants were maintained at 70% RH after IF injection. Vertical bars represent the SES.

changes in the accumulation of glutathione in response to the same treatments (Fig. 6, A and B). Significant increases in the level of total glutathione in response to challenge with an IF titer of 1 in 8 were measurable in both Cf2 and Cf9 8 h a.i. and were not drastically reduced compared to challenge with an IF titer of 1 in 2. At 24 h a.i., dilution of the IF from 1 in 2 to 1 in 8 resulted in a 38 and 20% reduction in the accumulation of total glutathione in Cf2 and Cf9, respectively. Dilution of the IF to 1 in 64 resulted in a delayed onset of total glutathione accumulation to 24 h a.i., whereas in Cf9 the same treatment resulted in measurable accumulation of total glutathione at 8 h. Nevertheless, despite the measurable increase in total glutathione in Cf9 cotyledons in response to challenge with an IF dilution of 1 in 64, this increase was only 64% of the increase in response to 1 in 8 dilution and 51% of the response to 1 in 2 dilution.

The ratio of GSSG:GSH in the *Cf*-containing lines also showed IF dose dependence (Fig. 6, C and D). GSSG was only measurable in extracts of Cf2 cotyledons 24 h a.i. of a 1 in 64 dilution of IF and represented only 41% of total glutathione. At a 1 in 8 dilution titer, increases in GSSG were detectable after 4 h and represented 78% of total glutathione after 24 h. In Cf9 cotyledons an increase in GSSG occurred by 4 h using a 1 in 8 dilution titer and after 8 h using the 1 in 64 dilution titer. After 24 h GSSG represented more than 50% of the total glutathione in Cf9 cotyledons injected with either concentration of IF.

Effects of IF on the Activities of Antioxidant Enzymes and Levels of LOX

It was previously demonstrated that in response to pathogen infection marked increases in the activity of specific SOD isoforms and in the transcription of their corresponding genes occurs (Buonaurio et al., 1987; Bowler et al., 1988). These events are of potential significance in the present investigation, since events leading to the formation of ROIs occur very shortly after IF challenge. We therefore examined qualitatively changes in the activity of SOD enzymes using nondenaturing polyacrylamide activity gels. No gross changes in the activity of any of the SOD enzymes (EC 1.15.1.1) was observed by this method (data not shown). Using activity staining in nondenaturing polyacrylamide gels we also investigated changes in the activity of CAT isoenzymes (EC 1.11.1.6). Again, no gross changes in the activity of CAT was observed by this method (data not shown).

The induction of transcription of LOX genes (Melan et al., 1993) and corresponding increases in the activity of these enzymes have been documented and roles for LOX in resistance against pathogens have been proposed (Hildebrand, 1989). These changes may play an important role in the release of specific lipid peroxide molecules, which are subsequently converted into bioactive molecules by a series of enzymatic steps (Vick and Zimmerman, 1987). These molecules have a wide variety of functions (Serhan et al., 1981; Samuelsson, et al., 1987; Farmer and Ryan, 1992). LOX activity has been shown to be induced by pathogens (Peever and Higgins, 1989; Koch et al., 1992; Melan et al., 1993), and the subsequent products of LOX activity have been shown to possess antibacterial (Croft et al., 1993) and signal transduction properties (Melan et al., 1993). Changes in LOX activity were found to increase in response to injection of IF, and this increase was found to be *Cf-Avr* gene dependent (Fig. 7A), dependent on the humidity of the incubation environment (Fig. 7B) and also on the dose of IF applied (Fig. 8). Injection of IF at titers of 1 in 2 and 1 in 8 into the cotyledons of Cf2 and Cf9 induced an increase in the activity of LOX detectable 8 h a.i., and LOX continued to increase thereafter. However, in the case of Cf9 treatments at 48 h, the extractable LOX activity was highly variable. These data are thought to reflect the fact that cotyledons at this stage were almost uniformly necrotic and inviable (Hammond-Kosack et al., 1996). In the absence of *Cf* genes, there was a slight increase in LOX activity by 24 h a.i. (Cf0, Fig. 7A). Dilution of the IF titer to 1 in 8 and 1 in 64 had a marked effect on the induction of LOX activity in Cf2 and to a lesser extent on Cf9 (Fig. 8). Twenty-four hours after injection of a 1 in 64 dilution of IF

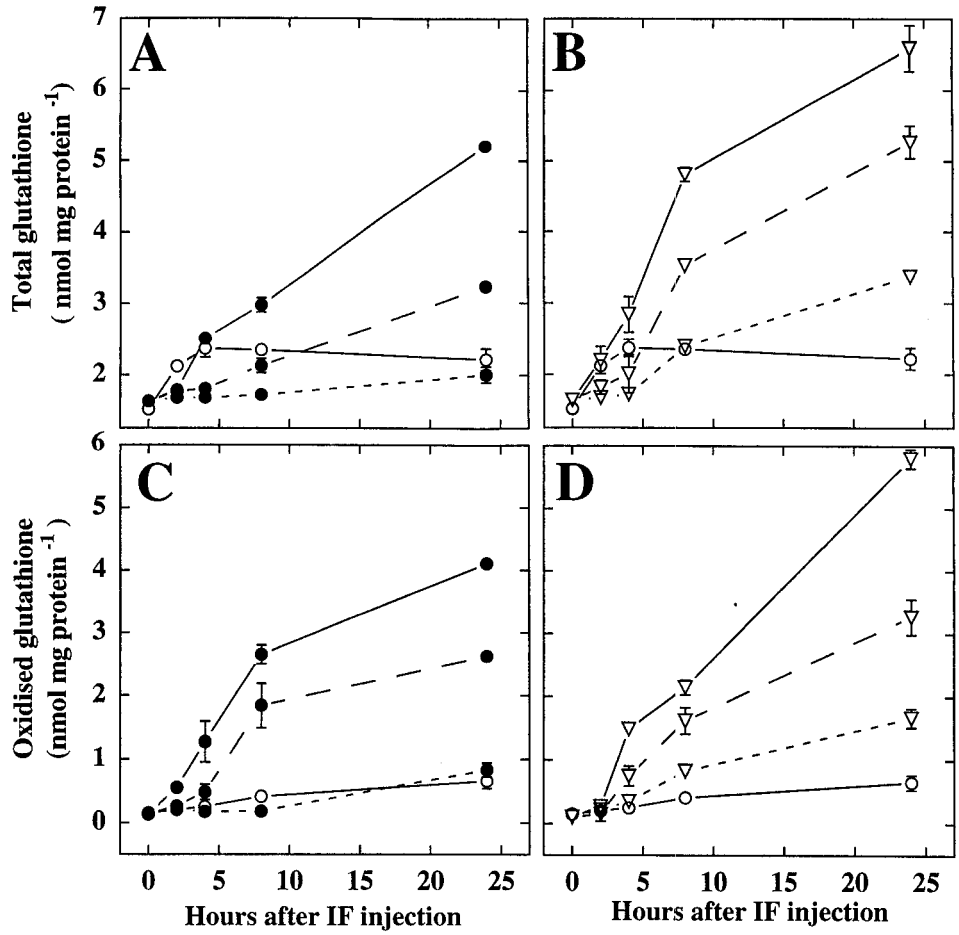

Figure 6. Effect of reducing the titer of the IF on the induction of total glutathione (A and B) and GSSG (C and D) in Cf2 (A and C) and Cf9 (B and D) tomato cotyledons. The IF titers injected were 1 in 2 (solid lines), 1 in 8 (long dashed lines), and 1 in 64 (short dashed lines). ○, Total glutathione levels induced in Cf0 cotyledons after challenge with IF at a 1 in 2 titer. The plants were maintained at 70% RH after IF injection. Vertical bars represent the SES.

into Cf2, the activity of LOX was 36% of the activity measured after injection of a 1 in 2 dilution. At this IF titer no significant increase in any of the other parameters measured was detected for Cf2 plants. A drastic delay in LOX induction occurred when plants injected with a 1 in 2 dilution of IF were subsequently incubated at 98% RH (Fig. 7B). No increases in the activity of LOX were measured before 24 h in Cf2 and Cf9 under these conditions and the activities were 62 and 12% lower, respectively. However, by 48 h the LOX activity was comparable in the two humidity regimes for both incompatible interactions. It is interesting that the activity of LOX in Cf0 plants treated in the same way and incubated in the same sealed chamber were significantly increased at 48 h (200% activity at 70% RH). This may be a reflection of the fact that all plants were incubated together and the evolution of volatile products from Cf2 and Cf9 plants in which LOX activity had been induced may have had an inducing effect on the activity of LOX in Cf0.

DISCUSSION

Three tomato NILs carrying either the resistance genes *Cf-2* or *Cf-9* or no *Cf* gene were infiltrated with *C. fulvum* race-specific elicitors and their responses were compared. Our objective was to define the temporal order of *Cf*-dependent biochemical events activated immediately after *Avr*-gene product recognition. The uniform genetic background of the NILs allowed a rigorous assessment of the *Cf-Avr* dependency of the biochemical responses and the subsequent physiological consequences of these events. By using the IF and not *C. fulvum* infections as the challenge, we could ascertain the dose dependency of each activated biochemical response. Markers for oxidative stress were particularly interesting, since a large body of evidence indicates that the formation of ROIs is one of the earliest measurable consequences of pathogen attack and may be involved in subsequent defense signaling (Levine et al., 1994; Mehdy, 1994; Yalpani et al., 1994; Baker and Orlandi, 1995). The onset of each response was both *Cf-Avr* gene dependent and IF dose dependent, whereas high humidity reduced the magnitude of the responses.

The earliest event occurring 2 or 4 h after IF injection in incompatible (Cf9 and Cf2, respectively, Fig. 1) but not the compatible interaction (Cf0) was the formation of superoxide anions ($O_2^{\cdot-}$). This indicates the rapid *Cf-Avr*-gene-

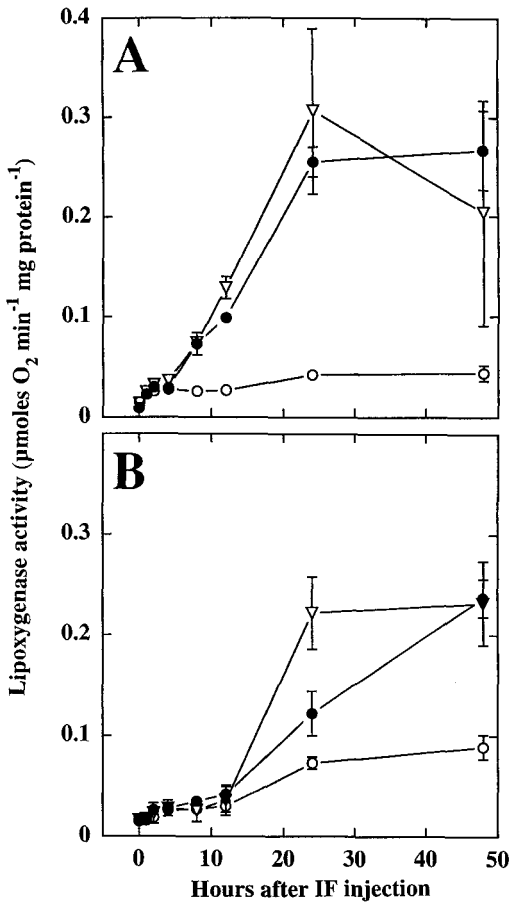

Figure 7. LOX activity in extracts from Cf0, Cf2, and Cf9 tomato cotyledons after injection with a 1 in 2 titer of *C. fulvum* race-specific IF prepared from race 0. The plants were maintained at either 70% RH (A) or 98% RH (B). The compatible interaction involved Cf0 (○), and incompatible interactions involved Cf2-Avr2 (●) and Cf9-Avr9 (▽). Vertical bars represent SES.

dependent induction of ROI synthesis. Similar observations have previously been made using a Cf5 tomato cell-suspension culture after challenge with an Avr5-containing *C. fulvum* elicitor preparation (Vera-Estrella et al., 1992). However, the slow and prolonged kinetics and dose dependency of this response in the whole-plant system are different from the rapid and transient responses previously measured in tomato cell-suspension cultures (Vera-Estrella et al., 1992, 1994a, 1994b) and soybean and bean suspension cultures in response to a fungal glucan and/or oligogalacturonide elicitor (Apostol et al., 1989; Legendre et al., 1993; Levine et al., 1994). The initial flooding of the cotyledon air spaces when the IF was injected may have altered the physiological status of the plant cells and the oxygen availability, delaying the onset of the oxidative burst. Alternatively, the superoxide could be diluted or rapidly dismuted in IF. Doke and Ohashi (1988) observed in a whole-plant system that the *N*-gene-mediated oxidative burst following tobacco mosaic virus infection was detected within minutes of the *N*-gene-mediated resistance response being activated by a temperature shift from 30 to 24°C. During *C. fulvum* ingress into tomato leaves the oxidative burst may be transient because only a few cells surrounding the penetrated substomatal cavity would come into contact with the Avr elicitor (Lazarovits and Higgins, 1976; de Wit, 1977; Hammond-Kosack and Jones, 1994). After IF infiltration, however, most cells would come into contact with the elicitor, allowing a more prolonged net $O_2^{\cdot-}$ formation.

A second documented consequence of *Cf-Avr*-gene-dependent elicitation of an oxidative burst in tomato cell-suspension cultures was the accumulation of the products of lipid peroxidation (Vera-Estrella et al., 1992). The *Cf-Avr*-gene-dependent induction of lipid peroxidation in tomato cotyledons was rapid and both IF dose and humidity dependent. A direct consequence of the peroxidation of lipids is structural damage to membranes. It has been proposed that the peroxidation of lipids is a key process in membrane alteration that underlies the symptoms of the hypersensitive response (Keppler and Novacky, 1986). Indeed, the measurement of *Cf-Avr*-gene-dependent increases in the level of TBARS precedes increases in solute leakage.

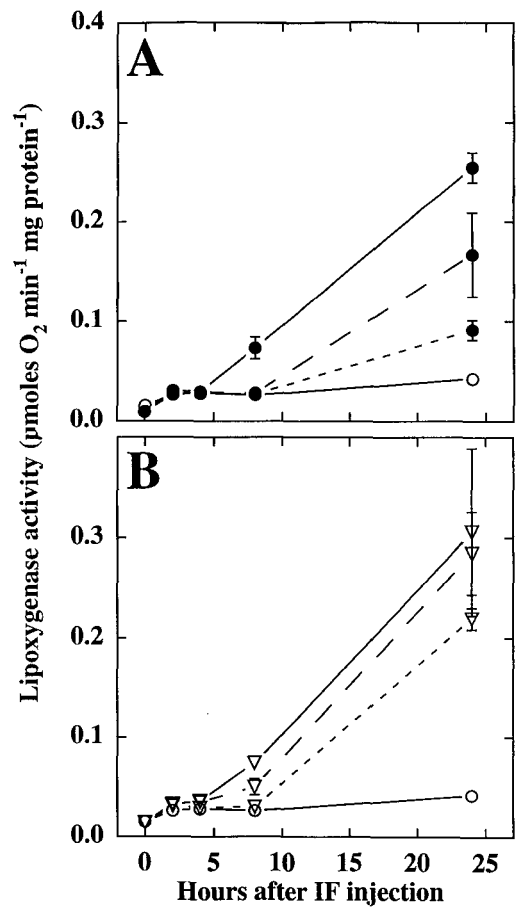

Figure 8. Effect of reducing the titer of the IF on the induction of LOX activity in incompatible interactions involving Cf2-Avr2 (A) and Cf9-Avr9 (B). The IF titers injected were 1 in 2 (solid lines), 1 in 8 (long dashed lines), and 1 in 64 (short dashed lines). ○, LOX activity induced in Cf0 cotyledons after challenge with IF at a 1 in 2 titer. The plants were maintained at 70% RH after IF injection. Vertical bars represent the SES.

Both lines of evidence support the hypothesis that Cf-protein activation has a profound impact on the loss of cellular homeostasis of Avr-elicitor-treated tissues containing the necessary *Cf* gene. These changes in turn preceded changes in cell viability (Hammond-Kosack et al., 1996) and are likely to play an important role in cell death. The whole-plant system used in this study allows correlations to be made between the effect of early responses to *Cf*-gene action (superoxide generation) on the physiology of the host. The increases in the levels of superoxide are likely to be responsible for the concomitant increases in lipid peroxidation, because increased LOX activity was not evident until later. Because the generation of superoxide was sustained, this radical may also contribute to solute leakage and to eventual cell death.

The generation of ROIs at the site of entry of an avirulent pathogen may play several important roles in prevention of pathogen spread but also has important consequences for the host plant. A role for H_2O_2 as an antimicrobial agent (Kim et al., 1988; Wu et al., 1995), a substrate for lignin synthesis (Gross et al., 1977), and a signal molecule for the hypersensitive cell death response (Levine et al., 1994), phytoalexin accumulation (Edwards et al., 1991; Guo et al., 1993), and SA biosynthesis (Leon et al., 1995) has already been demonstrated. But ROI levels must be controlled to avoid damage to the surrounding noninvaded tissue whose viability may be required for the coordinated defense response.

Coincident with *Cf*-gene-dependent/Avr-dose-dependent changes in lipid peroxidation, there were parallel increases in the level of total glutathione and a marked increase in the ratio of GSSG to GSH. We previously demonstrated that oxidative stress induces accumulation of glutathione (May and Leaver, 1993), and this study provides a further example. It may emerge that a general feature of effective disease resistance will be a mobilization of GSH and GSH-dependent detoxification processes at the sites of incompatible infections, to save plant cells from the consequences of their own defense mechanisms. Edwards et al. (1991) also observed a net accumulation of GSH in bean cells after challenging with a pathogen elicitor. GSH itself or alterations in GSH/GSSG redox balance may function in cellular protection in healthy tissue but also in the regulation of plant defense genes (Wingate et al., 1988; Zhang and Mehdy, 1994) and genes involved in the removal of ROI (Hérouart et al., 1993). However, an Arabidopsis mutant *cad2–1* with a total glutathione content of only 30% of the wild-type level still exhibited an almost normal resistance phenotype to the fungus *Peronospora parasitica* and a strain of bacterium *Pseudomonas syringae* pv *tomato* expressing *AvrB*. Since incompatibility in the *cad2–1* mutant resulted in a protracted stimulation of GSH synthesis, whereas in wild-type plants total GSH were only slightly elevated, this suggests that normal GSH levels were already sufficient to quench the induced oxidative stress (May et al., 1996).

Alterations to the ratio of GSSG:GSH (Alscher, 1989) in the IF-challenged tissue and their consequent changes in cellular redox status may be more important than absolute glutathione increases on the overall responses activated in the *Cf*-expressing plant cells. Causative roles for redox changes in the regulation of redox-sensitive enzymes (Ziegler, 1985), receptors (Malbon et al., 1987), signal transduction (Huang et al., 1994; Okazaki et al., 1994), cellular proliferation (Biguet et al., 1994), RNA stability (Zhang and Mehdy, 1994), and transcription factors (Schreck et al., 1991; Babiychuk et al., 1994; Westendorp et al., 1995) have already been proven. It is likely that a tightly controlled and delicate balance between the accumulation of ROIs and antioxidant activity will determine the full expression of the effective defense response.

The increase in LOX enzyme activity at 8 h in both incompatible interactions at low humidity indicates that a significant part of the lipid peroxidation detectable during the latter part of the time course was a consequence of both enzymatic and free radical damage. However, since the elevated levels of LOX activity from 12 h onward, in combination with the earlier oxidative burst, did not cause an overall increase in lipid peroxidation at high humidity, it can be concluded that cellular protectant mechanisms must be continuously active and effective in this situation.

The primary products of LOX-catalyzed reactions, fatty acid hydroperoxides, are metabolized into molecules with known or hypothesized regulatory activities. For plants these include traumatin and jasmonic acid, which may affect a variety of physiological processes (Siedow, 1991). For example, they induce defense-related genes, including those encoding Phe ammonia lyase (Grunlach et al., 1992), protease inhibitors (Farmer and Ryan, 1992), chalcone synthase and Pro-rich wall proteins (Creelman et al., 1992), and LOX (Bell and Mullet, 1993; Croft et al., 1993). Thus, the *Cf-Avr*-dependent increases in LOX activity may serve to provide the substrate for the synthesis of additional signaling molecules that coordinate and amplify the defense response to *C. fulvum*. Alternatively, the increase in LOX activity may result in a localized increase in volatile fatty acid products that may themselves possess antimicrobial activity, as revealed in other host-microbe interactions (Croft et al., 1993). The types of volatile fatty acids produced by tomato plants and their toxicity toward *C. fulvum* hyphae are not known.

High humidity dramatically reduced the onset and eventual magnitude of lipid peroxidation and changes to the glutathione pool but only altered the kinetics of increased LOX enzyme activity. For the challenged Cf9 plants, the effect of higher humidity can probably best be explained by the elimination of the desiccation process associated with the induced supraoptimal opening of stomata, reported by Hammond-Kosack et al. (1996). However, for Cf2 plants alternative explanations are required because of the lack of elicitor-induced stomatal opening. Possibly the increased hydration status of plant tissue results in a reduced concentration of ROIs at the plasma membrane surface that could participate in subsequent chemical reactions or enter the cell (H_2O_2 only). By analogy, the increased water content may enhance the possibility of contact with cell-wall-bound peroxidases and this could lower H_2O_2 concentrations at the cell surface. An increased water content would

also dilute solute and ion concentrations outside the plant cell. Neither $O_2^{\cdot -}$ nor H_2O_2 is energetic enough to initiate lipid peroxidation directly but needs to participate in the Fenton reaction to generate the hydroxyl radical, which is a powerful initiator of lipid peroxidation (Halliwell and Gutteridge, 1989, 1990). A reduction in the availability of transition metal catalysts (e.g. Fe^{2+}) outside the plant cell could significantly reduce the overall production of $OH^{\cdot}$, thereby decreasing the degree of lipid peroxidation and hence a requirement for the activation of cellular protection mechanisms based on glutathione. The effects of various chelating agents on modulating the IF-induced responses will be interesting to test in this context. The high-humidity data also indicate that a given reduction in cell viability is not associated with a specific change to the glutathione pool. Thus, changes in redox status alone are unlikely to induce cell death. Finally, because the eventual magnitude of LOX activity was unaffected by humidity changes, a distinct signaling cascade probably activates this response.

These studies support the use of a whole-plant-elicitor model in the analysis of plant-pathogen interactions. It is plausible that because the IF preparation injected was only partially purified other components in the solution in addition to the Avr peptides may also have contributed to or modified the *Cf*-gene-specific responses observed. Furthermore, although this study demonstrates that various oxidative events occurred subsequent to *Cf*-gene action, the magnitude of changes in an authentic fungal interaction may be different. Clearly, cell-specific markers for oxidative stress and methods for in vivo GSH determination are necessary to define changes in cells surrounding the site of pathogen ingress. Given the availability of NILs, the extensive genetic map of tomato, and the feasibility of a mutagenesis approach, the *C. fulvum*-tomato interaction offers many advantages for the analysis of resistance gene function. To this end we have already identified two genes that are required, in addition to the *Cf-9* gene, for the establishment of effective resistance (Hammond-Kosack et al., 1994b). Saturation mutagenesis will eventually reveal the identity of other genes involved in *Cf*-gene-mediated resistance. The concerted deployment of genetic, biochemical, and molecular techniques will be necessary to reveal how *Cf*-gene products activate effective defense upon *Avr*-gene product recognition. This point is further elaborated by Hammond-Kosack et al. (1996).

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LITERATURE CITED

Alscher RG (1989) Biosynthesis and antioxidant function of glutathione in plants. Physiol Plant **77:** 457–464

Apostol I, Heinstein PF, Low PS (1989) Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. Plant Physiol **90:** 109–116

Babiychuk E, Kushnir S, Van Montagu M, Inzé D (1994) The *Arabidopsis thaliana* apurinic endonuclease Arp reduces human transcription factors Fos and Jun. Proc Natl Acad Sci USA **91:** 3299–3303

Baker CJ, Orlandi EW (1995) Active oxygen in plant pathogenesis. Annu Rev Phytopathol **33:** 299–321

Bell E, Mullet JE (1993) Characterization of an *Arabidopsis* lipoxygenase gene responsive to methyl jasmonate and wounding. Plant Physiol **103:** 1133–1137

Biguet C, Wakasugi N, Mishal Z, Holmgren A, Chouaib S, Tursz T, Wakasugi H (1994) Thioredoxin increases the proliferation of human B-cell lines through a protein kinase C-dependent mechanism. J Biol Chem **269:** 28865–28870

Bond TET (1938) Infection experiments with *Cladosporium fulvum* Cooke and related species. Ann App Biol **25:** 277–307

Bowler CJ, Alliotte T, DeLoose M, Van Montagu M, Inzé D (1988) The induction of manganese superoxide dismutase in response to stress in *Nicotiana plumbaginifolia*. EMBO J **8:** 31–38

Bowles DJ (1990) Defense-related proteins in higher plants. Annu Rev Biochem **59:** 873–907

Bradley DJ, Kjellbom P, Lamb CJ (1992) Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. Cell **70:** 21–30

Brisson LF, Tenhaken R, Lamb C (1994) Function of oxidative cross-linking of cell wall structural proteins in plant disease resistance. Plant Cell **6:** 1703–1712

Buonaurio R, Della Torre G, Montalbini P (1987) Soluble superoxide dismutase (SOD) in susceptible and resistant host-parasite complexes of *Phaseolus vulgaris* and *Uromyces phaseoli*. Physiol Mol Plant Pathol **31:** 173–184

Christopher J, Pistorius E, Axelrod B (1970) Isolation of an isozyme of soybean lipoxygenase. Biochim Biophys Acta **198:** 12–19

Creelman RA, Tierney ML, Mullet JE (1992) Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. Proc Natl Acad Sci USA **89:** 4938–4941

Croft KPC, Juttner F, Slusarenko AJ (1993) Volatile products of the lipoxygenase pathway evolved from *Phaseolus vulgaris* (L.) leaves inoculated with *Pseudomonas syringae* pv *phaseolicola*. Plant Physiol **101:** 13–24

Delaney TP, Uknes S, Vernooij B, Friedrich L, Weymann K, Negrotto D, Gaffney T, Gut-Rella M, Kessmann H, Ward E, Ryals J (1994) A central role of salicylic acid in plant disease resistance. Science **266:** 1247–1250

de Wit PJGM (1977) A light and scanning-electron microscopic study of infection of tomato plants by virulent and avirulent races of *Cladosporium fulvum*. Neth J Plant Pathol **83:** 109–122

de Wit PJGM (1992) Molecular characterization of gene-for-gene systems in plant-fungus interactions and the application of avirulence genes in control of plant pathogens. Annu Rev Phytopathol **30:** 391–418

de Wit PJGM, Spikman G (1982) Evidence for the occurrence of race and cultivar-specific elicitors of necrosis in intercellular fluids of compatible interactions of *Cladosporium fulvum* and tomato. Physiol Plant Pathol **21:** 1–11

Dickinson MJ, Jones DA, Jones JDG (1993) Close linkage between the *Cf-2/Cf-5* and *Mi* resistance loci in tomato. Mol Plant-Microbe Interact **6:** 341–347

Dixon RA, Lamb CJ (1990) Molecular communication in interactions between plants and microbial pathogens. Annu Rev Plant Physiol Plant Mol Biol **41:** 339–367

Doke N, Ohashi Y (1988) Involvement of an $O_2^{\cdot -}$ generating system in the induction of necrotic lesions on tobacco leaves infected with tobacco mosaic virus. Physiol Mol Plant Pathol **32:** 163–175

Edwards R, Blount JW, Dixon RA (1991) Glutathione and elicitation of the phytoalexin response in legume cell cultures. Planta **184:** 403–409

Farmer EE, Ryan CA (1992) Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. Plant Cell **4:** 129–134

Flór HH (1946) Genetics of pathogenicity in *Melampsora lini*. J Agric Res **73:** 335–357

Gabriel DW, Rolfe B (1990) Working models of specific recognition in plant-microbe interactions. Annu Rev Phytopathol **28:** 365–391

Gross GG, Janse C, Elstner EF (1977) Involvement of malate, monophenols, and the superoxide radical in hydrogen peroxide formation by isolated cell walls from horseradish (*Armoracia lapathifolia* Gilib.). Planta **136:** 271–276

Grunlach H, Muller MJ, Kutchan TM, Zenk MH (1992) Jasmonic acid is a signal transducer in elicitor-induced plant cell culture. Proc Natl Acad Sci USA **89:** 2389–2393

Guo Z-J, Nakagawara S, Sumitani K, Ohta Y (1993) Effects of intracellular glutathione levels on the production of 6-methoxymellein in cultured carrot (*Daucus carota*) cells. Plant Physiol **102:** 45–51

Gutteridge JMC, Halliwell B (1990) The measurement and mechanism of lipid peroxidation in biological systems. Trends Biochem **15:** 129–135

Halliwell B, Gutteridge JMC (1989) Free Radicals in Biology and Medicine, Ed 2. Clarendon, Oxford, UK

Halliwell B, Gutteridge JMC (1990) Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol **186:** 1–85

Hammond-Kosack KE, Harrison K, Jones JDG (1994a) Developmentally regulated cell death on expression of the fungal avirulence gene *Avr9* in tomato seedlings carrying the disease-resistance gene *Cf-9*. Proc Natl Acad Sci USA **91:** 10445–10449

Hammond-Kosack KE, Jones DA, Jones JDG (1994b) Identification of two genes required in tomato for full *Cf*-9-dependent resistance to *Cladosporium fulvum*. Plant Cell **6:** 361–374

Hammond-Kosack KE, Jones JDG (1994) Incomplete dominance of tomato *Cf* genes for resistance to *Cladosporium fulvum*. Mol Plant-Microbe Interact **7:** 58–70

Hammond-Kosack KE, Jones JDG (1995) Plant disease resistance genes: unravelling how they work. Can J Bot **73:** S495–S505

Hammond-Kosack KE, Silverman P, Raskin I, Jones JDG (1996) 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. Plant Physiol **110:** 1381–1394

Hérouart D, van Montagu M, Inzé D (1993) Redox-activated expression of the cytosolic copper/zinc superoxide dismutase gene in *Nicotiana*. Proc Natl Acad Sci USA **90:** 3108–3112

Hilderbrand DF (1989) Lipoxygenases. Physiol Plant **76:** 249–253

Huang LE, Zhang H, Bae SW, Liu AY-C (1994) Thiol reducing reagents inhibit the heat shock response. J Biol Chem **269:** 30718–30725

Jones DA, Dickinson MJ, Balint-Kurti PJ, Dixon MS, Jones JDG (1993) Two complex resistance loci revealed in tomato by classical and RFLP mapping of the *Cf-2*, *Cf-4*, *Cf-5* and *Cf-9* genes for resistance to *Cladosporium fulvum*. Mol Plant-Microbe Interact **6:** 348–357

Jones DA, Thomas CM, Hammond-Kosack KE, Balint-Kurti PJ, Jones JDG (1994) Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. Science **266:** 789–793

Keen NT (1990) Gene-for-gene complementarity in plant-pathogen interactions. Annu Rev Genet **24:** 447–463

Keppler LD, Novacky A (1986) Involvement of membrane lipid peroxidation in the development of bacterially induced hypersensitive reaction. Phytopathology **76:** 104–108

Kim KK, Fravel DR, Papavizas GC (1988) Identification of a metabolite produced by *Talaromyces flavus* as glucose oxidase and its role in the biocontrol of *Verticillium dahliae*. Phytopathology **78:** 488–492

Klement Z (1982) Hypersensitivity. *In* MS Mount, GH Lacy, eds, Phytopathogenic Procaryotes. Academic Press, New York, pp 149–177

Koch E, Meier BM, Eiben H-G, Slusarenko A (1992) A lipoxygenase from leaves of tomato (*Lycopersicon esculentum* Mill.) is induced in response to plant pathogenic Pseudomonads. Plant Physiol **99:** 571–576

Lamb CJ, Lawton MA, Dron M, Dixon RA (1989) Signals and transduction mechanisms for activation of plant defences against microbial attack. Cell **56:** 215–224

Lazarovits GH, Higgins VJ (1976) Histological comparison of *Cladosporium fulvum* race 1 on immune, resistant, and susceptible tomato varieties. Can J Bot **54:** 224–233

Legendre L, Rueter S, Heinstein PF, Low PS (1993) Characterization of the oligogalacturonide-induced oxidative burst in cultured soybean (*Glycine max*) cells. Plant Physiol **102:** 233–240

Leon J, Lawton MA, Raskin I (1995) Hydrogen peroxide stimulates salicylic acid biosynthesis in tobacco. Plant Physiol **108:** 1673–1678

Levine AL, Tenhaken R, Dixon R, Lamb C (1994) H_2O_2 from the oxidative burst orchestrates the hypersensitive disease resistance response. Cell **79:** 583–593

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem **193:** 265–275

Malbon CC, George ST, Moxham CP (1987) Intramolecular disulfide bridges: avenues to receptor activation. Trends Biochem Sci **12:** 172–175

May MJ, Leaver CJ (1993) Oxidative stimulation of glutathione synthesis in *Arabidopsis thaliana* suspension cultures. Plant Physiol **103:** 621–627

May MJ, Parker JE, Daniels MJ, Leaver CJ, Cobbett CS (1996) An Arabidopsis mutant depleted in glutathione shows unaltered responses to fungal and bacterial pathogens. Mol Plant-Microbe Interact (in press)

Mehdy MC (1994) Active oxygen species in plant defense against pathogens. Plant Physiol **105:** 467–472

Melan MA, Dong X, Endara ME, Davis KR, Ausubel FM, Peterman TK (1993) An *Arabidopsis thaliana* lipoxygenase gene can be induced by pathogens, abscisic acid, and methyl jasmonate. Plant Physiol **101:** 441–450

Okazaki T, Chung U-I, Nishishita T, Ebisu S, Usuda S, Mishiro S, Xanthoudakis S, Igarashi T, Ogata E (1994) A redox factor protein, ref1, is involved in negative gene regulation by extracellular calcium. J Biol Chem **269:** 27855–27862

Oteiza PI, Bechara EJH (1993) 5-Aminolevulinic acid induces lipid peroxidation in cardislipin-rich liposomes. Arch Bioch Biophys **305:** 282–287

Peever TL, Higgins VJ (1989) Electrolyte leakage, lipoxygenase, and lipid peroxidation induced in tomato leaf tissue by specific and nonspecific elicitors from *Cladosporium fulvum*. Plant Physiol **90:** 867–875

Peterson GL (1977) A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. Anal Biochem **83:** 346–356

Raskin I (1992) Role of salicylic acid in plants. Annu Rev Plant Physiol Plant Mol Biol **43:** 439–463

Samuelsson B, Dahlen S-E, Lindgren JA, Rouzer CA, Serhan CN (1987) Leukotrienes and lipoxins: structures, biosynthesis, and biological effects. Science **237:** 1171–1176

Schreck R, Rieber P, Baeuerle PA (1991) Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κb transcription factor and HIV-1. EMBO J **10:** 2247–2258

Serhan C, Anderson P, Goodman E, Dunham P, Weissmann G (1981) Phosphatidate and oxidized fatty acids are calcium ionophores. J Biol Chem **256:** 2736–2741

Siedow JN (1991) Plant lipoxygenases: structure and function. Annu Rev Plant Physiol Plant Mol Biol **42:** 145–188

Smith IK (1985) Stimulation of glutathione synthesis in photorespiring plants by catalase inhibitors. Plant Physiol **79:** 1044–1047

Stevens MA, Rick CM (1988) Genetics and breeding. *In* JG Atherton, J Rudich, eds. The Tomato Crop. Chapman and Hall, London, pp 35–109

Sutherland MW (1991) The generation of oxygen radicals during host plant responses to infection. Physiol Mol Plant Pathol **39:** 79–93

Tigchelaar EC (1984) Collections of isogenic tomato stocks. Rep Tomato Genet Coop **34:** 55–57

Vera-Estrella R, Barkla BJ, Higgins VJ, Blumwald E (1994a) Plant defense response to fungal pathogens. I. Activation of host-plasma membrane H^+-ATPase by elicitor-induced enzyme dephosphorylation. Plant Physiol **104:** 209–215

Vera-Estrella R, Blumwald E, Higgins VJ (1992) Effect of specific elicitors of *Cladosporium fulvum* on tomato suspension cells. Plant Physiol **99:** 1208–1215

Vera-Estrella R, Higgins VJ, Blumwald E (1994b) Plant defense response to fungal pathogens. II. G-protein-mediated changes in host plasma membrane redox reactions. Plant Physiol **106:** 97–102

Vick BA, Zimmerman DC (1987) The lipoxygenase pathway. *In* PK Stumpf, JB Mudd, WD Nes, eds, The Metabolism, Structure and Function of Plant Lipids. Plenum, New York, pp 383–390

Westendorp MO, Shatrov VA, Schulze-Osthoff K, Frank R, Kraft M, Los M, Krammer PH, Droge W, Lehmann V (1995) HIV-1 Tat potentiates TNF-induced NF-κB activation and cytotoxicity by altering the cellular redox state. EMBO J **14:** 546–554

Wingate VPM, Lawton MA, Lamb CJ (1988) Glutathione causes a massive and selective induction of plant defense genes. Plant Physiol **87:** 206–210

Wu G, Shortt J, Lawrence EB, Levine EB, Fitzsimmons KC, Shah DM (1995) Disease resistance conferred by expression of a gene encoding H_2O_2-generating glucose oxidase in transgenic potato plants. Plant Cell **7:** 1357–1368

Yalpani N, Enyedi AJ, Leon J, Raskin I (1994) UV light and ozone stimulate accumulation of salicylic acid, pathogenesis-related proteins and virus resistance in tobacco. Planta **193:** 372–376

Zhang S, Mehdy MC (1994) Binding of a 50-kD protein to a U-rich sequence in an mRNA encoding a proline-rich protein that is destabilized by a fungal elicitor. Plant Cell **6:** 135–145

Ziegler DM (1985) Role of reversible oxidation-reduction of enzyme thiols-disulfides in metabolic regulation. Annu Rev Biochem **54:** 305–329