Plant Defense Response to Fungal Pathogens

II. G-Protein-Mediated Changes in Host Plasma Membrane Redox Reactions

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Elicitor preparations containing the avr5 gene products from races 4 and 2.3 of Cladosporium fulvum, and tomato (Lycopersicon esculentum L.) cells containing the resistance gene Cf5 were used to investigate the involvement of redox processes in the production of active oxygen species associated with the plant response to the fungal elicitors. Here we demonstrate that certain race-specific elicitors of C. fulvum induced an increase in ferricyanide reduction in enriched plasma membrane fractions of tomato cells. The addition of elicitors to plasma membranes also induced increases in NADH oxidase and NADH-dependent cytochrome c reductase activities, whereas ascorbate peroxidase activity was decreased. These results suggest that changes in the host plasma membrane redox processes, transferring electrons from reducing agents to oxygen, could be involved in the increased production of active oxygen species by the race-specific elicitors. Our results also show that the dephosphorylation of enzymes involved in redox reactions is responsible for the race-specific induced redox activity. The effects of guanidine nucleotide analogs and mastoparan on the activation of plasma membrane redox reactions support the role of GTP-binding proteins in the transduction of signals leading to the activation of the defense response mechanisms of tomato against fungal pathogens.

The association of active oxygen species with the induction of defense responses to plant pathogens has been demonstrated in numerous plant-pathogen interactions (Sutherland, 1991). The origin of active oxygen species, particularly the superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2), has been linked to redox changes at the plasma membrane (Crane and Barr, 1989; Rubinstein and Luster, 1993). In plants, NADH or NAD(P)H, used as sources of reducing equivalents, can react with oxidoreductases localized on both sides of the plasma membrane (Rubinstein and Luster, 1993). The involvement of NADH oxidation in the generation of active oxygen species and H_2O_2 has been established in different plant systems (Elstner, 1982; Muller and Lin, 1986). In radish plasma membrane vesicles, a Cyt c oxidoreductase and a NADPH oxidase were reported to transfer electrons across the plasma membrane from ascorbate to ferricyanide, generating superoxide anions (Vianello and Macri, 1989). Plasma membrane-bound NAD(P)H oxidase was suggested to generate superoxide radicals upon infection of potato tubers with incompatible races of Phytophthora infestans (Doke, 1985).

Changes in active oxygen species in pathogen-challenged plant tissue might also be affected by host antioxidant systems. In plants, the principle antioxidizing enzymes at the plasma membrane/cytosolic level are catalase and superoxide dismutase, which react with active forms of oxygen, and ascorbate peroxidase and glutathione reductase, which regenerate oxidized antioxidants. Ascorbate-specific peroxidase activity, together with glutathione reductase and dehydroascorbate reductase, remove H_2O_2 through a mechanism known as the Halliwell-Asada or ascorbate-GSH pathway (Nakano and Asada, 1981). In this pathway, ascorbate is oxidized to monodehydroascorbate, then ascorbate is regenerated in a glutathione (GSH)-dependent reaction catalyzed by dehydroascorbate reductase. Finally, the GSSG is reduced back to GSH in a reaction that involves glutathione reductase and NADH. Although this antioxidant pathway is best documented in the chloroplast, the presence of the necessary substrate and enzymes in other subcellular compartments indicates its activity elsewhere in the plant cell (Dalton et al., 1986). Changes in enzyme activities involved in this pathway could also be involved in the increased production of active oxygen species associated with plant defense responses against pathogens.

We previously reported an increase in active oxygen species and extracellular peroxidase activity upon incubation of tomato (Lycopersicon esculentum) cell suspensions with one race-specific elicitor, the putative product of the avirulence gene avr5 of the fungus Cladosporium fulvum (Vera-Estrella et al., 1992). This observed increase in active oxygen species suggested that changes in the redox processes at the plasma membrane may be involved in the plant defense response against pathogens.

Here we demonstrate that enriched plasma membrane fractions from a tomato cell line near-isogenic for the gene Cf5 treated with a race-specific elicitor of C. fulvum showed increased redox activity associated with changes in NADH oxidase, NADH-dependent Cyt c reductase, ascorbate per-
oxidase, and glutathione reductase activities. Since an elicitor-induced increase in ATPase activity was activated by G protein and involved enzyme dephosphorylation (Vera-Estrella et al., 1994), we also examine the possibility that the observed plasma membrane-bound redox components were under the same type of control, i.e., that the enzyme(s) are being dephosphorylated through the action of G proteins.

MATERIALS AND METHODS

Plant Material

Cell suspensions derived from a line of tomato (Lycopersicon esculentum L. cv Moneymaker) with the resistance gene C5/ were grown in 500-mL Erlenmeyer flasks containing 100 mL of Murashige and Skoog medium in the dark at 25°C on a rotary shaker at 120 rpm and subcultured weekly (Vera-Estrella et al., 1992). Cell suspensions used for all experiments were 3 to 4 d old.

Production of Specific Elicitors

IFs from tomato leaf tissue infected with Cladosporium fulvum were prepared according to the method of De Wit and Spikman (1982) with C5 incompatible races 2.3 or 4 and compatible races 2.4.5. 2.4.5.9. or 5 inoculated onto the cv Bonny Best (no known Cf genes). Control IF was obtained from uninoculated plants incubated under the same conditions as the inoculated plants. The IFs were precipitated with acetone (90%) and the pellet was then freeze-dried, suspended in distilled water to give the original volume, and stored at -20°C. The semi-crude preparations produced in this manner for each race, referred to as race IF, still contain specific elicitors for each of the Cf genes on which the particular race of C. fulvum is avirulent (i.e., causes a hypersensitive response). Concentrations of IF used in experiments with tomato cell suspensions or with enriched plasma membrane fractions were based on the minimum dilution of IF that induced necrosis on leaves of C.f9 plants. Generally, a ratio of IF to water of 1:64 was used and contained between 1.25 and 1.40 μg protein/μL.

Isolation of Plasma Membranes

Plasma membrane-enriched fractions were isolated by homogenization of 4-d-old C5/ tomato cells as described previously (Vera-Estrella et al., 1994). Purity of the membranes (95%) was tested as described (Blumwald and Poole, 1987). The membrane protein concentration was approximately 10 μg/μL suspension medium.

Quantification of Plasma Membrane-Associated Redox Activities

All of the enzyme activities were measured at 25°C with appropriate controls and the NADH- and/or H2O2-dependent oxidations were tested prior to the addition of the membrane protein and/or the elicitor preparation. Rates of ferricyanide reduction by plasma membranes in the presence or absence of NADH were measured spectrophotometrically with a diode array spectrophotometer (Hewlett-Packard model 8452A). The reaction was assayed as previously described by Pantoja and Wilmer (1991) in 1 mL of incubation medium containing 260 mM mannitol, 1.0 mM CaCl2, 10 mM Tris/Mes buffer, pH 6.5, 0.2 mM ferricyanide, and 25 μM NADH. Changes in optical density were measured at 420 nm for 8 to 20 min immediately after the addition of 25 μg of plasma membrane protein in the presence or absence of control or race IF. Rates of ferricyanide reduction were expressed as μmol mg⁻¹ membrane protein h⁻¹.

NADH oxidase was measured spectrophotometrically according to Askerlund et al. (1987) by the loss in A500 in a 1-mL assay medium containing 20 mM Tris/Mes, pH 6.5, 150 μM NADH, 1 μM KCN, and 25 μg of plasma membrane protein in the presence or absence of control IF or race IF. Rates of NADH oxidation were expressed as μmol mg⁻¹ membrane protein h⁻¹.

GSH reductase activity was measured according to Foyer and Halliwell (1976) by coupling NADH in the presence of 2.5 mM GSSG, 125 μM NADH, 50 mM Tricine buffer, pH 6.5, and 0.5 mM EDTA in the presence or absence of IF. The reaction was initiated by the addition of GSSG, and measurements of the enzyme activity were corrected for chemically oxidized GSH NADH oxidation.

Ascorbate peroxidase was measured by a modified spectrophotometric procedure based on the rate of decrease in ascorbate A250 during ascorbate peroxidation (Aasada, 1984). The assay was performed in a reaction medium containing 250 μM ascorbate, 50 mM phosphate buffer, pH 7.0, 10 mM H2O2, 3.8 μmol EDTA, 50 μg of plasma membrane protein in the presence or absence of IF. Corrections were made for nonenzymatic and H2O2-independent oxidation.

NADH-dependent Cyt c reductase was measured as an increase in A550 of a 1-mL reaction mixture containing 20 μM Cyt c, 10 mM potassium phosphate buffer, pH 6.5, 0.6 M mannitol, 10 μM NADH, 0.1 mM EDTA, 25 μg of plasma membrane-enriched protein fraction in the presence or absence of IF preparations. The specific activity of the enzyme was demonstrated by the lack of inhibition of its activity by superoxide dismutase. The values were expressed as μmol mg⁻¹ membrane protein h⁻¹. Total protein of plasma membrane fractions and IF preparations was measured by the method of Bradford (1976) with BSA as the standard.

Inhibitors

The following inhibitors were used on ferricyanide reduction assays: potassium cyanide (0.5 mM), an inhibitor of peroxidase activity; salicylhydroxamic acid (1 mM), an inhibitor of oxidase activity; and superoxide dismutase (100 units), which catalyzes the dismutation of O2- to H2O2 (O2- are produced during the oxidation of NADH by peroxidase and can act as reducing agents). The effects of 0.1 μM okadaic acid, an inhibitor of protein phosphatase 1 and 2A, and 10 μM staurosporine, an inhibitor of protein kinase C, were assayed in the presence of 3 mM Tris-ATP, pH 6.5.

Chemicals

Nucleotides [ADP(β)S, ATP(γ)S, GDP(β)S, and GTP(γ)S] were obtained as lithium salts from Boehringer Mannheim.
(Laval, Québec, Canada). Stauroporine (from Streptomyces sp.), mastoparan (from VespuIa leWisiI), okadaic acid, and glutathione reductase (from yeast) were from Sigma.

RESULTS
Effect of Race-Specific Elicitors on the Reduction of Ferricyanide

The possible involvement of the plasma membrane redox activities in the production of active oxygen species during the elicitation of defense responses in tomato was studied using a cell line of tomato containing the resistance gene Cf5 and race-cultivar-specific elicitors from C. fulvum. To determine whether tomato plasma membrane-enriched fractions retained the specific response to the elicitor previously demonstrated in whole cells (Vera-Estrella et al., 1992), ferricyanide reduction was measured in enriched plasma membrane fractions exposed to different race-specific elicitors contained in IFs from plants infected with one of a number of races of C. fulvum. If the race-cultivar specificity was retained, then only the incompatible combinations (race 4 and 2.3 IF, containing the avr5 gene product) would be expected to induce changes in ferricyanide reduction. Treatment of purified Cf5 plasma membranes with race 4 or 2.3 IF induced a 240% increase in ferricyanide reduction, whereas other IFs (all obtained using races compatible to Cf5 plants) did not affect the reduction of ferricyanide (Fig. 1). The same results were observed with protoplasts and cell suspensions (data not shown).

Effects of Race-Specific Elicitors on Plasma Membrane Oxidases, Peroxidases, and Reductases

The transport of electrons from cytoplasmic electron donors (e.g. NADH) to external acceptors (ferricyanide, ascorbate radical, and oxygen) are mediated by the plasma membrane. Oxygen is a natural electron acceptor, and the step-limiting transfer of electrons from NADH to oxygen is catalyzed by intrinsic plasma membrane enzymes such as NADH (quinone) oxidase and NADH-dependent Cyt c reductase. Incubation of plasma membranes for 10 min (Vera-Estrella et al., 1992) with catalase, superoxide dismutase, potassium cyanide, and salicylhydroxamic acid before the addition of IF inhibited the increase in the ferricyanide reduction observed in the presence of race 4 or 2.3 IF (Fig. 2). The effects of superoxide dismutase and catalase suggested that the reduction proceeded directly from NADH via oxygen reduction to the superoxide radical and H2O2, whereas the effects of potassium cyanide and salicylhydroxamic acid suggested the possible involvement of peroxidases and/or oxidases in the observed ferricyanide reduction. To determine if ascorbate peroxidase, NADH oxidase, NADH-dependent Cyt c reductase, and glutathione reductase were associated with the increased ferricyanide reduction, the activity of these enzymes was tested in the presence and absence of race 4 IF. The elicitor induced an increase in the activity of NADH oxidase (200%) and Cyt c reductase (150%), whereas the activity of ascorbate peroxidase was inhibited (200%) and the activity of glutathione reductase remained unchanged (Fig. 3).

Effect of Okadaic Acid and Staurosporine on the Activity of NADH Oxidase, Ascorbate Peroxidase, and the Reduction of Ferricyanide by the Race 4 Elicitor

Reversible changes in the phosphorylation state of enzymes are known to be fundamental in the mechanisms of receptor binding and transduction of signals. The phosphorylation and dephosphorylation of proteins by kinases and phosphatases are known to be responsible for the regulation of enzyme activities and are also known to serve as a cascade system for the amplification of signals (Ranjeva and Boudet, 1987). The possibility that elicitor-induced stimulation of ferricyanide reduction and NADH oxidase activity and reduction of ascorbate peroxidase activity were results of changes in the phosphorylated state of enzymes was tested using okadaic acid, a specific inhibitor of protein phosphatase 1 and 2A (Haystead et al., 1989), and staurosporine, an inhibitor of protein kinases (Herbert et al., 1990). Okadaic acid reduced by 50% the increase in ferricyanide reduction induced by race 4 (Fig. 4A) or 2.3 IF (not shown), and almost completely abolished the increase in NADH oxidase activity (Fig. 4B) and the inhibition of ascorbate peroxidase activity (Fig. 4C). In contrast, the presence of staurosporine did not affect ferricyanide reduction or NADH oxidase and ascorbate peroxidase activities (Fig. 4, A–C). The elicitor-induced increase in Cyt c reductase was not inhibited by either okadaic acid or staurosporine (not shown).

Effect of Guanidine Nucleotides and Mastoparan on Ferricyanide Reduction and NADH Oxidase and Ascorbate Peroxidase Activities

The increased NADH oxidase activity and ferricyanide reduction, as well as the inhibition of ascorbate peroxidase activity through a phosphatase, suggested a link between elicitor-receptor recognition and a direct signal-transduction pathway. The possible involvement of G-proteins in the
transduction pathways initiated by the binding of the elicitor to putative receptors in the tomato plasma membrane was tested by using guanine nucleotide analogs. GTP(γ)S, a GTP analog that locks G proteins in a GTP-bound active form (Kaziro et al., 1991), induced an increase in NADH oxidase activity and ferricyanide reduction and an inhibition of ascorbate peroxidase activity, comparable to that obtained in the presence of race 4 IF (Fig. 5, A–C). GDP(β)S, a GDP analog that locks G proteins in a GDP-bound inactive form (Kaziro et al., 1991), abolished the increase in NADH oxidase activity and the reduction of ferricyanide induced by race 4 IF (Fig. 5, A and B). The GDP(β)S analog also prevented the inhibition of ascorbate peroxidase activity (Fig. 5C). Incubation of plasma membranes with ADP(β)S and ATP(γ)S had no effect on the enzyme activities in the presence or absence of race 4 IF (Fig. 5, A–C). These results suggested that a G protein was involved in the activation of protein phosphatases, which in turn might dephosphorylate NADH oxidase and ascorbate peroxidase.

The possibility that the putative elicitor-receptor recognition activated other enzymes involved in the redox activity of the plasma membrane through the activation of G proteins was also tested. The activities of Cyt c reductase and GSH reductase were not affected by the addition of nucleotides (not shown), suggesting that these enzymes may be activated through a mechanism other than G protein activation. The role of G proteins in the transduction of the elicitor-receptor recognition signal was further confirmed using mastoparan, a peptide toxin from wasp venom (Higashijima et al., 1990). This peptide has been shown to promote nucleotide exchange by the G protein through a mechanism similar to that used by native receptors (Higashijima et al., 1990). The addition of mastoparan to a reaction medium containing plasma membrane vesicles induced an increase in NADH oxidase activity and ferricyanide reduction and inhibition of the ascorbate peroxidase activity similar to that obtained with GTP(γ)S and with race 4 IF (Fig. 5, A–C).

**DISCUSSION**

The tomato–C. fulvum interaction is an example of a host-pathogen relationship in which the involvement of racespecific elicitors has been extensively used in the study of
defense responses in compatible and incompatible interactions (De Wit, 1992). The extracellular production of active oxygen species in the incompatible interaction has been shown to be an important part of the reaction of tomato cells to race-specific elicitors of C. fulvum (Vera-Estrella et al., 1992).

Apoplast preparations (IF) from infected plants containing the avr5 elicitor (race 4 and 2.3) or lacking the avr5 elicitor (races 5, 2.4.5, and 2.4.5.9) and tomato cells with the resistance gene C/5 were used in the present study to investigate redox events following the treatment of plasma membrane with the elicitor. A significant increase in ferricyanide reduction was observed only in the incompatible combinations of race 4 or 2.3 IF (avr5 elicitor) and C/5 cells (Fig. 1), indicating that plasma membranes retained the specificity previously demonstrated in intact plants (De Wit and Spikman, 1982) and cell suspensions (Vera-Estrella et al., 1992). A number of studies have previously shown the increased production of active oxygen species in response to pathogens (e.g. Döke, 1985; Apostol et al., 1989; Kepler et al., 1989; Vera-Estrella et al., 1992), and it has been postulated that the source of the reducing equivalents might be either NADH (which can be provided from malate by malate dehydrogenase) or plasma membrane electron transport systems (Rubinstein et al., 1984).

Increased NADH-dependent ferricyanide reduction following the treatment of host plasma membranes with race-specific elicitors suggested that plasma membrane redox activity might result in the production of O$_2^-$ anions and the concomitant generation of H$_2$O$_2$. The possibility that these active oxygen species, peroxidases, and/or other oxidases were involved during the increased redox activity was demonstrated by the effects of catalase, superoxide dismutase, potassium cyanide, and salicylhydroxamic acid on the elicitor-induced increase in redox activities (Fig. 2).

The observed increase in NADH oxidase and Cyt c reductase activities upon elicitor treatment (Fig. 3) suggested that these two enzymes might be responsible for the increased production of active oxygen species previously reported in tomato cell suspensions (Vera-Estrella et al., 1992). In phagocytes a membrane-bound NAD(P)H oxidase complex can transfer electrons from NAD(P)H to molecular oxygen, generating superoxide anions (Morel et al., 1991). In plants, it has been reported that auxin-stimulated redox activity of soybean was related to an increase in activity of a plasma membrane-bound NADH oxidase (Brightman et al., 1988, 1992; Mörre and Brightman, 1991).

Ascorbate peroxidase is known to be involved in the reduction of H$_2$O$_2$ to H$_2$O and O$_2$ (Dalton et al., 1986). The observed changes of ascorbate peroxidase in race 4 IF-treated plasma membranes (Fig. 3) would indicate a change in the relative contribution of this enzyme to the detoxification of H$_2$O$_2$. The inhibition of the ascorbate peroxidase activity by the elicitor suggests that the previously reported increase in H$_2$O$_2$ induced by the elicitor (Vera-Estrella et al., 1992) could be due partly to the inhibition of the peroxide-scavenging mechanism.

The effects of okadaic acid on the elicitor-mediated changes in redox activity (Fig. 4) suggest that dephosphorylation is required for the increase and decrease in activities of NADH oxidase and ascorbate peroxidase, respectively. Elicitor-induced changes in Cyt c reductase were not affected by either okadaic acid or staurosporine (not shown), suggesting that the increased activity of this enzyme may be a consequence of a reaction(s) occurring after the activation of NADH oxidase.

The activation of NADH oxidase and the inactivation or suppression of ascorbate peroxidase activity via dephosphorylation suggested a link between the elicitor-receptor recognition and the activation of a phosphatase(s) involved in their regulation. From the effects of guanidine nucleotide analogs and mastoparan on the plasma membrane NADH oxidase, ferricyanide reduction, and ascorbate peroxidase activities, we propose that G proteins are responsible for the transduction of the signals leading to the activation of the tomato defense mechanisms against the fungal pathogen C. fulvum. This view is strengthened by the findings that auxin-stimulated plasma membrane NADH oxidase activity of soybean hypocotyls has been shown to be activated by guanine nucleotide analogs (Mörre et al., 1993), and that the activation of host plasma membrane H$^+$-ATPase by fungal elicitors was mediated by G proteins (Vera-Estrella et al., 1994).

In conclusion, our results are consistent with a model in which host plasma membrane receptors bind the fungal elicitor and trigger the activation of G proteins. These in turn transduce the signal by activating (directly or indirectly) a membrane-bound phosphatase(s), resulting in the stimulation of plasma membrane redox activity. The increased
plasma membrane NADH oxidase activity and the concomitant
decrease in ascorbate peroxidase activity are important
aspects in the initial response of tomato cells to specific
elicitors of C. fulvum. The increase in redox activities would
induce the production of active oxygen species, which appear
to be important components associated with the defense
response of plants to pathogens (Vera-Estrella et al., 1992).
The signal transduction pathway for these plasma membrane
redox changes is remarkably similar to that shown for elicitor-
induced changes in plasma membrane H^+-ATPase activity
(Vera-Estrella et al., 1994). The increased NADH oxidase
activity could induce the generation of superoxide anions,
which, after dismutation (spontaneously or mediated by su-
peroxide dismutase) and together with the increase in H^+
concentration in the cell wall compartment, could be the
primary source for the formation of H_2O_2. An elicitor-induced
increase in H_2O_2 will favor the formation of hydroxyl radicals,
which in turn will activate lipid peroxidation, leading to
irreversible membrane damage.

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