

Rapid, *Cf-9* and *Avr9*-Dependent Production of Active Oxygen Species in Tobacco Suspension Cultures

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The tomato *Cf-9* gene confers resistance to races of *Cladosporium fulvum* expressing the corresponding avirulence gene *Avr9*. The availability of transgenic tobacco lines carrying *Cf-9*, and the well-characterized 28 amino acid *Avr9* elicitor, make this an excellent system to study resistance gene function. In this paper we establish tobacco suspension cultures derived from transgenic tobacco plants containing *Cf-9*, as well as from plants without *Cf-9*. Cultures derived from *Cf9* tobacco produce active oxygen species (AOS) within 5 min of treatment with pure or synthetic *Avr9*. This enabled us to perform biochemical and pharmacological analysis in cell culture of the very earliest events in resistance gene function. In addition to AOS production, an increase in oxygen uptake was detected in the *Avr9*-treated *Cf9* cells. Both phenomena were inhibited by low concentrations of diphenyleneiodonium (DPI). Additional pharmacological inhibitor studies suggest that uptake of calcium, activation of protein kinases, and probably phospholipase A₂ (PLA₂) activity are intermediates in the *Cf-9*- and *Avr9*-dependent signaling pathway that leads to AOS production. Interestingly, those defense responses did not result in plant cell death.

Additional keywords: NADPH oxidase, oxidative burst, plant defense.

Most plants are resistant to most plant pathogens. In incompatible plant-pathogen interactions, resistance is frequently correlated with localized cell death, the synthesis of numerous antimicrobial proteins, and structural modifications to cell walls at the site of infection (Hammond-Kosack and Jones 1996). Defense mechanisms can be initiated as a result of the direct or indirect interaction of the product of a pathogen avirulence (*Avr*) gene with the product of the corresponding plant resistance (*R*) gene (Baker et al. 1997). Following the interaction, the R protein is presumed to activate a signaling cascade that prevents pathogen proliferation. An absence of

or mutation in either or both genes results in the inability of the plant to recognize the pathogen and then activate defense mechanisms, and thus leads to successful pathogen colonization.

Among the large variety of defense reactions activated in plant tissues or suspension cultures in response to pathogen attack, one of the earliest is the production of active oxygen species (AOS). This term includes the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\cdot\text{OH}$). They are produced by the sequential reduction of molecular oxygen. O_2^- is unstable in solution, and can dismutate to H_2O_2 either via superoxide dismutase (SOD), or nonenzymatically (Sutherland 1991). H_2O_2 is more stable in solution. However, it is the substrate for catalase and various peroxidases that are highly abundant in plants. The $\cdot\text{OH}$ is an extremely reactive chemical species and is capable of interacting with most organic molecules. $\cdot\text{OH}$ is produced by O_2^- and H_2O_2 in the presence of iron by the Fenton reaction (Sutherland 1991).

Identification of the enzyme(s) responsible for the production of AOS during plant defense is an intense area of research. Cell-wall-bound peroxidases, oxalate oxidase, xanthine oxidase, and NADPH oxidase have all been implicated as possible candidates. Several different lines of evidence support the involvement of an enzymatic complex similar to the mammalian NADPH oxidase in plant cells. This enzymatic complex found in mammalian neutrophils has two components located in the plasma membrane (gp91-phox and p22-phox) and becomes active when at least three proteins from the cytosol (p47-phox, p67-phox, rac) bind to the membrane components (Henderson and Chapel 1996; Jones 1994; Segal and Abo 1993). Genes that encode gp91 homologues have been identified in plants (Groom et al. 1996; Keller et al. 1998; Torres et al. 1998). In addition, the oxidative burst in plants is inhibited by an irreversible inhibitor of the mammalian NADPH oxidase, diphenyleneiodonium (DPI) (Auh and Murphy 1995; Desikan et al. 1996; Jabs et al. 1997; Levine et al. 1994).

Many studies on defense-related AOS production in plants have been conducted with plant suspension cultures. In some cases, these plant cultures have been elicited with bacterial cultures. Nonspecific elicitors such as chitosan, or fungal cell wall preparations, and a peptide derived from a glycoprotein from *Phytophthora sojae* in parsley suspension cultures, have received particular attention. However, there is no definitive

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proof that *R* gene-dependent defense responses are activated in the same way.

The *Cf-9/Avr9* system has some very useful features for the study of specific *R/Avr*-induced plant defense responses. The tomato *Cf-9 R* gene has been cloned (Jones et al. 1994). The deduced amino acid sequence predicts a mainly extracytoplasmic glycoprotein with a membrane anchor in the carboxy terminus and with 27 extracytoplasmic leucine-rich repeats (LRRs) carrying multiple putative glycosylation sites. In contrast, the *Pto* and NBS/LRR class of *R* gene products are intracellular. The complementary avirulence gene from *Cladosporium fulvum*, *Avr-9*, has also been cloned (Van den Ackerveken et al. 1992) and encodes a 63 amino acid preproteins that is processed by both plant and fungal proteases to the mature 28 amino acid peptide (Van den Ackerveken et al. 1993). The mature *Avr9* peptide forms a cystine-knot structure (Vervoort et al. 1997). Previous studies with nitroblue tetrazolium (NBT) staining indicated that production of AOS by *Cf9* tomato cotyledons is induced within 2 to 3 h in response to *Avr9* infiltration (May et al. 1996). Transgenic tobacco plants containing the tomato resistance gene *Cf-9* also respond to *Avr9* injection to produce NBT staining within 75 min followed by a hypersensitive response resulting in macroscopic gray necrosis (Hammond-Kosack et al. 1998). This result indicates that *Cf-9* retains its activity and specificity when transferred to tobacco.

We generated cell suspension cultures from these *Cf9* tobacco plant lines to study the earliest events induced by the *Cf-9/Avr-9* interaction. In this system we can add pure *Avr9* elicitor to the *Cf9* tobacco cells and study the events activated within 5 min, which is much easier and more reproducible than in leaves. We show that *Cf9* tobacco suspension cultures respond to *Avr9* and produce AOS within 5 min of elicitation whereas comparable nontransformed suspension cultures do not. This *Cf-9*- and *Avr9*-dependent system has allowed us to study the signal transduction components leading to the *Cf-9*-dependent activation of AOS accumulation. Other studies have looked at *R* gene function in cell cultures (Chandra et al. 1996b; Levine et al. 1994) but, in these examples, the *Avr* elicitor is delivered by bacterial type III secretion systems and the time course of *Avr* protein delivery is much more poorly defined, resulting in nonspecific AOS production over 0 to 2 h, followed by *Avr*-dependent AOS production over 2 to 8 h. The very rapid elicitation of AOS in the *Cf9/Avr9* tobacco cell cultures permitted a more detailed analysis of the requirements for elicitation, and a comparison with nonspecific elicitation by PEP13 in parsley cell cultures.

RESULTS

Tobacco suspension cultures produce AOS that is both *Cf-9* and *Avr9* dependent.

Hammond-Kosack et al. (1998) reported that *Cf-9* can function in transgenic tobacco. To establish a suspension culture, callus was initiated from self progeny of two different *Cf9* tobacco lines that had been shown to be active. Individual progeny that gave rise to calli were subsequently tested for whether they were homozygous or heterozygous. From individual calli, suspension cultures were initiated (see Materials and Methods). In all, seven suspension cultures were examined for *Avr9* responsiveness. Most responded to *Avr9* by pro-

duction of AOS (see below), but the cultures varied in the strength of their response. This variation was not strongly correlated with zygosity. These cultures have remained active for 2 years. Since all cultures showed the same qualitative *Avr9* and *Cf-9* dependence in AOS production, all experiments were carried out with the most active cell line.

One of the most rapid responses detected in *Cf-9*-containing tomato after *Avr9* infiltration is the production of AOS (May et al. 1996). Challenge of the *Cf9* tobacco suspension cultures with intercellular fluid (IF) obtained from transgenic plants that express the avirulence gene *Avr9* fused to a plant signal peptide (Hammond-Kosack et al. 1994) led to an accumulation of AOS in the media. This AOS accumulation either exhibited a pronounced biphasic profile or a mild phase shoulder (Fig. 1B). The first phase of net accumulation was detected as early as 5 min after elicitation and reached a maximum at approximately 50 to 60 min. After this peak, either AOS accumulation reached a plateau or the levels of AOS decreased slightly. The second phase of net AOS accumulation started at around 2 h after elicitation with maximal AOS levels detectable at 3.5 to 4 h. The *Cf9* cells responded to IF (*Avr9*⁻) obtained from control tobacco plants not expressing *Avr9*, with a small AOS accumulation coincident with the first phase. After this no further AOS production was detected. This response was observed with six out of seven different suspension culture lines generated from individual tobacco plants. The effect of both IF (*Avr9*⁺) and IF (*Avr9*⁻) was also assayed in three different control tobacco cultures lacking *Cf-9* (Fig. 1A). These cultures produced only very small amounts of AOS irrespective of whether *Avr9* was present or not.

The AOS accumulation described above is both *Cf-9* and *Avr9* dependent, since it was only observed in the *Cf-9*-containing tobacco challenged with IF containing *Avr9*. To rule out the possibility that other compounds present in the IF (*Avr9*⁺) were responsible for AOS production, purified *Avr9* peptide (a gift from Pierre de Wit) and synthetic *Avr9* peptide (see Materials and Methods) were added to the cultures. Synthetic *Avr9* peptide carrying the R8K mutation was used since it was reported to be a more active *Cf-9*-dependent elicitor than wild-type *Avr9* (Kooman-Gersmann et al. 1997). The responses obtained with both *Avr9* preparations were exactly the same as described above for IF (*Avr9*⁺). The accumulation of AOS in the *Cf9* cultures depended on the amount of elicitor added (Fig. 1C and D). These results demonstrate unequivocally that the AOS accumulation is both *Cf-9* and *Avr9* dependent.

The oxidative burst is probably produced by an NADPH oxidase complex.

Several different mechanisms have been proposed as the source of AOS production by plants upon elicitation. An enzymatic complex similar to the NADPH oxidase responsible for the oxidative burst in mammalian neutrophils is currently considered to be a likely mechanism. Therefore, the effect of DPI on the *Cf-9*- and *Avr9*-dependent AOS accumulation was investigated. DPI is an irreversible inhibitor of flavin-containing enzymes such as the mammalian neutrophil NADPH oxidase complex, and was found to inhibit AOS accumulation in a dose-dependent manner. Complete inhibition was obtained when a very low concentration of DPI (0.2 μ M) was added to the *Cf9* cells only 5 min prior to *Avr9* elicitation

(Fig. 2). When DPI was applied during the second phase of AOS accumulation, further AOS accumulation halted within 5 min (Fig. 2).

A cell wall peroxidase has been suggested to be responsible for AOS accumulation in French bean suspension cultures in response to fungal elicitor (Bolwell et al. 1995). This AOS accumulation, as well as the peroxidase enzyme activity, shows a pH optimum of 7.5, with almost no AOS produced at pH 6. However, the pH optimum for AOS accumulation in the Cf9 tobacco suspension cultures challenged with Avr9 was approximately 6.0 and AOS accumulation was not detected at pH higher than 7 (Fig. 3A). This indicates that the source of AOS in the Cf-9/Avr9-mediated pathway is different from what has been described in French bean in response to a crude elicitor derived from fungal cell walls.

When the assay was done in less buffered conditions (0.5 mM 2-(*N*-morpholino)ethanesulfonic acid [MES] instead of 5 mM), alkalization of the media was observed in the Avr9-

treated Cf9 cells (Fig. 3B). The increase of pH was only 0.25 U and was not inhibited by DPI pretreatment, which inhibits the oxidative burst.

Avr9 induces an increase in oxygen consumption in Cf9 cells.

The mammalian NADPH oxidase uses electrons from cytosolic NADPH to reduce external oxygen to $\cdot\text{O}_2^-$. This results in a considerable increase in the oxygen consumption associated with AOS production by neutrophils, which is described as the oxidative burst. The association between AOS accumulation and oxygen consumption by Cf9 tobacco cells in response to Avr9 was investigated with an oxygen electrode. Numerous controls were included to discriminate between the stress caused by stirring of the cells in the electrode chamber, which is a known inducer of AOS production (Legendre et al. 1993), and the specific response to Avr9. After a lag phase of 2 to 3 min a considerable increase in oxygen uptake was evi-

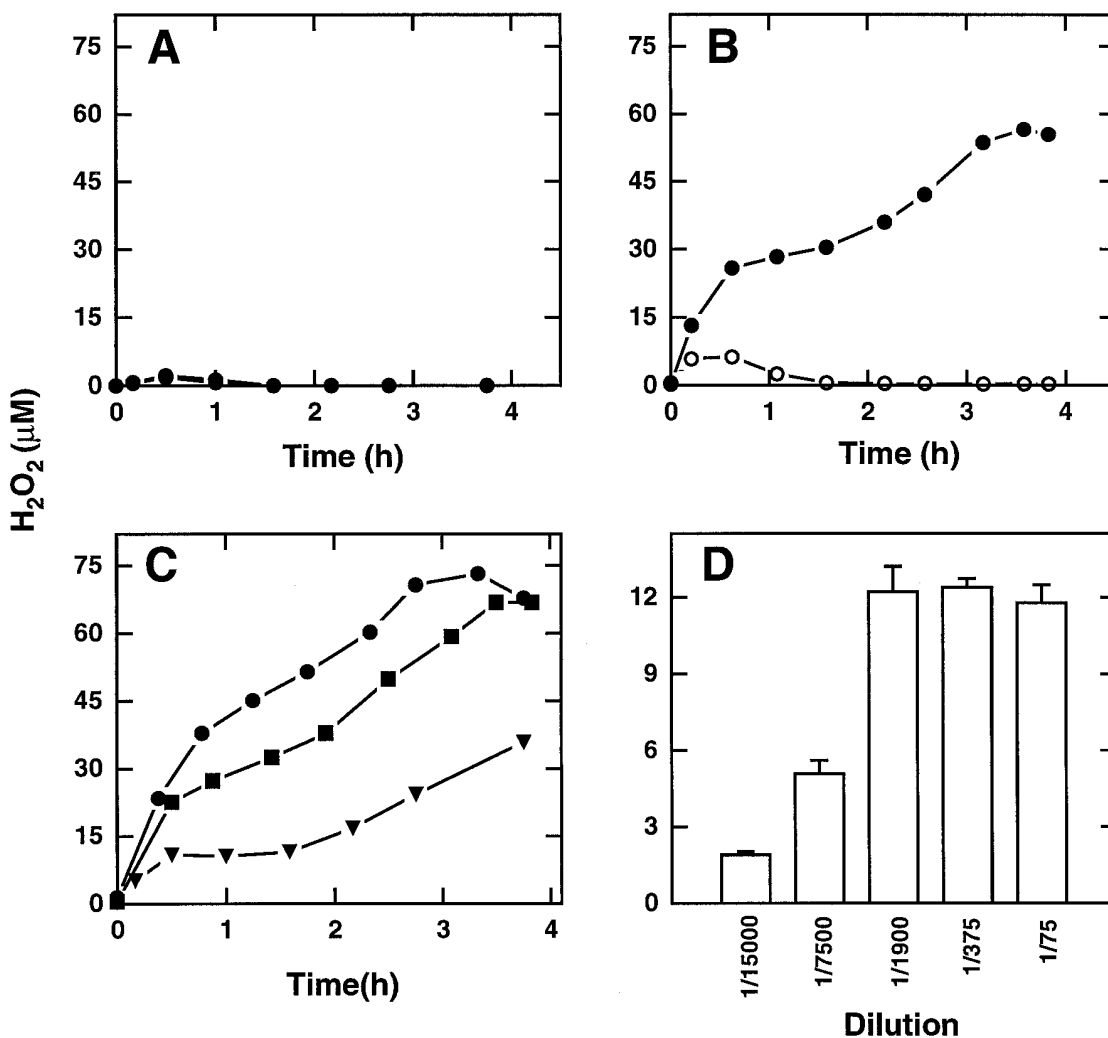


Fig. 1. Tobacco suspension cultures accumulate active oxygen species (AOS) in a Cf-9- and Avr9-dependent manner. **A**, Control Petit Havana (minus Cf-9) tobacco cultures were treated with intercellular fluid (IF) (Avr9⁺) (closed circles) or IF (Avr9⁻) (open circles) at time zero (final IF dilution 1/400). Open circles are not visible as the two time courses were indistinguishable. AOS concentration was determined at times indicated. **B**, As in **A** but with Petit Havana tobacco cultures expressing Cf-9 (Cf9 cultures). **C**, Cf9 tobacco cultures were treated with active synthetic R8K Avr9 at final concentrations of 6.5 ng/ml (closed triangles), 20 ng/ml (closed squares), and 60 ng/ml (closed circles) at time zero. AOS concentration was determined at times indicated. **D**, Cf9 tobacco cultures were treated with IF (Avr9⁺) at final dilutions indicated. AOS concentration was determined 50 min after treatment.

dent, specifically in the Cf9 cells to which IF (Avr9⁺) had been added (Fig. 4). Addition of water or IF (Avr9⁻) resulted in an increase in oxygen consumption of between 20 and 40% whereas IF (Avr9⁺) or pure Avr9 induced an increase of 80 to 130% (Fig. 4). Preincubation of the Cf9 cells with DPI completely abolished the increase in oxygen uptake induced by Avr9.

H₂O₂ is the major AOS detected.

The inhibition of AOS production by DPI, the increase in oxygen uptake on elicitation, and the pH optimum for the oxidative burst suggest that in the response of Cf9 cells to Avr9 an NADPH oxidase-like complex is responsible for the AOS production. This enzyme reduces oxygen to superoxide anion in mammalian neutrophils. Several attempts were made to detect superoxide anion in Avr9-treated Cf9 cells by the lucigenin and cytochrome c methods (Auh and Murphy 1995; Vera-Estrella et al. 1992) in the presence and absence of the SOD inhibitor diethyldithiocarbamic acid (DDC). Although some signal was detected in the Cf9 cells treated with IF (Avr9⁺) and not with IF (Avr9⁻), the incorporation of DDC did not result in an increase of the signal as has been reported in other systems (Auh and Murphy 1995; Jabs et al. 1997). All these "superoxide assays" also gave some signal with commercial H₂O₂ (Sigma, Poole, UK). Any Avr9-induced signal in a "superoxide assay" was inhibited by catalase, giving rise to some uncertainty about the specificity of this signal. We propose that in the tobacco Cf-9/Avr9 system, superoxide is dismutated too rapidly to be detected by these assays.

The AOS accumulation in Cf9 tobacco cells was detected by the chemiluminescence of luminol in a ferricyanide-catalyzed oxidation. Since this method is able to detect various AOS we tried to establish which compound is predominantly responsible for the signal. The effects of the enzymes catalase and SOD on the Avr9-induced AOS accumulation are shown in Table 1. Addition of catalase (50 U/ml) to the cells prior to Avr9 completely inhibited the signal in the luminol

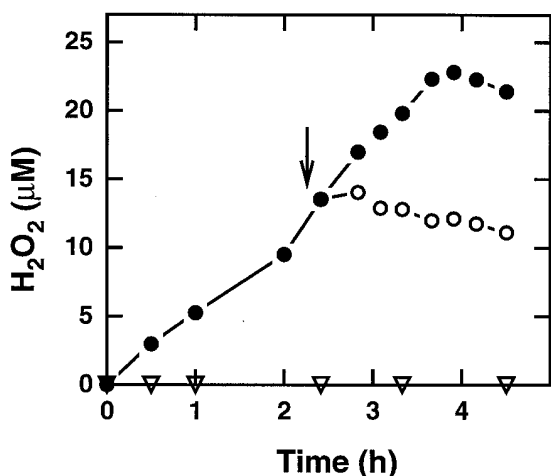


Fig. 2. Diphenyleneiodonium (DPI) inhibits the Avr9-induced active oxygen species (AOS) accumulation in Cf9 tobacco cultures. AOS concentration was determined at times indicated in Cf9 cells treated with intercellular fluid (IF) (Avr9⁺) at time zero (filled circles), DPI (0.2 µM) 5 min prior to IF (Avr9⁺) (open triangles), or DPI (0.2 µM) at the time indicated by the arrow (open circles).

assay (Table 1) whereas SOD (100 U/ml) did not have a significant effect on the signal. These results indicate that, in our hands, the luminol assay primarily detects H₂O₂.

Uptake of external calcium is required for AOS accumulation.

The production of AOS by Cf9 tobacco suspension cultures challenged with Avr9 was dependent on the presence of calcium in the medium. When calcium was removed from the medium by washing the cells and equilibration in calcium-free buffer in the presence of 0.5 mM EGTA for 3 h prior to Avr9 addition, very little AOS production was detected (Fig. 5A). The addition of 2.5 mM calcium to the EGTA-inhibited cells after washing completely restored the ability of the cells to produce an oxidative burst in response to Avr9 (Fig. 5A).

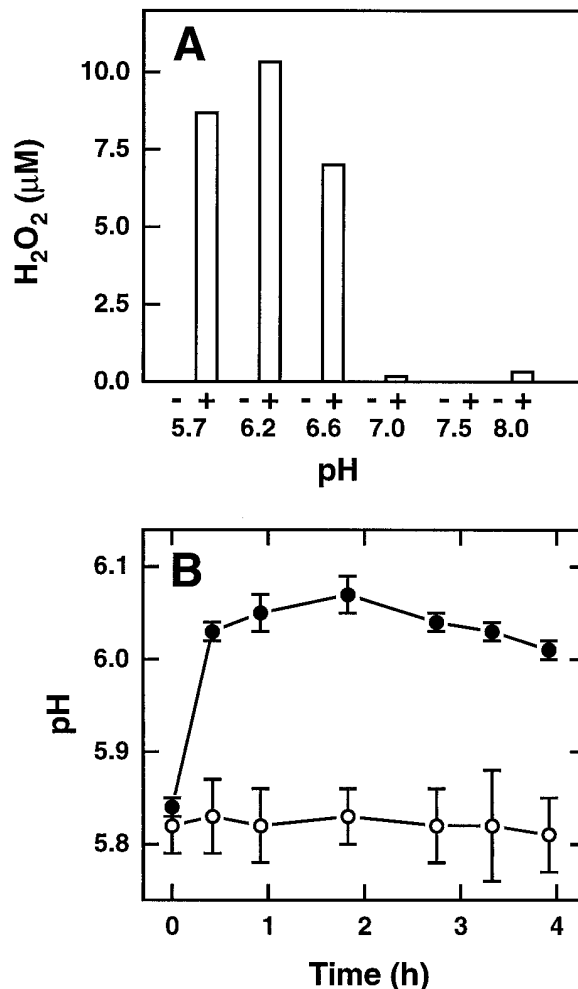


Fig. 3. pH optimum of the Cf-9-dependent response, and pH changes induced by the response. **A**, Cf9 cells were resuspended in buffer containing 5 mM morpholineethylsulfonic acid (MES) and 5 mM Tris at the various pH values indicated instead of the 5 mM MES pH 6 after the washing as described in Materials and Methods. After 3 h of equilibration, intercellular fluid (IF) (Avr9⁻) (-) or IF (Avr9⁺) (+) was added. Active oxygen species (AOS) concentration after 50 min of elicitation was determined. **B**, Cf9 cells were resuspended in 0.5 mM MES buffer instead of 5 mM. After 3 h of equilibration, water (open circles) or synthetic Avr9 (closed circles) was added and the extracellular pH determined at the times indicated.

Also, preincubation with two calcium channel blockers, lanthanum and gadolinium chloride, resulted in inhibition of the production of AOS in a dose-dependent manner, with 100% inhibition occurring at concentrations of 0.25 mM, for either lanthanum (Fig. 5B) or gadolinium (data not shown). Collectively, these data suggest that an uptake of extracellular calcium is required for accumulation of AOS induced by *Cf-9/Avr9*. Since calcium is involved in the signaling pathway, the effect of two calmodulin antagonists on the AOS accumulation was studied. Preincubation with trifluoperazine dimaleate (50 μ M) or W-7 (150 μ M) 15 min prior to *Avr9* addition completely inhibited the AOS accumulation (data not shown).

To determine if calcium uptake by the tobacco suspension cultures was itself sufficient to activate the signaling pathway, we tested the effect of the calcium ionophore A23187. A23187 has been demonstrated to increase intracellular calcium levels in different plant systems by facilitating calcium ion uptake from the extracellular medium. No burst of AOS production was caused by A23187 addition at concentrations up to 50 μ M in cells with extracellular calcium concentration of 0.5 mM. Also the *Avr9/Cf-9*-dependent oxidative burst was not restored in the gadolinium-inhibited cells when concentrations of up to 100 μ M of A23187 were applied (data not shown).

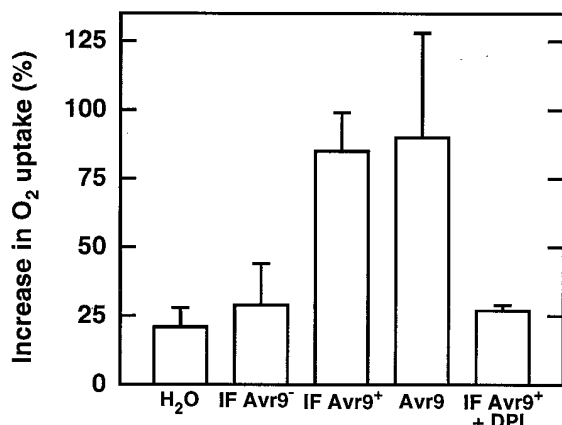


Fig. 4. *Avr9* induces an increase in oxygen uptake by *Cf9* cells. *Cf9* tobacco suspension cultures (2 ml, 0.02 g/ml) that had been washed and equilibrated (Materials and Methods) were placed in oxygen electrode chamber with or without diphenyleneiodonium (DPI). Oxygen uptake was continuously recorded; after 8 to 9 min, 50 μ l of H₂O, intercellular fluid (IF) (*Avr9*⁻), IF (*Avr9*⁺), or synthetic R8K *Avr9* (150 ng/ml) was added. Ratio or oxygen uptake before and after the additions was calculated.

Table 1. Effect of catalase and superoxide dismutase (SOD) on active oxygen species (AOS) accumulation^a

Treatment	IF (<i>Avr9</i> ⁻)	IF (<i>Avr9</i> ⁺)
Control	0	100
+ Catalase	0	0
+ Catalase boiled	14	110
+ SOD	31	118
+ SOD boiled	35	134

^a Catalase and SOD were added to *Cf9* tobacco cultures at final concentrations of 50 and 100 U/ml, respectively, half an hour before intercellular fluid (IF). The same enzymes were inactivated by boiling for 5 min. AOS was determined 50 min after IF addition for each treatment and compared with the control, to which only IF (*Avr9*⁺) was added.

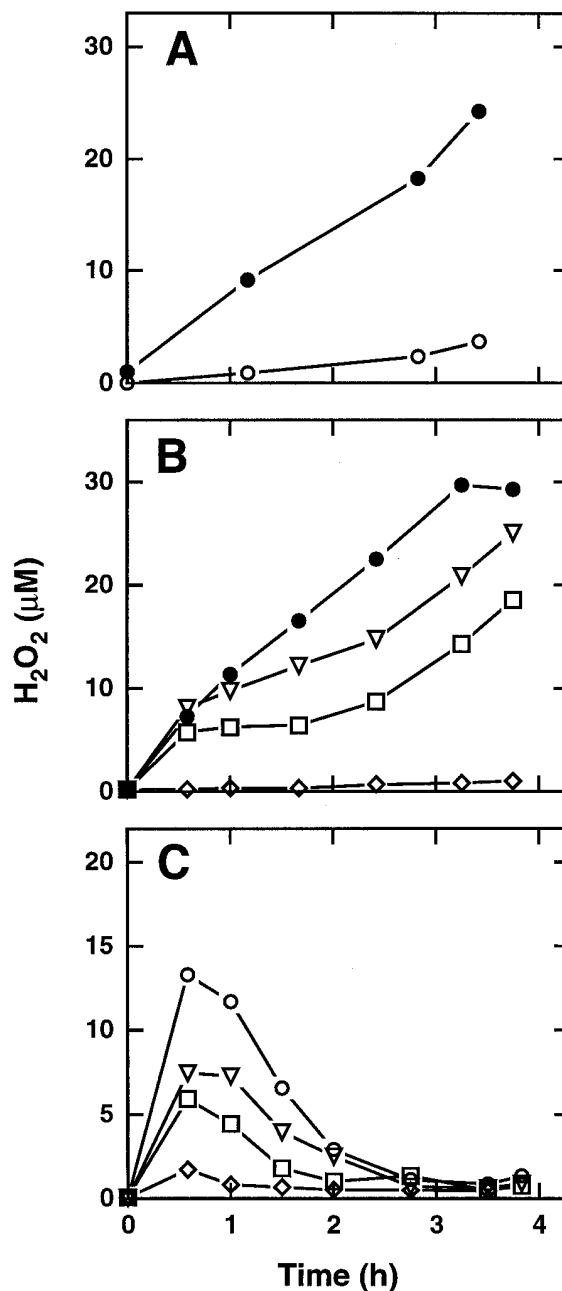


Fig. 5. Uptake of external calcium is required for active oxygen species (AOS) accumulation. **A**, *Cf9* cells were washed as described in Materials and Methods in buffer with 0.5 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) and without calcium ions. EGTA-treated cells were equilibrated in the same buffer (open circles) or 2.5 mM CaCl₂ was added to overcome the EGTA effect (closed circles). AOS concentration determined at time indicated after treatment with intercellular fluid (IF) (*Avr9*⁺) at time zero. **B**, *Cf9* cells were preincubated with 0 (closed circles), 5 μ M (open triangles), 25 μ M (open squares), or 250 μ M (open diamonds) lanthanum chloride 5 min prior IF (*Avr9*⁺) was added. **C**, Tobacco suspension cultures were treated with 0 (open diamonds), 25 μ M (open squares), 50 μ M (open triangles), or 100 μ M (open circles) of amphotericin B. **B** and **C**, AOS concentration determined at times indicated.

The antibiotic amphotericin B has been shown to induce ion fluxes (calcium uptake and export of potassium and chloride ions) in parsley suspension cultures in a manner similar to that observed in response to elicitors, and also to induce production of AOS, although with a delay in comparison with the elicitor (Jabs et al. 1997). The addition of amphotericin B to either Cf9 or untransformed tobacco cells induces the accumulation of AOS (Fig. 5C). However, in contrast to Avr9 elicitation of Cf9 cells, AOS accumulation after amphotericin B treatment was transient, peaking at approximately 45 min.

Protein phosphorylation is required for AOS production.

The involvement of protein phosphorylation in the signaling pathway leading to the production of AOS has been reported in several different plant systems (Suzuki and Shinshi 1996). In Figure 6 we show the effect of two protein kinase inhibitors, K-252a and staurosporine, on the Cf9- and Avr9-dependent oxidative burst. Both inhibitors at concentrations of 5 μM blocked AOS production, indicating that protein kinase activity is required. Small concentrations of either compound (1 μM) completely inhibited the first phase of production but not the second.

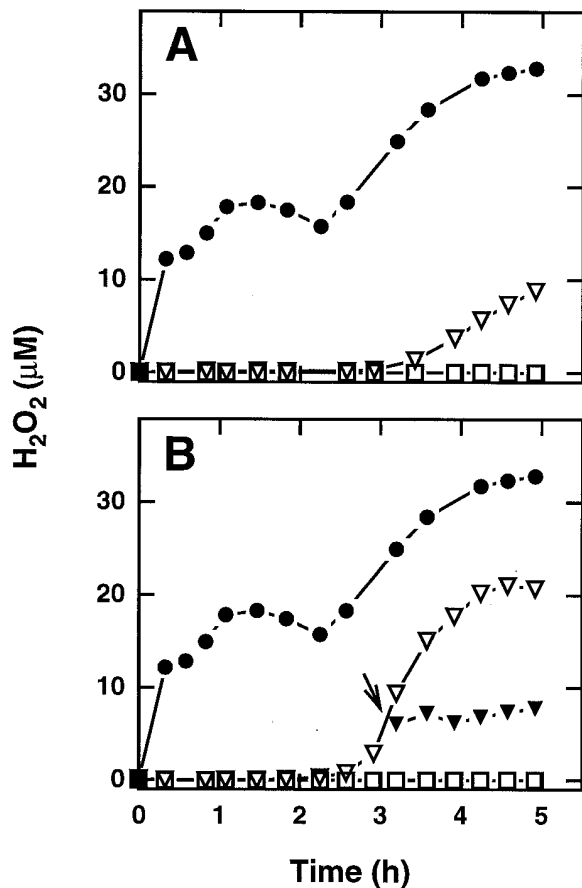


Fig. 6. Inhibition of active oxygen species (AOS) accumulation by protein kinase inhibitors. **A**, Cf9 tobacco suspension cultures were treated with 0 (closed circles), 1 μM (open triangles), or 5 μM (open squares) of staurosporine for 5 min before intercellular fluid (IF) (Avr9⁺) was added. AOS concentration determined at times indicated. **B**, As in **A** but with inhibitor K-252a. At time indicated by arrow, additional K-252a (equivalent to 1 μM) was added to cultures pretreated with 1 μM (closed triangles).

However, if an additional identical amount of inhibitor was added during the second phase this prohibited any further AOS production. These inhibitor studies indicate that protein kinase activity is continuously required for Cf9- and Avr9- dependent AOS synthesis. The protein kinase C inhibitors bisindolylmaleimide I and calphostin C (up to 500 nM) did not inhibit the Avr9-induced AOS accumulation (data not shown).

Increased protein phosphorylation can be produced by inactivation of protein phosphatases as well as by activation of protein kinases. Therefore, different inhibitors of protein phosphatase activity were tested for their ability to induce AOS accumulation in the absence of elicitor. The protein phosphatase 2A inhibitor cantharidin at concentrations as low as 1 μM induced H₂O₂ production in either tobacco cell type after a delay of approximately 1 h (Fig. 7A). To determine whether cantharidin and Avr9 act in the same signal transduction pathway, suboptimal concentrations of both were added to the Cf9 cells. Both a 1/10,000 dilution of IF (Avr9⁺), and a 0.2 μM concentration of cantharidin induced only very low levels of AOS production when used independently. When added together, a cooperative effect was observed (Fig. 7B).

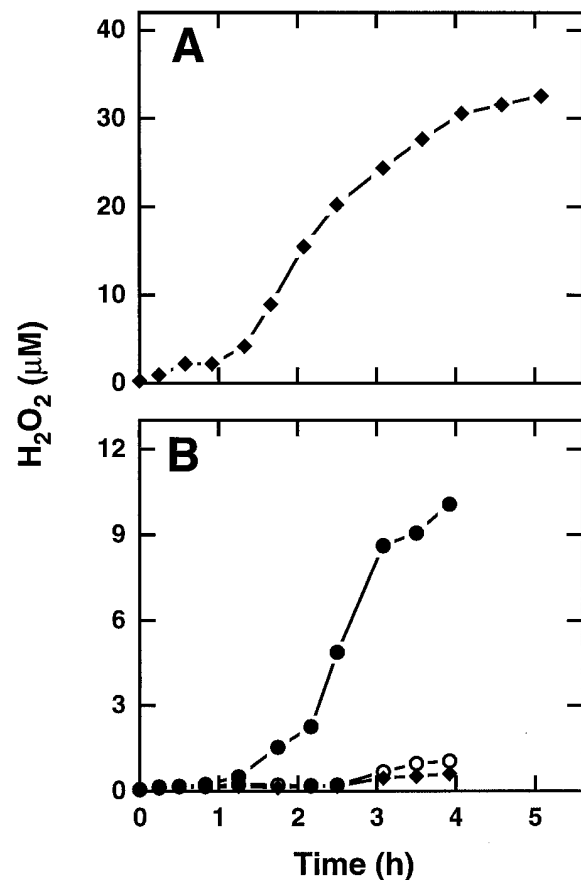


Fig. 7. Cantharidin (a protein phosphatase inhibitor) induces the Avr9-dependent active oxygen species (AOS) production. **A**, Tobacco suspension cultures were treated with cantharidin (1 μM) in the absence of elicitor at time 0; AOS concentration determined at times indicated. **B**, Cf9 tobacco suspension cultures were treated at time zero with 0.2 μM cantharidin (closed diamonds), a dilution 1:10,000 of intercellular fluid (IF) (Avr9⁺) (open circles) or both together (closed circles). AOS concentration determined at times indicated.

Another protein phosphatase 2A inhibitor, okadaic acid, also induced AOS accumulation by tobacco suspension cultures in the absence of Avr9, although higher concentrations were needed (3 μ M). The inhibitor of protein phosphatase 2B cypermethrin did not induce AOS accumulation at concentrations up to 5 μ M.

Phospholipase A₂ activity is probably required for AOS production.

Phospholipase A₂ (PLA₂) has been implicated as a signal intermediate in some of the pathways leading to an oxidative burst in neutrophils. Also, some increases in enzyme activity have been described in soybean suspension cultures treated with different elicitors (Chandra et al. 1996a). This enzyme hydrolyzes phospholipids at the position sn-2 to generate fatty acid and the corresponding lysolipid. The effects of different PLA₂ inhibitors are summarized in Table 2. 4-Bromophenacyl bromide, quinacrine, or chlorpromazine inhibited AOS accumulation by Cf9 tobacco suspension cultures in response to Avr9 in a dose-dependent manner. These data suggest that PLA₂-derived molecules may also be components of a Cf-9/Avr9-activated signal transduction cascade. Two fatty acids, arachidonic and linolenic acids, that could be the products of PLA₂ activity induced AOS production by the Cf9 tobacco cells, although the levels were low (approximately 10 to 25% of the Avr9-induced values) and only the first peak was induced (data not shown).

DISCUSSION

The biochemical processes underlying *R/Avr* gene-mediated plant defense responses are of immense intrinsic interest, and if better understood could lead to the development of novel methods of disease control. To improve our understanding of plant defense, we study the tomato *Cf-9* gene and how it confers resistance to *C. fulvum* races that carry Avr9. We attempted to use Cf9 tomato suspension cultures to study the Avr9 response but, like others (Honée et al. 1998), we did not obtain Avr9-responsive cultures. By using transgenic tobacco suspension cultures carrying *Cf-9* as the experimental material, we were able to specifically investigate *Cf-9*-dependent responses. *Cf-9*- and Avr9-dependent events have been studied previously in tomato cotyledons (Hammond-Kosack et al. 1996; May et al. 1996). However, to investigate the earliest events in the signaling pathway, the use of suspension cultures offers unique opportunities for synchronous cell challenge and

pharmacological and biochemical investigation. We show here that these Cf9 cultures rapidly produce AOS in response to Avr9 and that these cultures can be used to study the events that activate the components of the defense response. Furthermore, although there are certain parallels with other plant suspension culture systems based on either bacterial *Avr* gene-dependent elicitation or on elicitation by nonspecific elicitors, our work reveals unique features of the *Cf-9/Avr9*-dependent system. It is interesting to compare our analysis of the *Cf-9*-dependent defense response with the *Pto*-dependent response studied by Chandra et al. (1996b), the *Avr*-dependent response of soybean cell cultures to avirulent bacterial strains (Levine et al. 1994), and the response of various cultures to non-race-specific elicitors (see Scheel 1998).

AOS production: Kinetics and possible involvement of an NADPH oxidase-like enzyme.

In numerous reports, plant cell cultures have been shown to rapidly produce AOS in response to elicitors extracted from fungal cell walls, to oligogalacturonides isolated from plant cell walls, and more slowly to bacteria that carry avirulence genes. But this report together with that of the oxidative burst associated with *Pto* function (Chandra et al. 1996b) constitute the only examples of an oxidative burst produced by transgenic material in which the only difference is the presence or absence of the *R* gene. However, whereas the *Pto* cultures were treated with the whole bacteria, in our study a well-characterized elicitor was used.

In elicitor-treated plant cultures AOS can be detected within several minutes, with a maximum of between 20 and 40 min (Baker et al. 1993; Bolwell et al. 1995; Desikan et al. 1996; Jabs et al. 1997; Legendre et al. 1993; Levine et al. 1994; Nürnberger et al. 1994; Vera-Estrella et al. 1992; Viard et al. 1994). Treatment with bacteria or bacterial elicitor leads to a biphasic accumulation of AOS (Baker and Orlandi 1995; Baker et al. 1993; Chandra et al. 1996b; Levine et al. 1994). The first burst of AOS was described as nonspecific since it was induced by both virulent and avirulent bacteria, and a second was considered race specific since it was detected solely with the avirulent bacteria and may reflect the time required for the bacterial signal to be delivered to the plant cells. The observation that pure Avr9 also induces a biphasic AOS accumulation in Cf9 tobacco cells indicates that these two phases might have an intrinsic physiological basis. This resembles the results of Jabs et al. (1997) in which an elicitor evoked a biphasic AOS accumulation in parsley cell cultures. This could be due to a primary phase that is not dependent on gene induction and a secondary phase that is, or that is potentiated in some other way by the initial phase.

An enzyme complex similar to the well-characterized mammalian NADPH oxidase has been suggested as responsible for the AOS production in plant defense responses (Lamb and Dixon 1997; Wojtaszek 1997). This enzymatic complex in neutrophils transfers electrons from intracellular NADPH to extracellular molecular oxygen, producing the superoxide anion. Although the term oxidative burst was borrowed from neutrophils, the increase in oxygen uptake associated with AOS production in plant cultures has not always been studied. In the Cf9 tobacco, a significant increase in oxygen consumption was detected on elicitation, which was inhibited by DPI. The detection of superoxide

Table 2. Inhibition of active oxygen species (AOS) accumulation by phospholipase A₂ (PLA₂) inhibitors^a

Compound	Concentration (μ M)	Activity (%)
Bromophenacyl bromide	5	100
	15	0
Quinacrine	1	105
	10	59
	100	2
Chlorpromazine	5	67
	20	24
	50	0

^a Cf9 tobacco suspension cultures were treated with the compounds and concentrations indicated 5 min prior to when IF (Avr9⁺) was added. AOS concentration was determined 45 min after Avr9 treatment.

proved difficult in the *Cf-9/Avr9* system, probably because it dismutates immediately to H_2O_2 .

Increase in NADPH oxidase activity has been described in plasma membranes isolated from plant material after fungus or elicitor treatment. Potato tubers treated with incompatible *Phytophthora infestans* showed an enhanced NADPH-dependent superoxide generating activity that was SOD-inhibitable, whereas the plasma membranes from tubers treated with compatible *P. infestans* did not show enhanced activity (Doke and Miura 1995). An increase in NADPH oxidase activity has also been observed in membranes from Cf5 tomato cells treated with a crude IF containing Avr5 elicitor activity. In addition, with antibodies raised against the cytosolic components of human NADPH oxidase, a movement of the cytosolic cross-reacting materials to the membrane was detected (Xing et al. 1997). These two contributions provide additional evidence for a similarity with the mammalian neutrophil system, though the existence of p47 and p67 homologues in plants has still not been proven by gene isolation.

The rapid AOS detection in Cf9 tobacco cells contrasts to the situation in plants, in which the earliest AOS detection occurred 1 to 2 h after injection with Avr9 (Hammond-Kosack et al. 1998; May et al. 1996). The flooding with IF could dilute the superoxide, such that it is detectable only when the leaf or cotyledon dries. Alternatively, the different environment in leaves, which may carry more catalase, peroxidase, or SOD, could make the AOS more unstable, so that it takes longer for sufficient AOS to be produced to be detectable. Another possibility is that the Avr9 elicitor diffuses much more quickly through the relatively uncross-linked cell wall of suspension culture cells, but in leaves it reaches the plant cell membrane more slowly.

Intermediates in the signaling pathway.

(i) Ion fluxes. Changes in ion fluxes across the plasma membrane have been reported in elicitor-treated plant systems as one of the most rapid responses (Yang et al. 1997). These include efflux of K^+ and Cl^- and influx of Ca^{2+} and H^+ . Alkalinization of the media was observed in Avr9-treated Cf9 tobacco cells. This could be either the result of H^+ uptake or a consequence of $\cdot O_2^-$ dismutation to H_2O_2 . This extracellular alkalinization was not inhibited by DPI levels that inhibit the AOS production. Therefore, it must be concluded that this was not due to production of H_2O_2 but to H^+ uptake by the cells. Alkalinization has also been reported in other elicitor-treated plant systems (Bolwell et al. 1995; Jabs et al. 1997) with the exception of Cf5 tomato cells, in which an extracellular acidification was reported as a consequence of an increase in H^+ -ATPase activity (Vera-Estrella et al. 1994).

(ii) Calcium. The inhibition of the *Cf-9-* and *Avr9-*dependent AOS accumulation by EGTA, lanthanum, and gadolinium suggests that uptake of extracellular calcium is required as an intermediate component in the pathway. An elicitor-inducible influx of calcium necessary for the oxidative burst has been postulated in several plant systems (Jabs et al. 1997; Schwacke and Hager 1992; Tavernier et al. 1995), and recently elicitor-activated plasma membrane calcium channels have been detected in both tomato and parsley protoplasts by patch-clamp techniques (Gelli et al. 1997; Zimmermann et al. 1997). With aequorin-transformed tobacco cells it was possible to measure cytoplasmic Ca^{2+} upon different treatments

(Chandra and Low 1997). Treatment of these aequorin-containing tobacco cells with oligogalacturonic acid induced both the influx of Ca^{2+} and oxidative burst, whereas treatment with harpin induced an oxidative burst but not influx of Ca^{2+} . This result shows that different elicitors can induce the AOS production by the use of different signaling components. This was also suggested in soybean cultures, in which the pathway activated by oligogalacturonic acid appeared to involve G-proteins and phospholipase C, whereas elicitation by a cell wall preparation from *Verticillium dahliae* involved PLA₂ (Chandra et al. 1996a). The fact that different elicitors induce AOS production with different components reemphasizes the need to compare the data on non-race-specific elicitors with the *R/Avr*-dependent pathway. It is now clear that there may be multiple *R/Avr*-dependent pathways that can be distinguished in *Arabidopsis thaliana* by their differential requirement for *EDS1* and *NDR1* (Aarts et al. 1998).

Although those approaches suggest the involvement of Ca^{2+} , little is known about the calcium receptors. The inhibitory effect observed with calmodulin antagonist (TFP, W-7) in the Avr9-induced AOS accumulation suggests involvement of a calmodulin-like protein in the pathway leading to activation of the oxidase. Similar inhibitory effects have been described in potato tubers (Miura et al. 1995) and tomato cultures (Xing et al. 1997). In addition, transgenic tobacco expressing a dominant-acting calmodulin mutant gene showed a stronger oxidative burst than control plants in response to mechanical stress and elicitors such as cellulose and harpin (Harding et al. 1997).

(iii) Protein kinases. Several lines of evidence have implicated protein phosphorylation as playing a key role in transducing elicitor signals in plant defense (Suzuki and Shinshi 1996). The inhibitory effect of staurosporine and K-252a suggests that the *Cf-9-* and *Avr9-*dependent AOS production also requires protein phosphorylation. The AOS production stimulated by the protein phosphatase inhibitor cantharidin suggests that there is a delicate balance between kinase and phosphatase action and that, by inhibiting phosphatase, proteins can stay in the phosphorylated form, activating AOS production. Furthermore, the cooperative effect observed between cantharidin and Avr9 suggests that both act in the same pathway. Cantharidin has been shown to induce AOS accumulation in other plant cultures (Levine et al. 1994; Mathieu et al. 1996) and a cooperative effect between cantharidin and salicylic acid has been reported recently (Shirasu et al. 1997). The activation of the mammalian NADPH oxidase system is associated with phosphorylation of the regulating proteins p47 and p67. It has been postulated that these cytosolic components are phosphorylated mainly by a protein kinase C (PKC) although the oxidase can also be activated in a PKC-independent pathway (Jones 1994; Morel et al. 1991; Segal and Abo 1993). PKC inhibitors had no effect on the *Avr9-* and *Cf-9-*dependent oxidative burst, suggesting no involvement of this kinase. Similar results have been reported in Cf5 tomato cells (Xing et al. 1997). It is likely that, after elicitor recognition, a complicated network of phosphorylation is activated, and some protein kinases could be involved in the activation of the oxidase and others could follow an independent pathway.

(iv) Phospholipase signaling. The putative involvement of phospholipase activities, implicated in the activation of the oxidative burst in neutrophils, has received less attention in

studies on the signaling leading to AOS accumulation in plants. The AOS accumulation by Cf9 cells in response to Avr9 was inhibited by PLA₂ inhibitors shown to be effective against the oxidative burst induced in neutrophils in response to phorbol 12-myristate 13-acetate (PMA) or opsonized zymosan (OZ) (Dana et al. 1994; Henderson et al. 1989). Chandra et al. (1996a) demonstrated that some of these compounds also inhibit PLA₂ activity in soybean cultures, confirming its inhibitory effect in plant cells. In this study, soybean cultures produced an oxidative burst in response to an elicitor from *Verticillium dahliae* that was dependent on PLA₂ activity, but the same cultures mounted an oxidative burst in response to OGA that was independent of PLA₂ (Chandra et al. 1996a). Treatment of neutrophils with both PMA and OZ resulted in a translocation of PLA₂ from the cytosol to the membrane (Hazan et al. 1997). It has been hypothesized that in neutrophils the role of PLA₂ products is to modulate positively the NADPH oxidase in a parallel pathway to the phosphorylation and translocation to the membrane of p47 and p67 since, in the presence of PLA₂ inhibitors, NADPH oxidase activity was not detected but these two components translocated normally to the membrane (Dana et al. 1994). It would be desirable in future work to directly detect activation of PLA₂ enzyme activity, and identify any released signaling fatty acids.

AOS and cell death: A contrast to other systems.

It has been suggested that AOS could promote plant cell death leading to the hypersensitive response (Tenhaken et al. 1995). Infiltration of Cf9 tomato cotyledons with Avr9 resulted in cell death. This was partially inhibited in high humidity, suggesting a role for dehydration as well as AOS (Hammond-Kosack et al. 1996). Treatment of Cf9 suspension cultures with Avr9 did not induce cell death after 15 to 20 h of treatment (data not shown), which is inconsistent with the idea that AOS is sufficient to cause cell death, although it is possible that in the cell culture assay the stability and concentration of AOS are different, resulting in a less toxic effect. Similarly, elicitor-treated tomato (Vera-Estrella et al. 1992) and parsley (Jabs et al. 1997) cell cultures did not die although AOS accumulation was detected. In contrast, host cell death has been described in harpin-treated *A. thaliana* (Desikan et al. 1996), bacteria-treated soybean cells (Levine et al. 1994), and xylanase-treated tobacco cells (Yano et al. 1998). It has been suggested that uptake of Ca²⁺ induced by H₂O₂ is necessary and sufficient to trigger apoptosis in soybean cultures, the sustained levels of H₂O₂ being responsible for this cell death (Levine et al. 1996). However, in Cf9 tobacco cultures and parsley cultures (Jabs et al. 1997) the levels of H₂O₂ reach levels similar to or higher than those reached in soybean cultures (Shirasu et al. 1997), for a similar duration, without cell death. These data suggest that H₂O₂ production is not sufficient to cause cell death, and that other mechanisms must be activated for cell death to occur during the plant defense responses.

Models for Cf-9 action in response to Avr9:

Signaling and defense.

The majority of the isolated *R* genes possess an LRR domain that could be involved in the recognition of *Avr* gene-dependent ligands by protein-protein interactions (Hammond-Kosack and Jones 1997), and following this pathogen recog-

niton, the *R* protein presumably activates signaling cascade(s) that coordinate plant defense responses. Most of the *R* genes have putative signaling domains. The tomato *Cf* gene sequences predict a mainly extracytoplasmic protein with numerous LRRs and a very short C-terminal membrane anchor without an obvious signaling domain (Hammond-Kosack and Jones 1997). It still remains unclear how this putative transmembrane receptor initiates the signaling pathway. The binding of *Avr* to *Cf* could result in conformational changes that alter the membrane properties and initiate the signaling, perhaps by permitting calcium uptake by the cells. Alternatively, the *Cf* protein could interact with another transmembrane protein with LRR-kinase domains similar to, for example, *A. thaliana* RLK5 (Walker 1993) or to Xa21 (Song et al. 1995) and in this way activate the phosphorylation cascade. A third possibility has been proposed, in which *Avr9* does not bind to *Cf-9* but to some other receptor (Kooman-Gersmann et al. 1996). In this study, *Avr9* was clearly shown to bind at high affinity to tomato membranes independently of the presence or absence of *Cf-9*. This *Avr9* binding protein is widespread in Solanaceous species. However, *Cf-9* is required for resistance of tomato to races of *C. fulvum* carrying *Avr9*, which supports the idea of an important role for *Cf-9* in recognition. At present it is not clear whether the receptor that binds *Avr9* detected by Kooman-Gersmann et al. (1996) is a pathogenicity target of *Avr9*, or plays a role in *Cf-9* function, or both.

We conclude that *Cf-9* confers on tobacco suspension cultures the capacity to respond to *Avr9* with a very rapid (5 min) production of H₂O₂. After the interaction of *Avr9* with *Cf-9* or other proteins, a *Cf-9*-dependent cascade of signaling events is activated. Uptake of calcium, calmodulin-like proteins, kinase, and PLA₂ activities are probably intermediate components. However, it is probable that the network of signaling intermediates is complex and involves extensive cross talk. Whatever the signaling pathway, Cf9 tobacco cell cultures provide an excellent system to analyze the biochemical basis of *R* gene-dependent signaling.

MATERIALS AND METHODS

Generation and maintenance of tobacco suspension cultures.

Leaf pieces taken from tobacco plants of the genotypes 34.1B, 34.1I (Hammond-Kosack et al. 1998), Petit Havana, and Petit Gerard growing in tissue culture were plated on Murashige and Skoog (MS) medium supplemented with sucrose (30 g/liter), B5 vitamins, MES (0.59 g/liter), agarose (6 g/liter), 2,4-dichlorophenoxyacetic acid (1 mg/liter), and kinetin (0.1 mg/liter) at pH 5.7, and incubated in the dark at 25°C. Once callus tissue developed, this was chopped and transferred into liquid medium (as above but without MES and agarose) at pH 5.7 and incubated in a rotary shaker (Model G-53, New Brunswick Scientific, Edison, NJ) at 110 rpm, 25°C, in the dark. Cells were transferred to fresh medium every 2 weeks.

Elicitors.

IFs from Petit Havana tobacco plants, either nontransgenic or expressing the *Avr9* peptide (SLJ6201A) (Hammond-Kosack et al. 1994) grown under the same conditions, were extracted after infiltration with distilled water as described by

De Wit and Spikman (1982) and frozen at -20°C until use. The IF from Avr9-expressing plants was designated IF (Avr9⁺), and the IF from control plants IF (Avr9⁻).

R8K Avr9 peptide (Kooman-Gersmann et al. 1997) was synthesized by 9-fluorenylmethyloxycarbonyl (fmoc) solid-phase chemistry on a model 432A Synergy peptide synthesizer from Applied Biosystems (Warrington, UK) and purified by high-performance liquid chromatography (HPLC) with prepLC C18 delta-pack cartridges in a 626 LC system with 996 photodiode array (Waters, Watford, UK). After solubilization in distilled water, the peptide was incubated in 100 mM Tris buffer at pH 8 for 4 h at room temperature and 22 h at 4°C . The activated peptide was frozen at -20°C until use.

Treatment of the cells.

Three- to 6-day-old cultures were collected by centrifugation ($1,500 \times g$ for 10 min) and resuspended in assay buffer (5 mM MES pH 6, 175 mM mannitol, 0.5 mM CaCl_2 , 0.5 mM K_2SO_4) and incubated in a controlled environment incubator shaker with a glass top (Model G-25, New Brunswick Scientific) at 180 rpm, 22 to 24°C for 30 min. This washing procedure was repeated two more times. Then, cells were resuspended in the assay buffer at a density of 0.025 g/ml. After at least 3 h of equilibration, the experiments were started. Thus, all experiments were conducted in the light, on material that had been previously grown in the dark.

Determination of AOS.

The production of AOS was measured by chemiluminescence from the ferricyanide-catalyzed oxidation of luminol as described by Schwacke and Hager (1992). Briefly, at the times indicated 0.2-ml aliquots were removed and mixed in a cuvette with 0.6 ml of potassium phosphate buffer (50 mM, pH 7.9) and 0.1 ml of luminol 1.1 mM prepared in the phosphate buffer. The reaction was started by addition of 0.1 ml of 14 mM ferricyanide prepared in water and after a delay of 5 s the emission of light was determined during 20 s with a luminometer (Lumat LB 9501; Berthold, St. Albans, UK). All the inhibitors were tested to ensure that they did not alter the chemiluminescence assay.

Determination of oxygen uptake.

Oxygen uptake was determined with an oxygen electrode from Hansatech (King's Lynn, UK). Briefly, 2 ml of cell suspension washed and equilibrated as indicated above was placed in the oxygen electrode chamber with or without DPI. The oxygen uptake was monitored continuously. After approximately 8 to 9 min, 50 μl of control or elicitor solution was added. The increase in the rate of oxygen uptake between the rates prior to addition and after each addition was calculated.

Determination of cell death.

Cell death was determined as described by Levine et al. (1994). Briefly, cultures were incubated with 0.025% Evans blue for 10 min and then washed repeatedly with water until no more dye was obtained in the supernatant. The dye bound to dead cells was solubilized in 50% methanol with 1% sodium dodecyl sulfate (SDS) for 30 min at 50°C and then the absorbance was determined at 600 nm.

Chemicals.

Catalase, SOD from bovine erythrocytes, Evans blue, calcium ionophore A23187, K-252a, staurosporine, 4-bromophenacyl bromide, arachidonic acid, cantharidin, amphotericin B, linolenic acid, chloropromazine, W-7 (*N*-[6-aminohexyl]-5-chloro-1-naphthalenesulfonamide, HCl), MES, diphenyleneiodonium, ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), potassium ferricyanide, luminol, and salicylic acid were purchased from Sigma. Cypermethrin, okadaic acid, bisindolylmaleimide I, calphostin C, and trifluopromazine dimaleate were purchased from CalBiochem (Nottingham, UK). Gadolinium(III) chloride hexahydrate, and lanthanum chloride heptahydrate were purchased from Aldrich (Gillingham, UK).

Experimental design.

For each genotype / treatment combination at least two replica flasks were used in a single experiment, and each experiment was performed on at least three independent occasions. The variation between replica was always lower than 10%, although the magnitude of the response was slightly different between independent experiments. Data from each experiment are expressed as the means between all the replica flasks.

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