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Early Events in the Signal Pathway for the Oxidative Burst in Soybean Cells Exposed to Avirulent *Pseudomonas syringae* pv *glycinea*[1]

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Soybean (*Glycine max*) cv Williams 82 suspension cultures exhibit an oxidative burst approximately 3 h after challenge with *Pseudomonas syringae* pv *glycinea* (*Psg*) harboring the *avrA* (avirulence) gene. Pretreatment with the tyrosine (Tyr) kinase inhibitor herbimycin A or the serine/threonine kinase inhibitor K252a abolished the burst and subsequent induction of glutathione *S*-transferase. However, imposition of a 45-min rest period between pathogen challenge and subsequent addition of the kinase inhibitors resulted in escape from inhibition by herbimycin A, whereas inhibition by K252a persisted. Suramin, a G-protein inhibitor, inhibited the burst if added up to 90 min after pathogen challenge. The burst was also induced by the ion channel generator amphotericin B, and this induction was sensitive to suramin and K252a. Conversely, the ion channel blocker anthracene-9-carboxylate inhibited the *Psg:avrA*-induced burst. *Psg*:*avrA* rapidly induced Tyr phosphorylation of several proteins, and this was inhibited by herbimycin A or anthracene 9-carboxylic acid. These data suggest that the activation of ion channels is followed by an upstream Tyr kinase before the serine/threonine kinase-dependent steps in the signal pathway leading to the oxidative burst. *Psg:avrA*-dependent induction of phenylalanine ammonia-lyase was not inhibited by herbimycin or suramin, suggesting the operation of different signal pathways for the oxidative burst and phenylpropanoid-derived defense responses.

Plant cells respond to challenge with avirulent pathogens by mounting a multi-component defense response called the HR. Characteristic features of the HR include an oxidative burst leading to generation of H_2O_2 (Lamb and Dixon, 1997), localized cell death and cell wall cross-linking (Bradley et al., 1992; Levine et al., 1994), and synthesis of antimicrobial phytoalexins (Dixon and Harrison, 1990). The HR results from direct or indirect recognition of a microbial avirulence gene product by the product of a host resistance gene (de Wit, 1997; Parker and Coleman, 1997). Many avirulence and resistance genes have now been identified at the molecular level (Staskawicz et al., 1995; de Wit, 1997). In addition, biochemical studies have defined a number of microbial elicitor molecules that induce some or all of the responses induced by avirulent microbes (Ebel and Mithöfer, 1998). Such elicitors may or may not be direct products of microbial avirulence genes.

The oxidative burst in challenged plant cells resembles that exhibited by human neutrophils (Baggiolini and Wymann, 1990), producing H_2O_2 that originates from superoxide generated by a plasma membrane-associated NADPH oxidase (Lamb and Dixon, 1997). The major component (a gp91 homolog) of the plant oxidase complex has been cloned (Groom et al., 1996; Keller et al., 1997; Torres et al., 1998), and pharmacological experiments have shown that activation of the oxidase involves a protein kinase cascade that can be blocked by the Ser/Thr kinase inhibitor K252a (Levine et al., 1994; Yang et al., 1997).

Many studies over the past several years have reported the effects of pharmacological agents known to affect signaling in mammalian cells on the defense responses of cultured plant cells (for review, see Dixon et al., 1994; Yang et al., 1997; Ebel and Mithöfer, 1998). On the basis of such studies, ion fluxes (Jabs et al., 1997), calcium uptake (Stäb and Ebel, 1987), G-proteins (Legendre et al., 1992, 1993a), kinase cascades (Schwacke and Hager, 1992; Levine et al., 1994; Suzuko and Shinshi, 1996), and polyphosphoinositides (Legendre et al., 1993b) have been implicated as signal transduction components in the induction of phytoalexins or the oxidative burst. More recently, studies have reported the involvement of plant functional homologs of mammalian MAP kinases in plant defense signal transduction (Suzuki and Shinshi, 1995; Adam et al., 1997; Ligterink et al., 1997; Stratmann and Ryan, 1997; Zhang and Klessig, 1998). A feature of these kinases is their phosphorylation on both Ser/Thr and Tyr residues. However, little is known concerning Tyr phosphorylation in plants.

A large proportion of the pharmacological studies on plant defense signal transduction have utilized non-race-specific elicitor molecules as primary inducing agents.

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Abbreviations: DPI, diphenylene iodonium; GST, glutathione *S*-transferase; HR, hypersensitive response; MAP, mitogen-activated protein; PAL, Phe ammonia-lyase; *Psg*, *Pseudomonas syringae* pv *glycinea*.

With the exception of the tomato:*Pseudomonas syringae* pv *tomato* system (Lamb, 1994; Zhou et al., 1995, 1997), little biochemical information is available on signal transduction leading to the HR or defense gene induction in response to resistance-gene-mediated recognition of bacterial avirulence genes. We have developed a soybean cell culture system that responds, in a race-specific manner, to *Pseudomonas syringae* pv *glycinea* (*Psg*) carrying the *avrA* avirulence gene that is recognized by the corresponding *Rpg2* resistance gene in the soybean (*Glycine max*) cv Williams 82. Treatment of the cells with *Psg:avrA* results in a strong oxidative burst and isoflavonoid phytoalexin accumulation (Levine et al., 1994; Shirasu et al., 1997; Guo et al., 1998). The burst can be potentiated by physiological concentrations of the endogenous signal molecule salicylic acid (Shirasu et al., 1997), and also by chemical inhibitors of Ser proteases (Guo et al., 1998). Thus, this system is an excellent one for pharmacological dissection of *avrA*-mediated signal transduction.

In the present study, we investigate the potential involvement of Tyr phosphorylation in signal transduction utilizing the soybean: *Psg* cell culture system. We also examine the relative position in the signal transduction pathway of Tyr phosphorylation in relation to ion channel activity, G-protein activation, and Ser/Thr kinase(s). Our results indicate that Tyr phosphorylation is an early event in the induction of the oxidative burst by avirulent bacteria in soybean cells, and that it precedes the events requiring Ser/Thr phosphorylation.

MATERIALS AND METHODS

Chemicals

All protein kinase inhibitors were obtained from Calbiochem. GTP analogs, amphotericin B, and anthracene-9-carboxylate were purchased from Sigma. Protein kinase inhibitors, GTP analogs, and anthracene-9-carboxylic acid were dissolved in DMSO and maintained in 100× concentrated stocks. Control cultures were always treated with an equivalent amount of DMSO.

Maintenance and Inoculation of Soybean Cell Suspension Cultures

Soybean (*Glycine max* cv Williams 82) cell suspensions were subcultured every 7 d by 1:5 dilution in fresh Murashige and Skoog medium (Murashige and Skoog, 1962) containing 3% (w/v) Suc, 0.5 mg/L 2,4-D, and 0.5 mg/L 6-benzylaminopurine, pH 5.7. All experiments were performed starting 3.5 d post subculture. One-milliliter aliquots of suspension culture were transferred to 12-well tissue culture plates (1 mL/well) and maintained with circular rotation at 80 to 90 rpm.

Pseudomonas syringae pv *glycinea* (*Psg*) race 4 harboring the plasmid pLAFR1 carrying the *avrA* or *avrC* avirulence genes (Keen and Buzzell, 1991) was grown overnight in King's B medium (20 g of protease peptone, 10 mL of glycerol, 2.25 g of K_2HPO_4, and 1.5 g of $MgSO_4 \cdot 7H_2O$ L^{-1}, pH 7.2) supplemented with streptomycin (30 μg/mL) or kanamycin (50 μg/mL), respectively. The bacteria were then centrifuged, re-suspended in sterile water, and added to soybean suspension cultures at a final inoculum of 10^8 cfu/mL.

For conditioning experiments, the bacteria grown overnight were centrifuged gently, washed once with sterile distilled water, and re-suspended in filter-sterilized conditioned medium from 3.5-d-old soybean suspension cultures at 10^8 cfu/mL. The bacteria were grown in this medium in the presence or absence of various inhibitors for 90 min. They were then collected by centrifugation, washed twice with sterile distilled water, re-suspended in the sterile distilled water, and used as conditioned bacteria.

Measurement of H_2O_2 Accumulation and Cell Death

H_2O_2 was measured by monitoring the destruction of scopoletin fluorescence, and cell death was measured by Evan's blue staining, as described previously (Shirasu et al., 1997). All data represent the mean and SE from three or four independent experiments.

Protein Extraction, Immunoprecipitation, and Western Analysis

Total proteins were extracted from 1 mL of suspension cultures in ice-cold cell lysis buffer containing 50 mM Tris, pH 7.5, 2 mM EDTA, 2 mM EGTA, 150 mM NaCl, 1% NP40, 0.25% sodium deoxycholate, 1 mM DTT, 1 mM PMSF, 10 μg/mL each of leupeptin, pepstatin, aprotenin, 4 mM each of Na_3VO_4 and NaF, and one protease inhibitor cocktail tablet per 25 mL (Boehringer Mannheim). The protein extracts were clarified by centrifugation at 1,000*g* and were then either used for immunoprecipitations and/or directly for western analysis. In the immunoprecipitation reactions, 4 μg of mouse anti-(phospho-Tyr) monoclonal IgG2bκ 4G10 (Upstate Biotechnology, Lake Placid, NY) were employed in 1-mL reactions with gentle rocking overnight in the cold room. Immuno complexes were captured using protein A-agarose (Santa Cruz Biotech, Santa Cruz, CA), washed once with lysis buffer followed by three times with PBS, and re-suspended in 2× Laemmli sample buffer (Laemmli, 1970). Equal amounts of proteins were processed for SDS-PAGE analysis using Tris-Gly gels (Novex, San Diego).

In competition experiments, the complete immunoprecipitation reactions were incubated with 1 to 2 mM phospho-DL-Tyr, 1 mM phospho-DL-Thr, or 1 mM phospho-DL-Ser (Sigma). Western analysis of the proteins resolved by SDS-PAGE was carried out following transfer to a PVDF membrane (Immobilon-P, Millipore) and incubating with phospho-Tyr-specific monoclonal antibody or rabbit antiserum against maize GST (kindly supplied by Dr. Klaus Kreuz, Ciba Geigy, Basel). The primary antibody cross-reactions were detected by peroxidase-conjugated secondary antibodies against rabbit IgG from goat (1:10,000 dilution) (Bio-Rad) or against mouse IgG from sheep (1:2,000 dilution) (Amersham) and visualized by chemiluminescence (ECL detection system, Amersham). The blots were then exposed to x-ray film (X-OMAT, Kodak).

Extraction and Analysis of RNA

Total RNA was isolated from 200 mg fresh weight of tissue using the TRI-reagent method according to the manufacturer's instructions (Molecular Research Center, Cincinnati). For northern analysis, 20 μg of total RNA from each sample was run on a 1% agarose-formaldehyde gel and transferred to a Hybond-N membrane. The membranes were UV cross-linked in a Stratalinker (Stratagene). Hybridizations were carried out overnight at 65°C using a ^{32}P-labeled soybean PAL1 probe or a *Glomus versiforme* 18S rRNA probe using standard protocols (Sambrook et al., 1989). The 929-bp PAL1 fragment (Frank and Vodkin, 1991) was amplified (forward primer 5′CCAAGGAACCCCTATTGG3′; reverse primer 5′CCATTCCACTCCCCAAGG3′) from cv Williams 82 genomic DNA using standard PCR conditions in a Robocycler 96 (Stratagene). A 590-bp *G. versiforme* 18S rRNA RT-PCR fragment (Simon et al., 1992) was provided by Dr. Ignacio E. Maldonado-Mendoza (Noble Foundation). The membranes were washed twice at 65°C in 0.5× SSC and 0.2% (w/v) SDS, and exposed to x-ray film with intensifying screens

RESULTS

Effects of Inhibitors of Tyr and Ser/Thr Kinases on the Oxidative Burst Induced by *Psg:avrA*

The soybean cv Williams 82 contains the *Rpg2* resistance gene that recognizes the *avrA* avirulence gene of *Psg*, but not the *Rpg3* resistance gene that recognizes the *avrC* avirulence gene (Keen and Buzzell, 1991). Thus, challenging Williams 82 suspension cultures with *Psg:avrA* results in a sustained oxidative burst and subsequent cell death, whereas *Psg:avrC* induces only a very weak, early, nonspecific oxidative burst and no resultant cell death (Levine et al., 1994). Although somewhat variable between cell culture batches, the oxidative burst in response to *Psg:avrA* usually occurs around 180 min after contact with the bacteria, and reaches maximum levels of H_2O_2 release after 240 to 350 min (Shirasu et al., 1997).

It has previously been shown that the oxidative burst induced in soybean cv Williams 82 cells by *Psg:avrA* is strongly reduced by co-treatment with the protein Ser/Thr kinase inhibitor K252a (Levine et al., 1994; Guo et al., 1998), a compound that is highly effective in inhibiting protein Ser/Thr kinases in plant cell cultures, with an IC_{50} of 100 nM for in vivo inhibition of tomato cell culture defense responses, and a Ki of 15 nM for in vitro inhibition of tomato protein kinase (Grosskopf et al., 1990). Herbimycin and the isoflavone genistein have been widely used as pharmacological agents for inhibition of protein Tyr kinases in mammalian cells (Akiyama et al., 1987; Uckun et al., 1991; Riordan et al., 1998). Administration of herbimycin A (1.0–1.5 μM, IC_{50} 0.84 μM) to soybean cells 15 min prior to challenge with *Psg:avrA* considerably inhibited the extent of the subsequent oxidative burst (Fig. 1A). Under the same conditions, herbimycin A alone had no effect.

Preliminary experiments with genistein (50–100 μM) gave a less reproducible inhibition of the oxidative burst,

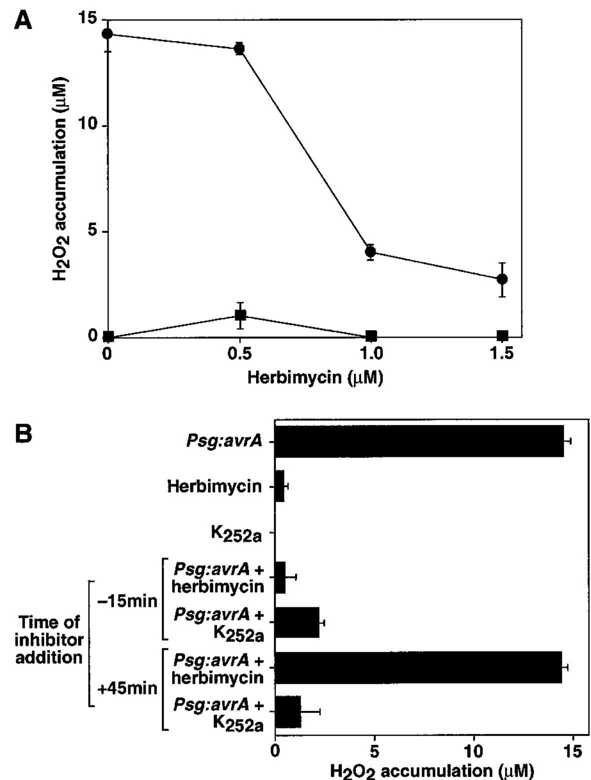

Figure 1. A herbimycin-inhibited step precedes a protein Ser/Thr kinase step in the oxidative burst signal pathway. A, Dose response for inhibition of the *Psg:avrA*-induced oxidative burst by herbimycin. Soybean suspension cells were treated for 4.5 h with H_2O (■, control), *Psg:avrA* (●, 10^8 cfu/mL), or *Psg:avrA* and various concentrations of herbimycin added 15 min prior to exposure to the bacteria. B, Escape from inhibition by herbimycin but not K252a. H_2O_2 accumulation was measured 4.5 h after treatment of cells with *Psg:avrA* or inhibitors (1 μM) alone, *Psg:avrA* plus inhibitors added 15 min prior to bacteria, or *Psg:avrA* added at zero time with inhibitors added 45 min later.

and variable inhibition was also seen with daidzein, the 5-deoxy-derivative of genistein routinely used as a negative control in mammalian Tyr kinase studies. Because genistein and daidzein are major isoflavonoid natural products in soybean, occurring as intermediates in an infection-induced phytoalexin defense pathway in this species (Graham et al., 1990), we decided not to pursue genistein further as a pharmacological agent.

Herbimycin A and K252a are similarly effective in blocking the oxidative burst if added 15 min prior to exposure to *Psg:avrA* (Fig. 1B). However, if the inhibitors are added 45 min after exposure to *Psg:avrA*, the cells completely escape from inhibition by herbimycin A, but the level of inhibition by K252a remains unaltered. We show later that inhibition by K252a is retained at least up to the period of H_2O_2 release. These data suggest that the event(s) inhibited by herbimycin A occur earlier in the signal pathway than the Ser/Thr kinase(s) inhibited by K252a, and that Ser/Thr phosphorylation is continuously required for the oxidative burst, whereas the requirement for putative Tyr phosphorylation is more transient.

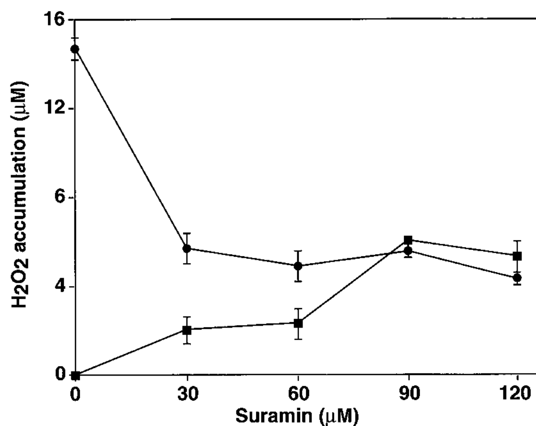

Figure 2. Dose response for the effects of the G-protein inhibitor suramin on the oxidative burst. Suramin was added alone or 15 min prior to exposure of soybean cells to *Psg:avrA*, and H_2O_2 accumulation was determined after 4.5 h. ●, *psg:avrA*; ■, control.

Involvement of G-proteins in the Oxidative Burst Induced by *Psg:avrA*

It has been suggested that heterotrimeric G-proteins function downstream of elicitor reception in the induction of the oxidative burst (Legendre et al., 1992, 1993a). To assess the involvement of G-proteins in our system, we first tested the effects of GTP homologs. GTP-γ-S, a nonhydrolyzable GTP analog that locks G-proteins in the active state, reproducibly doubled the amount of H_2O_2 released when added at 50 to 100 μM (EC_{50} 37.5 μM) 15 min prior to challenge with *Psg:avrA*. This effect of GTP-γ-S was *avr* gene dependent, because the compound did not induce or potentiate H_2O_2 production when added in conjunction with *Psg:avrC*. However, GDP-β-S, which locks G-proteins in the inactive state, was totally inactive in these assays, suggesting that there may be uptake problems for GTP analogs in DMSO-treated soybean cells, and that the extent of the response to GTP-γ-S may be an underestimate.

Suramin interferes with the GTP binding site of the alpha subunit of G-proteins (Chahdi et al., 1998) and also inhibits binding of growth factors to their receptors in mammalian systems (Mills et al., 1990). Between 30 and 120 μM (I_{50} 26 μM), suramin caused an approximately 60% decrease in H_2O_2 production when added 15 min prior to challenge with *Psg:avrA*. Increasing the concentration of suramin did not significantly reduce H_2O_2 production further, presumably because suramin itself can induce a detectable oxidative burst at concentrations above 30 μM (Fig. 2).

We next used pharmacological inhibition studies to help delineate the relationships between potential Tyr phosphorylation, G-protein activation, and Ser/Thr kinase involvement in the oxidative burst. The oxidative burst potentiated by GTP-γ-S was completely blocked by 1 μM K252a or 5 μM DPI, the latter being a suicide inhibitor of the NADPH oxidase (O'Donnell et al., 1993) with an I_{50} value of 2 μM for inhibition of the oxidative burst in soybean cells (Levine et al., 1994). This suggests that G-protein mediated events are upstream of the Ser/Thr kinase-mediated steps in the signal transduction pathway to activation of the NADPH oxidase (Fig. 3). Potentiation of the *Psg:avrA* induced oxidative burst by GTP-γ-S was completely blocked by suramin (Fig. 3), which is consistent with the effect of GTP-γ-S being mediated via a receptor-coupled G-protein. However, in the presence of GTP-γ-S, suramin did not reduce the burst below the level observed in the presence of *Psg:avrA* alone. Herbimycin A further reduced the burst potentiated by GTP-γ-S to a level below that obtained with *Psg:avrA* alone, suggesting that Tyr phosphorylation is somehow linked to G-protein action in this system.

Although the inhibitory effect of herbimycin A was lost when the compound was added 45 min after challenge with *Psg:avrA* (Fig. 1B), suramin inhibited the inducible oxidative burst when added at least 90 min after pathogen challenge (Fig. 4). Under the same experimental conditions, K252a and DPI completely inhibited the *Psg:avrA*-inducible oxidative burst when added at least 180 min after pathogen challenge. These results suggest that G-protein-mediated events occur between the Tyr and the Ser/Thr phosphorylation events in the signal pathway.

Effect of Ion Channel Generators and Blockers on the Oxidative Burst Induced by *Psg:avrA*

Defense responses can be induced in parsley suspension cultures by treatment with amphotericin B, a compound that forms artificial ion channels. Furthermore, a MAP kinase is phosphorylated in this system in a manner reversible by an ion channel blocker (Jabs et al., 1997; Ligterink et al., 1997). To test the involvement of ion channels in signaling in the soybean cells, we first studied the effect of amphotericin B as an inducer of the oxidative burst in the absence of bacteria. Preliminary experiments indicated that 50 to 100 μM amphotericin B was optimal for inducing the oxidative burst, with an EC_{50} of approximately 25 μM. Herbimycin A, suramin, K252a, and DPI each inhibited the induction of the oxidative burst by 50 μM amphotericin B in

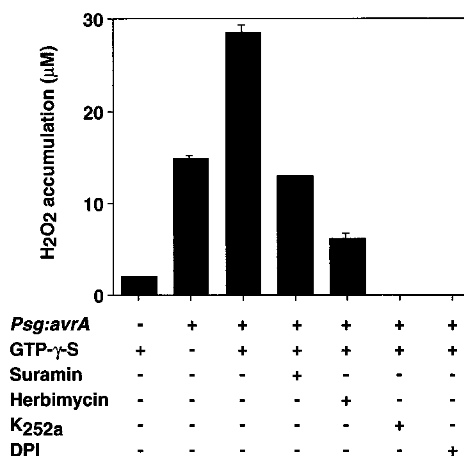

Figure 3. Effects of protein kinase inhibitors and suramin on the oxidative burst induced by *Psg:avrA* in the presence of GTP-γ-S. GTP-γ-S, herbimycin (1 μM), suramin (90 μM), K252a (1 μM), or DPI (5 μM) were added 15 min prior to treatment of cells with *Psg:avrA*, and H_2O_2 accumulation was determined 4.5 h after challenge.

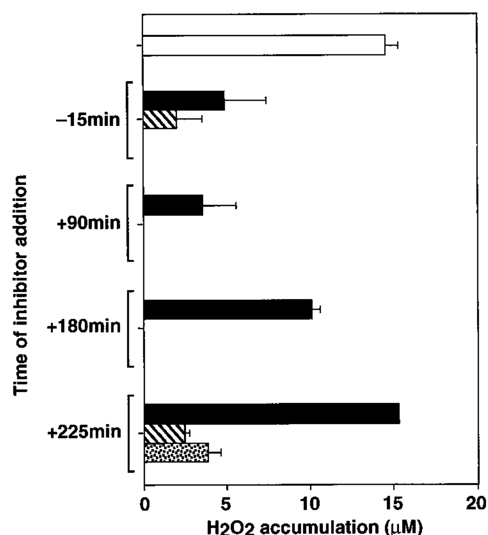

Figure 4. Escape of the *Psg:avrA* induced oxidative burst from inhibition by suramin. Suramin (90 μM), K252a (1 μM), or DPI (5 μM) was administered at the times shown to soybean suspension cultures treated with *Psg:avrA*, and accumulation of H_2O_2 was determined 4.5 h after exposure to the pathogen. White bars, *Psg:avrA*; black bars, *Psg:avrA* plus suramin; striped bars, *Psg:avrA* plus K252a; stippled bars, *Psg:avrA* plus DPI.

a manner comparable to their inhibition of the *Psg:avrA* induced burst (Fig. 5). Anthracene 9-carboxylate, an ion channel blocker, inhibited the *Psg:avrA* inducible oxidative burst at concentrations between 300 and 400 μM (I_{50} 340 μM). These results are consistent with changes in ion channel activity preceding the early Tyr phosphorylation-dependent step(s) in the signal pathway to the oxidative burst.

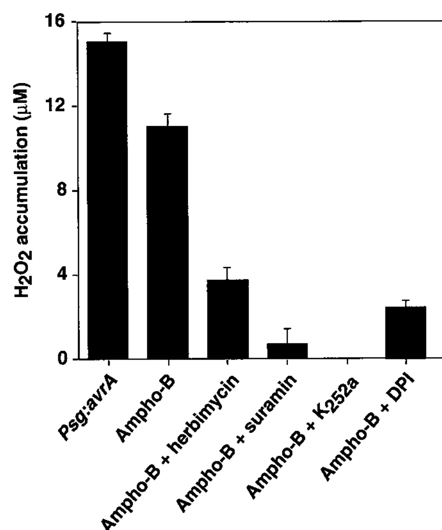

Figure 5. Inhibition of the oxidative burst induced by amphotericin B. Soybean suspension cells were treated with *Psg:avrA* alone, amphotericin B alone (Ampho-B, 50 μM), or amphotericin B preceded 15 min earlier by herbimycin (1 μM), suramin (90 μM), K252a (1 μM), or DPI (5 μM). The accumulation of H_2O_2 was determined 4.5 h after inducer treatment.

To ensure that the effects of the above inhibitors were not simply the results of cellular toxicity, we measured the level of cell death in the soybean suspensions by Evan's blue staining 5 h post application for each inhibitor (herbimycin, suramin, K252a, DPI, or anthracene 9-carboxylate) at the concentrations used in the above studies (1.2–10 times the I_{50} value depending on the inhibitor) in the presence of *Psg:avrA* relative to control levels. At this time, the level of cell death in bacterially induced cells was approximately 20% above control values, and the level of cell death in all bacteria plus inhibitor treatments was within this value and the control value (data not shown). However, higher concentrations of the inhibitors did exhibit cellular toxicity within the time periods of the experiments, and were not used.

Pre-Conditioning of Bacteria and Effects of Pharmacological Agents on *Psg:avrA*

Psg:avrA pre-incubated in conditioned soybean cell culture medium for 90 min induced an earlier onset of the oxidative burst (by approximately 90 min) than bacteria pre-incubated in water (Fig. 6). This effect was not observed if *Psg:avrA* were incubated in fresh Murashige and Skoog medium (data not shown). To verify that the inhibitors employed in the present investigation do not exert their effects on *Psg:avrA* itself, we grew bacteria in conditioned medium from soybean suspension cultures for 90 min in the presence of the same concentrations of herbimycin, suramin, K252a, and anthracene 9-carboxylate shown to inhibit the oxidative burst in the cell cultures, and then washed the bacteria prior to addition to soybean cells. Addition of inhibitors during the incubation did not affect the ability of the bacteria to induce an early oxidative burst (all values for H_2O_2 production were 75%–100% of the value obtained with non-pretreated bacteria). However, treatment with the bacterial protein synthesis inhibitor lin-

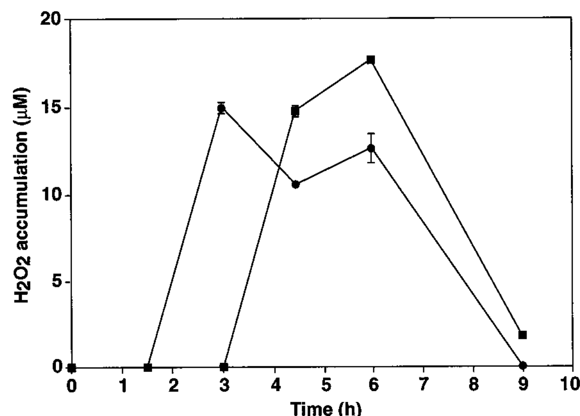

Figure 6. Effect of preconditioning *Psg:avrA* with culture medium from soybean suspension cultures. Bacteria were re-suspended in culture medium filtrate from 3.5-d-old soybean cells, and incubated for 90 min (●, conditioned). Unconditioned bacteria (■) were re-suspended in water and incubated for 90 min. Bacteria were then added to soybean suspensions, and H_2O_2 production was measured at the times shown.

comycin strongly inhibited the ability of the bacteria to induce the burst (H_2O_2 production was reduced to 15% of the value obtained with non-pretreated bacteria).

Effects of Inhibitors on Downstream Defense Response Gene Induction

GST is an anti-oxidant-response enzyme that is induced directly in soybean cells by the H_2O_2 produced in the oxidative burst (Levine et al., 1994). We utilized an anti-serum against maize GST (Flury et al., 1995) to determine the effects of the various pharmacological reagents on a downstream response enzyme as further confirmation of their effects on signal transduction in soybean. Figure 7 shows a western blot of total proteins extracted from soybean cells 4.5 h after exposure to *Psg*:*avrA* in the presence of the different inhibitors. Control cells and cells exposed to *Psg*:*avrC* showed no signal at 39 kD following development of blots with anti-(GST) serum and secondary antibody, whereas a strong signal was seen in cells treated with *Psg*:*avrA*. This response was inhibited by herbimycin A, suramin, and K252a. Perhaps surprisingly, amphotericin B alone did not induce GST, whereas anthracene 9-carboxylate increased the level of GST induction in response to avirulent bacteria.

Northern analysis revealed that, of all the inhibitors used in the present work, only K252a significantly inhibited induction of PAL transcripts in response to *Psg*:*avrA* (Fig. 8). This indicates that the herbimycin- and suramin-sensitive events are in a separate signal pathway from that which activates expression of phenylpropanoid-based defenses. However, unlike GST, PAL induction was stimulated by the addition of amphotericin B alone.

Effects of Inhibitors on Protein Tyr Phosphorylation

To provide direct evidence that *Psg*:*avrA*-mediated signal transduction involves Tyr phosphorylation, we used anti-(phospho-Tyr) monoclonal antibodies to immunoprecipitate and western blot proteins from soybean cells treated with *Psg* in the presence or absence of the various inhibitors. Four Tyr-phosphorylated proteins were revealed by this analysis. One approximately 19-kD protein was present at low levels in extracts from untreated cells and was strongly induced by *Psg*:*avrA* (Fig. 9). A slightly larger protein of approximately 20 kD was not present in uninduced cells, and was induced to relatively low levels in

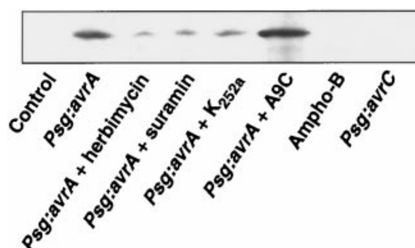

Figure 7. Effects of inhibitors on induction of GST by *Psg*:*avrA*. Cells were treated with *Psg*:*avrA* alone or *Psg*:*avrA* following pretreatment 15 min earlier with herbimycin (1 μM), suramin (90 μM), K252a (1 μM), amphotericin B (Ampho-B, 50 μM), or anthracene 9-carboxylic acid (A9C, 400 μM). Cells were untreated (control), or were treated with amphotericin B alone or with *Psg*:*avrC*. Cells were harvested 4.5 h after challenge, and proteins were extracted and resolved by SDS-PAGE, followed by western blotting and development of the blot with anti-(maize GST) serum.

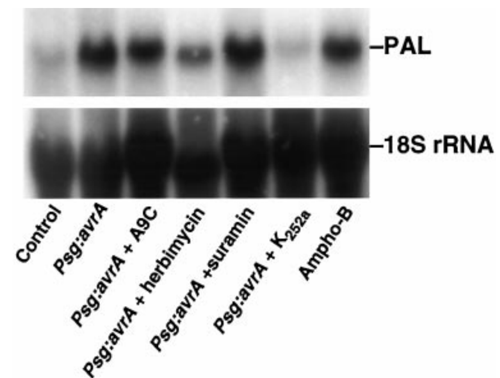

Figure 8. Effects of inhibitors on induction of PAL transcripts by *Psg*:*avrA*. Cells were treated with *Psg*:*avrA* alone or *Psg*:*avrA* following pretreatment 15 min earlier with anthracene 9-carboxylic acid (A9C, 400 μM), herbimycin (1 μM), suramin (90 μM), K252a (1 μM), or amphotericin B (50 μM). Cells were untreated (control), or were treated with amphotericin B (Ampho-B) alone. Cells were harvested 4.5 h after challenge, and total RNA was extracted and subjected to northern analysis using soybean PAL as a probe. Blots were then stripped and re-hybridized with *G. versiforme* rRNA (loading and transfer control).

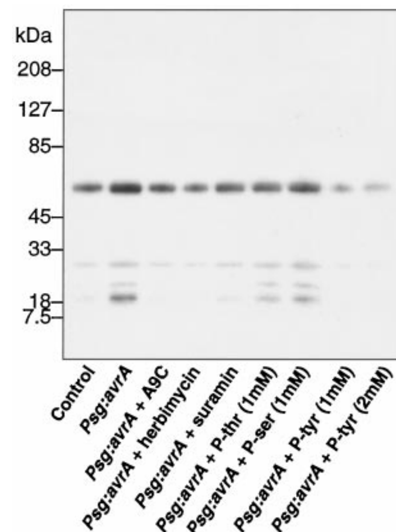

Figure 9. Immunoprecipitation/western blot analysis of phospho-Tyr-containing proteins. Cells were treated for 30 min with *Psg*:*avrA* alone or *Psg*:*avrA* following pretreatment 15 min earlier with anthracene 9-carboxylic acid (400 μM), herbimycin (1 μM), or suramin (90 μM). Extracts were prepared and immunoprecipitated with anti-(phospho-Tyr) monoclonal antibodies. Immunoprecipitations were also carried out on extracts from cells treated with *Psg*:*avrA* alone, in the presence of the competitor amino acids phospho-Ser (1 mM), phospho-Thr (1 mM), or phospho-Tyr (1 mM or 2 mM). Immunoprecipitates were resolved by SDS-PAGE and the gels were subjected to western analysis using anti-(phospho-Tyr) monoclonal antibodies.

response to *Psg:avrA*. The two other proteins, approximately 28 and 55 kD, were present in control cells at low and high levels, respectively, and were induced to approximately twice the control levels by bacteria. To test specificity, the immunoprecipitations were also carried out in the presence of phospho-Thr, phospho-Ser, or phospho-Tyr at 1 and 2 mM. Phospho-Tyr competed out the immunoprecipitation of the three lower M_r proteins and strongly reduced the amount of precipitated 55-kD protein, whereas phospho-Ser and phospho-Thr did not. This confirms the specificity of the antibodies for proteins with Tyr phosphorylation (Suzuki and Shinshi, 1995; Stratmann and Ryan, 1997).

Pretreatment of *Psg:avrA* treated cells with herbimycin A or anthracene 9-carboxylic acid completely prevented the appearance of the two lower M_r phosphoprotein bands and inhibited the increase in the two higher M_r proteins (Fig. 9). This confirms that these inhibitors do indeed block protein Tyr phosphorylation in the soybean cells, and also suggests that ion channel activity may be upstream of the Tyr kinase inhibitor-sensitive step(s). Suramin also reduced the levels of these proteins, but did not completely block the increases.

DISCUSSION

Most pharmacological studies on defense response signal transduction in plant cell cultures have used microbial or synthetic elicitors as the inducer (Dixon et al., 1994; Ebel and Mithöfer, 1998). In these cases, the oxidative burst is almost immediate (Legendre et al., 1993a; Jabs et al., 1997), indicating rapid elicitor-receptor recognition and signaling of downstream events. In contrast, if the inducing agent is a bacterial strain harboring a recognized avirulence gene, the oxidative burst does not start until after a lag period of around 3 to 4 h post infection (Levine et al., 1994; Shirasu et al., 1997; Guo et al., 1998). This lag period is presumably necessary for the bacteria to synthesize the avirulence gene product and to deliver this to the plant cells via the *hrp* (hypersensitive response and pathogenicity)-gene-mediated type III secretory system (Van den Ackerveken and Bonas, 1997). Specific nutritional conditions that satisfy requirements for induction and overcoming catabolite repression, rather than host-specific factors, are required for expression of the *avrB* gene (Huynh et al., 1989). This is presumably also the case for the *avrA* gene; thus, in the present system, a 90-min pre-incubation of bacteria in conditioned plant culture medium reduced the lag period for induction of the oxidative burst by a corresponding 90 min, whereas growth in fresh Murashige and Skoog medium did not.

Assuming that the onset of the oxidative burst starts very soon after perception of the *avrA* gene product in the plant cells, events occurring in the host cells within the 1st h post infection may not be dependent on *avrA* gene product recognition. For example, an early, small, and transient oxidative burst occurs in soybean cv Williams 82 cells in response to exposure to *Psg* harboring *avrC*, which is not recognized by this cultivar (Levine et al., 1994). This early, nonspecific burst is strongly potentiated by Ser protease inhibitors (Guo et al., 1998). It also occurs in cells exposed to *Psg:avrA*, and is presumably activated via a signaling pathway that operates prior to avirulence gene product recognition. It is known that plants recognize and respond to common microbial surface components such as chitin in fungal cell walls (Baureithel et al., 1994), and such recognition may lead to early defense responses (Felix et al., 1993). However, it is not known whether such early responses that do not involve *avr* gene product recognition by the host cells are necessary for subsequent responses that do require *avr* gene product recognition.

Pharmacological experiments should always be interpreted with caution. In particular, nonspecific effects are always possible, particularly if reagents are used at relatively high concentrations. The I_{50} values in the soybean cell system for the effects of the various inhibitors on the oxidative burst were all within the concentration ranges previously employed in signal transduction studies in plant or mammalian cells. Furthermore, we can in some cases rule out blanket effects on cell metabolism in view of inhibitor specificity. For example, suramin is an effective inhibitor of the oxidative burst and resulting GST induction, but does not inhibit PAL expression. DPI is not only an inhibitor of the NADPH oxidase, but also inhibits macrophage nitric oxide synthase at concentrations similar to those reported here (Stuehr et al., 1991). Nitric oxide is now known to function as a signal molecule in plant defense responses (Delledonne et al., 1998; Durner et al., 1998). We cannot therefore formally rule out the possibility that DPI affects nitric oxide production in soybean cells. However, nitric oxide, while potentiating induction of downstream defense genes such as *PAL* and chalcone synthase, is not an effective inducer of *GST*, a gene that is responsive to H_2O_2 (Delledonne et al., 1998). Thus, it is unlikely that the effects of DPI on the oxidative burst are mediated via inhibition of NOS.

The only paradoxical results we obtained from the inhibitor studies concerned the effects of ion channel reagents on GST induction. Amphotericin B appeared unable to induce GST, although this reagent strongly induced the oxidative burst, and GST induction in soybean cells is known to be linked causally to H_2O_2 generation (Levine et al., 1994). We could not evaluate higher concentrations of amphotericin B in this experiment because of cellular toxicity. Likewise, anthracene 9-carboxylate appeared to increase the level of GST protein in response to *Psg:avrA*. Clearly, GST protein levels cannot be viewed simply as a reporter for active oxygen generation in soybean cells.

The inhibition escape kinetics for herbimycin A, to which the plant cells are only sensitive within the first 45 min following exposure to bacteria, indicate that this compound inhibits a signaling event that is necessary for the *avrA*-mediated oxidative burst but which may be upstream of *avrA* gene product recognition. Similarly, the effectiveness of suramin as an inhibitor of the oxidative burst diminishes gradually when the compound is added at different times during the 3-h lag period prior to the onset of the burst. In contrast, K252a is an effective inhibitor of the burst for a much longer period, which is consistent with the conclusion that a Ser/Thr kinase cascade operates downstream of avirulence gene product recognition (Le-

vine et al., 1994) and has to remain in the "on position" for downstream defense responses to be expressed (Felix et al., 1991, 1994).

The observation that induction of GST and PAL is differentially affected by signal transduction inhibitors confirms previous results indicating that phytoalexin production, which is associated with increased PAL activity in soybean (Bhattacharyya and Ward, 1986), and the oxidative burst are regulated via distinct signal transduction pathways. Although the oxidative burst is necessary for *avrA*-dependent phytoalexin accumulation in soybean cells, treatments that induce a strong oxidative burst do not necessarily induce a phytoalexin response (Guo et al., 1998), which is consistent with the previous genetic demonstration that the overall HR and phytoalexin production are not causally linked (Jakobek and Lindgren, 1993). As mentioned above, the differential effects on gene expression also provide an internal control for blanket effects of herbimycin and suramin, the activities of which have not been described extensively in plant systems.

Herbimycin A is a potent inhibitor of Tyr phosphorylation and subsequent downstream events in mammalian cells, and as such has been widely used as a reagent to demonstrate Tyr kinase involvement in signaling (Einsphar et al., 1991; Uckun et al., 1991; Asslan et al., 1998; Riordan et al., 1998). Based on its effects on soybean phosphoproteins, we conclude that herbimycin A also inhibits Tyr kinase activity in soybean cells. Although the vast majority of studies on plant protein kinases have dealt with Ser/Thr kinases (Stone and Walker, 1995), inhibitor studies have suggested a role for Tyr phosphorylation in auxin transport and ABA-mediated events in plants (Bernasconi, 1996; Heimovaara-Dijkstra et al., 1996), and several recent reports have documented the involvement of Tyr-phosphorylated proteins in plant defense signaling (Suzuki and Shinshi, 1995; Adam et al., 1997; Ligterink et al., 1997; Stratmann and Ryan, 1997; Zhang and Klessig, 1998). In each case, the proteins were closely related in properties to the mammalian MAP kinases, which are phosphorylated on Tyr and Ser/Thr residues and which can themselves phosphorylate myelin basic protein as an in vitro substrate. Their natural plant substrates are not known.

The MAP kinase-related proteins are all 45 to 49 kD, whereas the Tyr-phosphorylated proteins observed in the present study to be rapidly induced at least 2.5 h prior to the *avrA*-mediated oxidative burst were of 19, 20, 28, and 55 kD. These proteins (with the possible exception of the 20 kD protein, which could be of bacterial origin) are therefore novel plant phosphoproteins. It must be stressed that, although our results clearly indicate that herbimycin inhibits Tyr phosphorylation of proteins in soybean cells, they do not prove that any of the Tyr-phosphorylated proteins revealed in Figure 9 is causally involved in regulation of the oxidative burst.

Based on the above observations, we propose the following model for the early signaling events in the oxidative burst in cultured soybean cells challenged with avirulent *Psg*. First, specific nutritional conditions associated with the conditioned plant culture medium that mimic similar conditions in planta induce expression of the *avrA* gene. Before or during the transfer of the *avrA* gene product to the host cells, recognition of a bacterial component(s) initiates a signal transduction pathway involving activation of ion channels and Tyr phosphorylation on one or more critical proteins. This is followed by coupling of G-proteins to receptors, which may be directly or indirectly associated with Tyr-phosphorylated proteins.

These events are essential for the full oxidative burst response following recognition of the *avrA* gene product, but not for phenylpropanoid-derived defenses such as the isoflavonoid phytoalexin response. Thus, Tyr-phosphorylated proteins may function as upstream components in an *avrA* gene product reception process that, assuming *avrA* product recognition occurs after 45 min post infection in the soybean cell system, is "pre-primed" by an earlier recognition process. Interestingly, these events find parallels in mammalian neutrophils, where herbimycin inhibits an early signaling step for the activation of NADPH oxidase following stimulation by peptides (Zhang et al., 1998), and Tyr kinase activity may be necessary for activation of downstream kinases involved in phosphorylation of the oxidase component p47-phox (Yaname et al., 1999).

There are two caveats to the above model. First, we cannot exclude the possibility that the Tyr-phosphorylation events are *avrA* gene dependent, even though their timing is suggestive of their occurring prior to recognition of the *avrA* gene product in the host cells. In this regard, we have not been able to demonstrate increased protein Tyr phosphorylation in soybean cv Williams 82 cells in response to *Psg:avrC*. Second, we do not know exactly when the *avrA* gene product first activates the signal pathway for the oxidative burst; the above model is based on assumptions supported by comparisons of the kinetics of the burst in response to bacteria or isolated elicitors (Guo et al., 1998). The sequence of events proposed above can only be rigorously tested once reagents become available to determine directly the exact timing of production of the *avrA* gene product and its transfer to or association with the host cells.

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