Potentiation of the Oxidative Burst and Isoflavonoid Phytoalexin Accumulation by Serine Protease Inhibitors[1]

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Treatment of soybean (*Glycine max* L. cv Williams 82) cell-suspension cultures with *Pseudomonas syringae* pv *glycinea* (*Psg*) harboring an avirulence gene (*avrA*) or with yeast elicitor resulted in an oxidative burst characterized by the accumulation of H_2O_2. This burst, and the resultant induction of glutathione *S*-transferase transcripts, occurred more rapidly and was more prolonged if cells were simultaneously treated with serine protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF) or diisopropylfluorophosphate. PMSF and diisopropylfluorophosphate potentiate a large oxidative burst in cells exposed to *Psg* harboring the *avrC* avirulence gene, which is not recognized by the soybean cultivar used in this study. The potentiated burst was inhibited by diphenylene iodonium, an inhibitor of NADPH oxidase, and by the protein kinase inhibitor K252a. PMSF treatment of elicited cells or cells exposed to *Psg:avrA* caused a large increase in the accumulation of the isoflavonoid phytoalexin glyceollin; however, this was not associated with increased levels of transcripts encoding key phytoalexin biosynthetic enzymes. Glyceollin accumulation was inhibited by diphenylene iodonium; however, the oxidative burst in cells treated with *Psg:avrC* and PMSF was not followed by phytoalexin accumulation. We conclude that active oxygen species from the oxidative burst are necessary but not sufficient for inducing isoflavonoid phytoalexin accumulation in soybean cells.

Avirulent pathogens trigger a hypersensitive response characterized by the rapid production of active oxygen species, usually measured as H_2O_2 (Sutherland, 1991; Doke et al., 1996; Mehdy et al., 1996; Lamb and Dixon, 1997). This response, known as the oxidative burst, can also be mimicked in suspension-cultured cells exposed to elicitors of microbial origin (Apostol et al., 1989; Levine et al., 1994). There is currently intense interest concerning the role of the oxidative burst as a generator of signals for cell death (Levine et al., 1994), activation of antioxidant defenses (Levine et al., 1994), induction of phytoalexin biosynthesis (Apostol et al., 1989; Devlin and Gustine, 1992; Park et al., 1995), and establishment of systemic acquired resistance (Alvarez et al., 1998).

The oxidative burst in plants is believed to share mechanistic similarities to that exhibited by mammalian neutrophils. It involves activation of a plasma membrane-bound NADPH oxidase, and components of the plant oxidase complex have recently been characterized at the biochemical (Van Gestelen et al., 1997) and molecular (Groom et al., 1996; Keller et al., 1998) levels. A significant body of pharmacological evidence points to the involvement of a protein kinase cascade as part of the signal transduction pathway between molecular recognition of elicitors or avirulent bacteria and activation of the oxidase (Schwacke and Hager, 1992; Chandra and Low, 1995; Shirasu et al., 1997). SA, a key chemical regulator of local and systemic resistance in plants (Klessig and Malamy, 1994; Ryals et al., 1995), acts as an agonist-dependent gain control for activation of the oxidative burst via its regulatory kinase cascade (Shirasu et al., 1997).

Reports linking the oxidative burst to phytoalexin biosynthesis have often been contradictory, even with respect to experiments performed on the same plant species. For example, treatment of soybean (*Glycine max*) cell cultures with catalase immediately before elicitation was shown to inhibit phytoalexin accumulation (Apostol et al., 1989), an observation interpreted as indicating a causal involvement of H_2O_2 as an inducer of the phytoalexin response. That this might be an oversimplification was apparent from a subsequent observation that certain biotic elicitors induce an oxidative burst in soybean cells, with no resultant phytoalexin production (Davis et al., 1993), and that catalase does not inhibit phytoalexin production in white clover cells (Devlin and Gustine, 1992).

H_2O_2 is a rapid, direct primary signal for the induction of transcripts encoding the antioxidant enzyme GST, whereas it is only a weak inducer of transcripts encoding the phytoalexin biosynthetic enzymes PAL and chalcone synthase in soybean cell cultures (Levine et al., 1994). This was supported by a recent demonstration that DPI, a suicide inhibitor of NADPH oxidase (O'Donnell et al., 1993), inhibits the oxidative burst in soybean cells exposed to a fungal glucan elicitor without inhibiting elicitor-induced phytoalexin accumulation (Mithofer et al., 1997). It remains to be resolved whether H_2O_2 is itself the endogenous signal for activation of the phytoalexin response after pathogen recognition.

Abbreviations: DFP, diisopropylfluorophosphate; DPI, diphenylene iodonium; GST, glutathione *S*-transferase; IFR, isoflavone reductase; PAL, L-Phe ammonia-lyase; SA, salicylic acid.

While attempting to bioassay a proteinaceous elicitor from plant cells, we observed that inclusion of Ser protease inhibitors in the culture medium greatly and synergistically stimulated the elicitor-mediated oxidative burst in cultured soybean cells. Such reagents represent novel tools for the pharmacological dissection of the events controlling the oxidative burst and related processes. We describe the effects of protease inhibitors on the oxidative burst induced by biotic elicitors and virulent or avirulent bacteria, and the consequent effects on phytoalexin biosynthesis and accumulation. Our results reveal the involvement of a protease-inhibitor-sensitive, negative regulatory step in the signal transduction pathway(s) leading to both the oxidative burst and the accumulation of phytoalexins. Furthermore, potentiation of the oxidative burst by protease inhibitors has allowed us to define the relationship between the production of active oxygen species and the phytoalexin response.

MATERIALS AND METHODS

Plant Materials

Cell-suspension cultures of soybean (*Glycine max* L. cv Williams 82) were grown at 25°C in the dark in Murashige and Skoog medium as described previously (Guo et al., 1997). Cells were transferred to fresh medium every 7 d and were used 3 to 4 d after transfer.

Chemicals

Antipain, DFP, and DPI were from ICN. Pyranine was purchased from Molecular Probes (Eugene, OR). Other chemicals were from Sigma.

Elicitors and Bacteria

Yeast elicitor was isolated according to published procedures (Schumacher et al., 1987) and was dialyzed exhaustively (Spectra/por; cutoff, 3.5 kD; Spectrum, Houston, TX) against distilled water. Its final concentration in the plant-cell culture medium is given in micrograms of Glc equivalents per milliliter.

Pseudomonas syringae pv *glycinea* (*Psg*) race 4 containing plasmids with either the *avrA* or *avrC* avirulence genes (Keen and Buzzell, 1991) was grown 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 $MySO_47H_2O$ L^{-1}, pH 7.2) overnight with streptomycin or kanamycin. The bacteria were collected by centrifugation and resuspended in sterile water for inoculation of cultured cells.

Measurement of the Oxidative Burst

The oxidative burst was determined by measuring H_2O_2 production by quenching of pyranine fluorescence at 512 nm with excitation at 405 nm (Apostol et al., 1989) using a luminescence spectrophotometer (model LS50B, Perkin-Elmer). One-milliliter batches of cells in 12-well microtiter plates were exposed to proteinase inhibitors 30 min before

the addition of yeast extract or bacteria. For proteinase inhibitors that required DMSO for solubilization, an equivalent amount of DMSO (0.1%, v/v) was added to control cultures. This concentration of DMSO did not induce the oxidative burst. For K252a- or DPI-inhibition experiments, 10 μM K252a or 1 μM DPI, respectively, was added immediately before the addition of proteinase inhibitors.

Inhibition of Soybean Ser Protease in Vitro

Soybean Ser protease was purified to homogeneity and assayed in the presence of various protease inhibitors, as described previously (Guo et al., 1998).

Determination of Isoflavonoids

Treated soybean cells were harvested by vacuum filtration and the cells and culture medium were collected separately. Cells were extracted with acetone, the acetone was removed under a stream of nitrogen, and the dried residue was dissolved in a small volume of methanol before reverse-phase HPLC analysis on a C_{18} column using a gradient of CH_3CN (A) and water containing 1% H_3PO_4 (B). The gradient was 12% to 30% A for 35 min and 30% to 65% A for 20 min, and absorbance of the eluent was monitored by a diode array detector at 287 nm. For determination of glyceollin in the culture medium, the medium was partitioned twice against 2 volumes of ethyl acetate, dried under nitrogen, and the residue was analyzed as described above.

Northern-Blot Analysis

Total RNA was isolated and hybridized in northern blots according to standard procedures (Sambrook et al., 1989). cDNA hybridization probes were soybean PAL (Frank and Vodkin, 1991), soybean GST (Czarnecka et al., 1988), Arabidopsis rRNA (Pepper et al., 1994), and alfalfa IFR (Paiva et al., 1991).

RESULTS

Ser Protease Inhibitors Potentiate the Oxidative Burst in Soybean Cells in Response to Elicitors or Avirulent or Virulent Bacteria

Exposure of suspension-cultured soybean cells to *Psg* harboring the *avrA* avirulence gene resulted in an oxidative burst characterized by extracellular accumulation of H_2O_2 and increased cell death (Levine et al., 1994; Shirasu et al., 1997). In contrast, soybean cv Williams 82 does not contain a resistance gene for recognition of the *avrC* avirulence gene, and *Psg* harboring *avrC* did not induce a sustained oxidative burst, although a small burst is often seen very soon after exposure to the bacteria (Shirasu et al., 1997).

Treatment of soybean cells with *Psg:avrA* in the presence of 0.5 mM PMSF, a strong inhibitor of soybean Ser protease (Guo et al., 1998), resulted in striking changes in the kinetics of the oxidative burst (Fig. 1a). Rather than a small burst after approximately 1 h followed by a strong burst peaking at 4 h, a strong burst was apparent by 30 min, and only
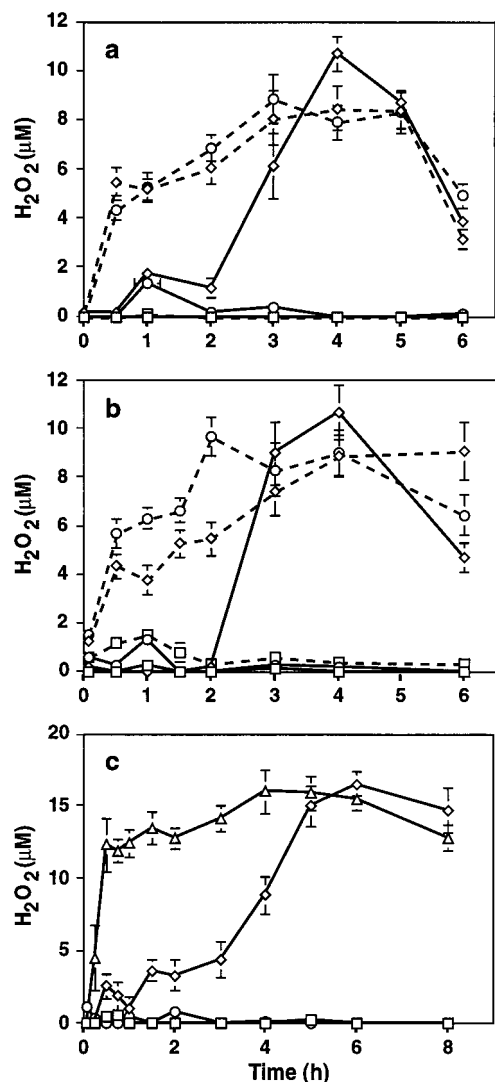
Figure 1. Ser protease inhibitors potentiate the oxidative burst in soybean suspension-cultured cells in response to elicitor and virulent or avirulent bacteria. a, Kinetics of the effects of PMSF (0.5 mM) on H_2O_2 generation in soybean cells after exposure to *Pseudomonas syringae* pv *syringae* (3×10^7 colony-forming units mL^{-1}) harboring the *avrA* gene (incompatible interaction) or the *avrC* gene (compatible interaction), or in untreated controls. All cells were treated with DMSO (final concentration, 0.1% [v/v]; continuous lines) or PMSF dissolved in DMSO (dashed lines). □, No bacteria; ◇, plus *Psg:avrA*; ○, plus *Psg:avrC*. b, Same conditions as in a, but using DFP (0.5 mM) in place of PMSF. Symbols are as in a, but DFP replaces PMSF. c, Effects of DFP on H_2O_2 generation in response to yeast elicitor (50 μg Glc equivalents mL^{-1}). □, DMSO alone; ○, DMSO plus DFP; ◇, DMSO plus yeast elicitor; △, DMSO plus yeast elicitor plus DFP. Results represent the means ± SD of three independent experiments.

declined after 5 h. Strikingly, an almost identical burst was seen in cells exposed to *Psg:avrC* in the presence of PMSF, indicating that the burst potentiated by the protease inhibitor in response to *Psg:avrA* was the early, nonspecific burst, rather than the effect being a more rapid expression of the later, avirulence-gene-specific burst. PMSF itself (or DMSO in which PMSF was dissolved) did not induce H_2O_2

production in the absence of bacteria. Bacteria that had been pretreated with PMSF and then washed to remove the protease inhibitor induced the same level of H_2O_2 as untreated bacteria, whereas washing PMSF-treated soybean cells immediately before the addition of bacteria did not prevent potentiation of the burst (data not shown). These results indicate that the effect of PMSF is on the plant cells and not on the inducing bacteria.

An identical potentiation of the oxidative burst in response to avirulent or virulent bacteria was observed with the Ser protease inhibitor DFP (Fig. 1b), although this inhibitor alone produced a small early burst of similar magnitude to that obtained with *Psg:avrC*. Similar results were obtained with 0.5 mM 3,4-dichloroisocoumarin, although this inhibitor alone induced a larger oxidative burst than DFP (data not shown).

Exposure of soybean cells to yeast elicitor resulted in an oxidative burst with kinetics similar to those induced by *Psg:avrA*, and this burst was also potentiated by DFP (Fig. 1c). Neither the aminopeptidase inhibitor bestatin (0.1 mM) nor the acidic aspartyl protease inhibitor pepstatin A (0.1 mM) had any effect on the oxidative burst, either alone or in combination with bacteria or elicitor (data not shown).

We recently purified the major Ser protease from suspension-cultured soybean cells (Guo et al., 1998). To determine whether this protease might be the site of action of the various Ser protease inhibitors as potentiators of the oxidative burst, we compared the effects of a panel of protease inhibitors on the burst in vivo and on the activity of the purified protease in vitro. In this and subsequent experiments, the burst was measured at 2 h after elicitation, at which time H_2O_2 production was still low in the absence of the potentiator. The results shown in Table I indicate that in most cases compounds that inhibited the protease in vitro also potentiated the oxidative burst in response to yeast elicitor. Nearly identical results were obtained when measuring the effects of the inhibitors on H_2O_2 production after exposure of cells to *Psg:avrA* or *Psg:avrC* (data not shown). However, there was no direct correlation between the effectiveness of the compounds to inhibit the protease in vitro and their ability to induce the oxidative burst in vivo. For example, leupeptin was one of the most potent inhibitors of the soybean protease, but it only weakly potentiated the burst at 2 h after elicitation. This result could be explained by poor uptake of the inhibitor into the cells, but PMSF, a strong potentiator of the burst, only weakly inhibited the protease in vitro, suggesting that the Ser protease in the soybean cell culture was not the target (or at least was not the only target) through which Ser protease inhibitors potentiate the oxidative burst.

H_2O_2 generated through the oxidative burst is an inductive signal for expression of antioxidant defenses, including the accumulation of GST transcripts (Levine et al., 1994). GST transcripts were weakly induced after exposure of soybean cells to PMSF alone; however, transcript levels induced in response to yeast elicitor, *Psg:avrA*, or *Psg:avrC* increased earlier and were maintained longer in cells exposed at the same time to PMSF (Fig. 2). Therefore, Ser protease inhibitors potentiate both the oxidative burst and a downstream gene-induction response.

Table I. *Comparison of the effects of a range of protease inhibitors on the activity of purified soybean Ser protease in vitro and on the oxidative burst in vivo*

Concentrations of inhibitors used in vitro are given. Inhibitors were applied to cells at a final concentration of 0.5 mM (0.2 mM for antipain), and H_2O_2 was measured 2 h after exposure to water (control) or yeast elicitor (200 μg Glc equivalents mL^{-1}). Results show the average $\pm$ SD from three independent experiments.

Inhibitor	Protease Activity		Oxidative Burst	
	Inhibitor concentration	Protease activity	Control	Yeast elicitor
	μM	%	μM H_2O_2	
Control (DMSO)	0	100	0.00 $\pm$ 0.10	2.10 $\pm$ 0.44
Antipain	20	0	4.55 $\pm$ 0.43	13.2 $\pm$ 1.17
DCI[a]	1000	26	1.03 $\pm$ 0.27	12.4 $\pm$ 1.49
DFP	1000	2	1.34 $\pm$ 0.17	9.46 $\pm$ 1.03
Leupeptin	15	0	0.13 $\pm$ 0.11	5.90 $\pm$ 0.34
PMSF	1000	81	0.11 $\pm$ 0.06	12.4 $\pm$ 1.41
TLCK[b]	1000	61	0.10 $\pm$ 0.13	3.52 $\pm$ 0.22
TPCK[c]	1000	47	5.86 $\pm$ 0.60	8.90 $\pm$ 1.07

[a] DCI, 3,4-dichloroisocoumarin. [b] TLCK, Nα-p-tosyl-L-lysine chloromethyl ketone. [c] TPCK, n-tosyl-L-phenylalanine chloromethyl ketone.

Oxidative Burst Potentiated by Ser Protease Inhibitors Is Blocked by Inhibitors of NADPH Oxidase and Protein Ser/Thr Kinase

The signal transduction pathway for H_2O_2 generation in soybean and other plant cells in response to avirulent pathogens or elicitors involves a protein Ser/Thr kinase cascade that leads to activation of the plasma membrane-associated NADPH oxidase complex (Levine et al., 1994; Chandra and Low, 1995; Shirasu et al., 1997; Xing et al., 1997). Treatment of soybean cells with DPI strongly inhibited the accumulation of H_2O_2 in response to PMSF added along with *Psg:avrA*, *Psg:avrC*, or yeast elicitor (Fig. 3). Therefore, the H_2O_2 produced by potentiation of the early, nonavirulence-gene-specific oxidative burst is generated by the NADPH oxidase complex previously implicated in the specific avirulence-gene-mediated response.

Treatment of soybean cell suspensions with the protein Ser/Thr kinase inhibitor K252a almost completely inhibited the oxidative burst in response to *Psg:avrA*, *Psg:avrC*, or yeast elicitor in the presence of PMSF (Fig. 4). The fact that the inhibition was well below the value in the presence of bacteria or elicitors alone suggests that all of the H_2O_2 produced during the potentiated early oxidative burst arose from the protein-kinase-dependent pathway.

The protein-phosphatase inhibitor cantharidin can induce the oxidative burst at high concentrations (10 μM), and can act synergistically with the potentiator SA at concentrations that alone do not induce H_2O_2 accumulation (Shirasu et al., 1997). When applied to soybean cell-suspension cultures at a noninductive concentration of 2 μM, cantharidin acted synergistically with DFP or PMSF to induce the oxidative burst (Fig. 5), confirming the operation of a protein kinase cascade for the potentiated burst.

In agreement with previous results (Shirasu et al., 1997), SA by itself did not induce the oxidative burst, but strongly potentiated the burst in cantharidin-treated cells, in a manner similar to DFP (Fig. 5). No oxidative burst was observed in cells exposed to PMSF and SA in the absence of

cantharidin (data not shown). The effect of adding protease inhibitors and SA to cantharidin-treated cells was additive.

Role of Active Oxygen Species in Phytoalexin Induction

Soybean cell-suspension cultures produce low amounts of the isoflavonoid phytoalexin glyceollin after exposure to yeast elicitor (Guo et al., 1997) (Fig. 6). The addition of PMSF or DFP alone to cell cultures did not induce glyceollin (data not shown); however, both PMSF and DFP had strong synergistic effects with yeast elicitor on glyceollin accumulation. Treatment with 0.5 mM DFP and 200 μg Glc equivalents mL^{-1} yeast elicitor led to the accumulation of 10 times more glyceollin than accumulated in response to yeast elicitor alone (Fig. 6). Treatment of cells with 0.5 mM PMSF plus 20 μg mL^{-1} yeast elicitor, which individually induced no glyceollin, resulted in the accumulation of nearly 100 μg glyceollin g^{-1} fresh weight of cells.

The effects of PMSF on glyceollin induction by yeast elicitor were associated with a delay in the appearance of maximal levels of transcripts encoding PAL and IFR, two key enzymes of isoflavonoid biosynthesis (Fig. 7). Treatment with PMSF inhibited the limited appearance of PAL and IFR transcripts in cells exposed to *Psg:avrC*, whereas the timing of PAL and IFR transcript appearance in cells exposed to *Psg:avrA* was unaffected. PMSF reduced the level of IFR transcripts in cells exposed to *Psg:avrA*. These data indicate that the striking increase in glyceollin accumulation in elicited cells exhibiting a rapid and prolonged oxidative burst after exposure to PMSF was not the result of increased induction of these phytoalexin biosynthetic enzymes.

Glyceollin accumulation in response to yeast elicitor or *Psg:avrA* and the corresponding responses potentiated by PMSF were strongly inhibited by DPI, suggesting that active oxygen species produced by the oxidative burst are necessary for phytoalexin accumulation (Fig. 8). However, although PMSF treatment led to nearly identical production of H_2O_2 in soybean cells exposed to either *Psg:avrA* or

GST

rDNA
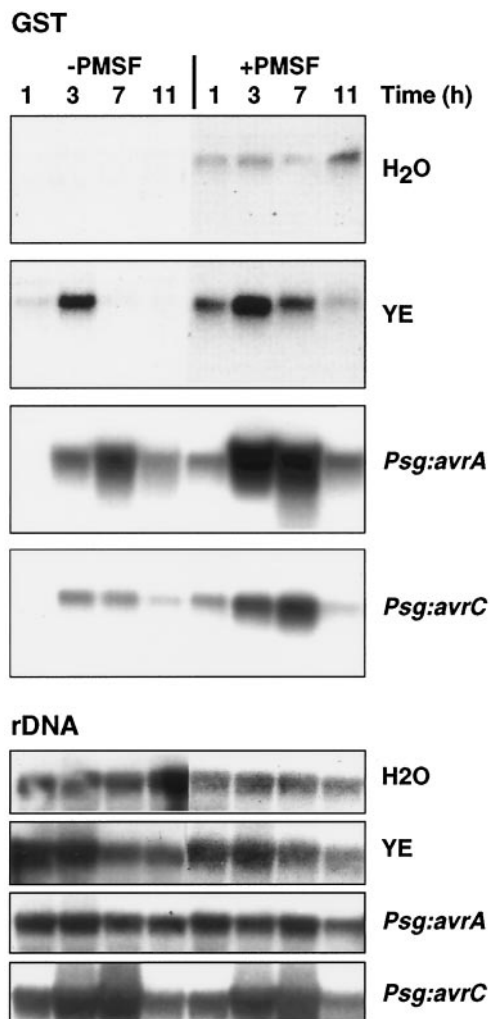
Figure 2. GST transcript levels in soybean suspension-cultured cells exposed to yeast elicitor (YE; 200 μg Glc equivalents mL^{-1}) or *Psg* harboring the *avrA* or *avrC* avirulence genes (3 × 10^7 colony-forming units mL^{-1}) in the presence or absence of PMSF (0.5 mM in DMSO). Controls were treated with DMSO alone. Samples were harvested at the times shown, and RNA was extracted and subjected to northern-blot analysis using a soybean GST cDNA (Czarnecka et al., 1988) as a probe. Loading and transfer efficiency were checked by reprobing the blots with a rDNA probe.

Psg:avrC (Fig. 1a), significant phytoalexin accumulation was observed only in response to *Psg:avrA* (Fig. 8). Therefore, the use of protease inhibitors reveals that H$_2$O$_2$ is necessary but not sufficient for glyceollin induction.

DISCUSSION

### Source of H$_2$O$_2$ from the Oxidative Burst Potentiated by Ser Protease Inhibitors

Although there is considerable evidence that the H$_2$O$_2$ produced in the oxidative burst arises via a plasma membrane NADPH oxidase (Groom et al., 1996; Keller et al., 1997; Mithofer et al., 1997; Van Gestelen et al., 1997), alternative sources such as extracellular peroxidases and amine
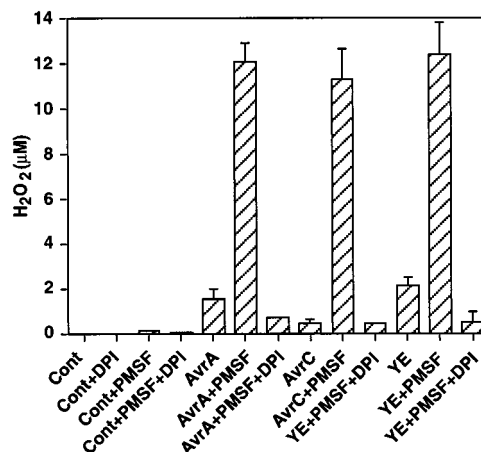

Figure 3. The oxidative burst in response to elicitor or virulent/avirulent bacteria potentiated by PMSF is blocked by the NADPH oxidase inhibitor DPI. Soybean suspension-cultured cells exposed to yeast elicitor (YE; 50 μg Glc equivalents mL^{-1}), *Psg:avrA* (AvrA), or *Psg:avrC* (AvrC) (3 × 10^7 colony-forming units mL^{-1}) were treated with PMSF (0.5 mM in DMSO) or DMSO alone (Cont). Production of H$_2$O$_2$ at 2 h after elicitation was measured in the presence or absence of DPI (10 μM). Results represent the means ± SD of three independent experiments.

oxidases have also been implicated under certain conditions (Bolwell, 1996; Allan and Fluhr, 1997). The fact that the potentiation of the oxidative burst by protease inhibitors is completely inhibited by DPI (Fig. 3), a suicide inhibitor of the NADPH oxidase and other flavoproteins in mammalian cells (O'Donnell et al., 1993), strongly suggests that protease inhibitors act upstream in the signal transduction pathway leading to activation of the oxidase, and are unlikely to affect processes such as the turnover of a
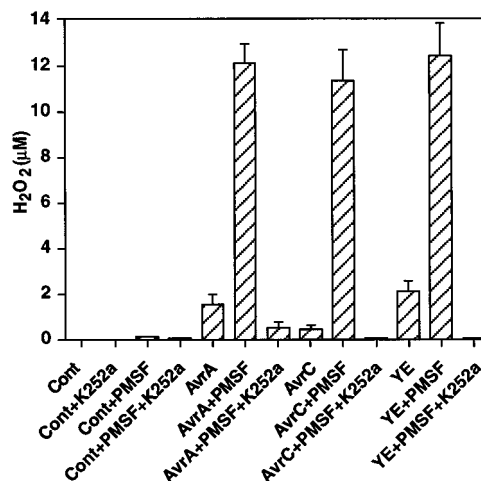
Figure 4. The oxidative burst in response to elicitor or virulent/avirulent bacteria potentiated by PMSF is blocked by the protein kinase inhibitor K252a. Soybean suspension-cultured cells exposed to yeast elicitor (YE), *Psg:avrA* (AvrA), or *Psg:avrC* (AvrC) were treated with PMSF (0.5 mM in DMSO) or DMSO alone (Cont). Production of H$_2$O$_2$ at 2 h after elicitation was measured in the presence or absence of K252a (1 μM). Results represent the means ± SD of three independent experiments.
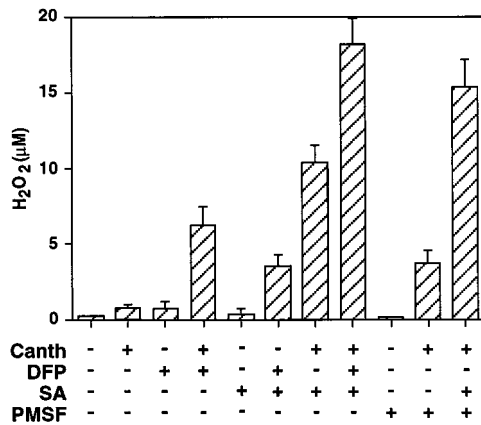
Figure 5. Ser protease inhibitors act synergistically with the Ser phosphatase inhibitor cantharidin (Canth) to induce the oxidative burst. Cells were treated with cantharidin (2 μM) and DFP or PMSF (0.5 mM in DMSO) singly or in combination, and H_2O_2 release was measured 60 min later. Effects of the addition of SA (50 μM) alone or in combination with cantharidin, protease inhibitors, or both were also determined. Results represent the average $\pm$ SD of two independent experiments.

peroxidase or amine oxidase. Such a conclusion is also consistent with the inhibition of Ser protease-mediated potentiation of the oxidative burst by K252a (Fig. 4) and with the ability of Ser proteases to act synergistically with cantharidin (Fig. 5), suggesting the involvement of a protein kinase cascade in the Ser protease inhibitor-mediated potentiation of H_2O_2 accumulation. These observations parallel a significant body of evidence implicating a protein Ser/Thr kinase cascade in the signal transduction pathway leading to activation of the NADPH oxidase (Schwacke and Hager, 1992; Chandra and Low, 1995; Shirasu et al., 1997).

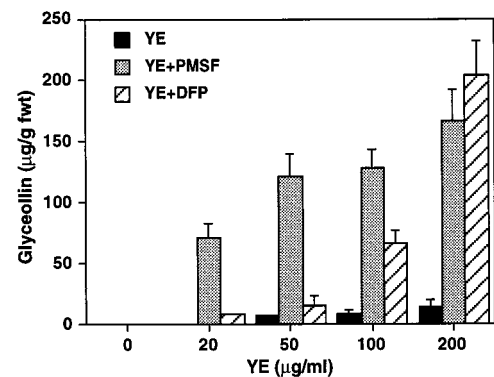## Site of Action of Ser Protease Inhibitors

The observation that several chemically distinct Ser protease inhibitors (Fig. 1; Table I) but not the aminopeptidase inhibitor bestatin or the aspartyl protease inhibitor pepstatin A act to potentiate the burst indicates that the most likely site of action is an endogenous Ser protease. However, this is unlikely to be the Ser protease recently purified from soybean cell cultures (Guo et al., 1998) because PMSF, a potent potentiator of the burst, failed to inhibit this enzyme significantly.

Bestatin is itself a powerful inducer of defense genes for wound responses in tomato leaves (Schaller et al., 1995), and it has been suggested that it might act to inhibit a regulatory protease that might control the levels of a transcription factor (Schaller et al., 1995). Two important differences distinguish the effects of bestatin in tomato from those reported here. First, the effects of most of the Ser protease inhibitors in the present work are agonist dependent and the compounds are therefore true potentiators, i.e. they are not strong inducers when added alone. Second, Ser protease inhibitors do not induce significantly the expression of the defense response genes PAL or IFR in
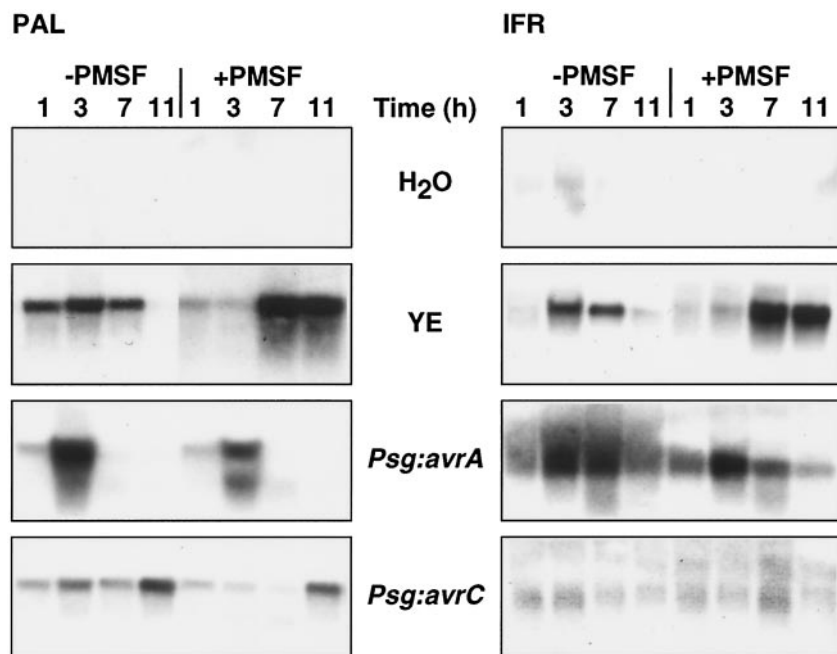
soybean cells (Fig. 7). Elevation of GST transcripts in response to elicitor or *Psg* plus PMSF can be explained as a response to the more rapid accumulation of H_2O_2 under these conditions (Levine et al., 1994).

The observation that Ser protease inhibitors are strong potentiators of the NADPH oxidase-dependent oxidative burst in soybean suspension-cultured cells is in striking contrast to the reported inhibitory effects of protease inhibitors on NADPH oxidase activation in mammalian cells. For example, chymotrypsin inhibitors block H_2O_2 formation by activated human polymorphonuclear leukocytes (Frenkel et al., 1987), and the irreversible Ser protease inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride inhibits superoxide production from guinea pig peritoneal macrophages (Diatchuk et al., 1997). In the latter study, a range of other Ser protease inhibitors, including PMSF, 3,4-dichloroisocoumarin, n-tosyl-L-phenylalaninechloromethyl ketone, and leupeptin, were inactive, and it was concluded that 4-(2-aminoethyl)benzenesulfonyl fluoride acted directly to interfere with the binding of the p47[phox] and/or the p67[phox] subunits to the catalytic Cyt b_{559} component of the oxidase (Diatchuk et al., 1997). These results indicate that Ser protease inhibitors such as 4-(2-aminoethyl)benzenesulfonyl fluoride and PMSF may have previously unforeseen sites of action, and this qualification must be borne in mind when considering the results of the present study.

We have previously shown that SA is a strong, agonist-dependent potentiator of the oxidative burst at physiological concentrations (Shirasu et al., 1997). The present results implicate proteolysis of a regulatory component in the signal pathway leading to the oxidative burst as a further site of control. SA probably acts at a different site from PMSF, in view of the observations that the agonist-dependent effects of SA and Ser protease inhibitors are additive (Fig. 5) and that SA potentiates the appearance of PAL transcripts in response to *Psg:avrA* (Shirasu et al., 1997), whereas PMSF does not (Fig. 7). Therefore, a picture emerges of the involvement of multiple endogenous signals, which need to be perceived together for the commitment of the plant to a program of metabolic changes that

Figure 6. Ser protease inhibitors potentiate the accumulation of the phytoalexin glyceollin in response to yeast elicitor (YE). Cells were treated with the concentrations of yeast elicitor shown in the presence or absence of PMSF or DFP (0.5 mM in DMSO), and cells were harvested for analysis of glyceollin levels after 24 h. Results represent the means $\pm$ SD of three independent experiments. fwt, Fresh weight.
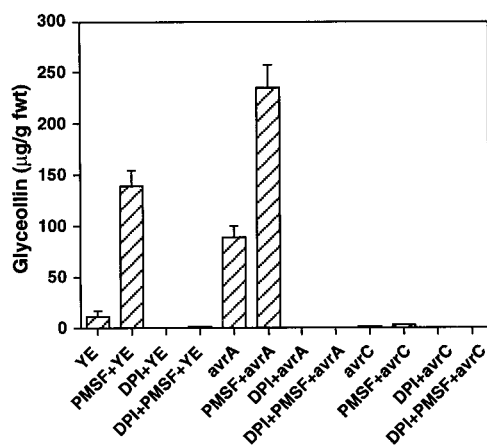
PAL

IFR

involve generation of potentially toxic active oxygen species and phytochemicals for defense.

Relationship between the Oxidative Burst and Phytoalexin Accumulation

Our data show that generation of H_2O_2 via the oxidative burst is necessary but not sufficient for phytoalexin accu-

Figure 8. Relationship between glyceollin induction and release of active oxygen species. Data show the effects of DPI (10 μM) on the production of glyceollin in soybean suspension-cultured cells in response to yeast elicitor (YE; 200 μg Glc equivalents mL^{-1}), *Psg:avrA*, or *Psg:avrC* in the presence or absence of PMSF (0.5 mM). Cells and culture media were harvested 24 h after elicitation, and glyceollin levels were analyzed by HPLC. In cells treated with yeast elicitor, all of the glyceollin accumulated intracellularly, whereas glyceollin was found in both cells and culture medium in cells treated with *Psg:avrA*. The glyceollin in the culture medium is included in the values given. Results represent the means ± SD of three independent experiments. fwt, Fresh weight.

mulation in soybean cells. For example, DPI inhibits the phytoalexin response but the potentiated burst obtained with *Psg:avrC* in the presence of PMSF does not lead to phytoalexin accumulation (Figs. 1 and 8). Clearly, active oxygen species must act in concert with another signal specified by the interaction of the *avrA* gene with the *Rpg3* resistance gene present in soybean before phytoalexin accumulation can occur. However, consistent with previous observations that H_2O_2 is not itself an inducer of genes encoding phytoalexin biosynthetic enzymes in soybean cells (Levine et al., 1994; Shirasu et al., 1997), increased phytoalexin accumulation after the early and sustained accumulation of H_2O_2 in protease-inhibitor-potentiated cells does not result from increased expression of PAL or IFR. If H_2O_2 were the signal for phytoalexin biosynthetic gene activation, one would expect a more rapid appearance of the PAL and IFR transcripts in the presence of PMSF, whereas the converse was observed (Fig. 7).

In conclusion, phytoalexin accumulation in soybean cell cultures requires, at minimum, the oxidative burst acting in concert with a second signal generated by the interaction of a race-specific or nonspecific elicitor with its cognate receptor(s). Our results also suggest that phytoalexin accumulation in the soybean cell system is controlled by mechanisms additional to the transcriptional induction of the phytoalexin biosynthetic machinery.

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