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Salicylic Acid Potentiates an Agonist-Dependent Gain Control That Amplifies Pathogen Signals in the Activation of Defense Mechanisms

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The phenylpropanoid-derived natural product salicylic acid (SA) plays a key role in disease resistance. However, SA administered in the absence of a pathogen is a paradoxically weak inductive signal, often requiring concentrations of 0.5 to 5 mM to induce acquired resistance or related defense mechanisms or to precondition signal systems. In contrast, endogenous SA accumulates to concentrations of <70 μM at the site of attempted infection. Here, we show that although 10 to 100 μM SA had negligible effects when administered to soybean cell suspensions in the absence of a pathogen, physiological concentrations of SA markedly enhanced the induction of defense gene transcripts, H_2O_2 accumulation, and hypersensitive cell death by an avirulent strain of *Pseudomonas syringae* pv *glycinea*, with optimal effects being at ~50 μM. SA also synergistically enhanced H_2O_2 accumulation in response to the protein phosphatase type 2A inhibitor cantharidin in the absence of a pathogen. The synergistic effect of SA was potent, rapid, and insensitive to the protein synthesis inhibitor cycloheximide, and we conclude that SA stimulates an agonist-dependent gain control operating at an early step in the signal pathway for induction of the hypersensitive response. This fine control mechanism differs from previously described time-dependent, inductive coarse control mechanisms for SA action in the absence of a pathogen. Induction of H_2O_2 accumulation and hypersensitive cell death by avirulent *P. s. glycinea* was blocked by the phenylpropanoid synthesis inhibitor α-aminooxy-β-phenylpropionic acid, and these responses could be rescued by exogenous SA. Because the agonist-dependent gain control operates at physiological levels of SA, we propose that rapid fine control signal amplification makes an important contribution to SA function in the induction of disease resistance mechanisms.

INTRODUCTION

Avirulent pathogens induce a localized hypersensitive response (HR) in which a challenged cell undergoes rapid collapse, accompanied by the activation of various defenses in the challenged cell and surrounding cells. In addition, systemic acquired resistance (SAR) to normally virulent pathogens gradually develops throughout the plant. Salicylic acid (SA), a product of the phenylpropanoid pathway, has long been implicated as a signal in SAR (Raskin, 1992), and SAR is compromised in transgenic plants expressing the bacterial *nahG* gene encoding SA hydroxylase (Gaffney et al., 1993). Because of this well-established role, the mechanism of SA action has hitherto been investigated by examining the effects of exogenous SA administered to naive plant tissues or cells in the absence of a pathogen (Raskin, 1992; Ryals et al., 1994). In these assays, a high concentration of SA and a long preincubation time are often required to induce SAR or markers such as pathogenesis-related (PR) proteins.

Pretreatment of naive cells or tissues with SA can also induce signaling components that condition the system to respond more strongly to subsequent elicitation. Thus, preincubation of parsley suspension cultures with SA enhances the induction of phenylalanine ammonia–lyase (PAL), xanthotoxol *O*-methyl transferase, and furanocoumarin phytoalexins in response to subsequent treatment with a fungal elicitor (Kauss et al., 1992). Preincubation with SA also enhances both spontaneous and elicitor-induced production of H_2O_2, although the optimal effect required >500 μM SA and >24 hr of preincubation (Kauss and Jeblick, 1995). Likewise, tobacco plants hydroponically fed with 1 to 2 mM SA for 1 to 7 days exhibit enhanced defense gene expression after wounding or pathogen infection (Mur et al., 1996). The requirement for long preincubations with SA suggests that in these systems, SA is mediating an inductive response, and

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indeed, SA preconditioning of abraded cucumber hypocotyls is blocked by protein synthesis inhibitors (Fauth et al., 1996).

Transgenic plants expressing *nahG* also exhibit weakened resistance or in some cases full susceptibility to attack by avirulent pathogens (Delaney et al., 1994). Thus, SA is involved in the expression of localized hypersensitive resistance as well as SAR, and production of SA may be the major function for phenylpropanoid synthesis stimulated in the early stages of the HR (Mauch-Mani and Slusarenko, 1996). Defenses activated in the HR include toughening of cell walls by oxidative cross-linking and elaboration of transcription-dependent responses, such as phytoalexin synthesis, lignin deposition, and deployment of hydrolytic enzymes (Briggs, 1995; Staskawicz et al., 1995). There is a temporal hierarchy of defense gene activation such that some genes, for example, those encoding PAL, the first enzyme of phenylpropanoid synthesis, exhibit rapid, localized activation, whereas many PR protein genes undergo slower activation both locally and then systemically throughout the plant (Dixon and Harrison, 1990; Ryals et al., 1994).

Another feature of the HR is a rapid burst of oxidative metabolism leading to the generation of superoxide and subsequent accumulation of H_2O_2 (Doke, 1983; Mehdy, 1994; Auh and Murphy, 1995). H_2O_2 from the oxidative burst contributes to key aspects of the HR, not only as a substrate for oxidative cross-linking (Bradley et al., 1992; Brisson et al., 1994) but also as a diffusible signal for the induction of genes encoding enzymes with cellular protectant functions, for example, glutathione *S*-transferase (GST), and as a localized, threshold trigger of hypersensitive cell death (Levine et al., 1994; Tenhaken et al., 1995). Whereas chronic elevation of H_2O_2 levels may also contribute to induction of PR protein genes (Chen et al., 1993), H_2O_2 from the oxidative burst does not appear to be a direct signal for localized activation of early defense genes such as *pal* (Levine et al., 1994).

Recent reports that SA functions not only in SAR but also in localized, genetically determined resistance to avirulent pathogens prompted us to test SA activity in the presence of a pathogen. Using this alternative assay, we show that SA potentiates an early step in the signal pathway for stimulation of H_2O_2 production, defense gene expression, and hypersensitive cell death in soybean cells inoculated with an avirulent strain of *Pseudomonas syringae* pv *glycinea*. Potentiation was optimal at physiological concentrations (50 μM) of SA, and no preincubation or conditioning was required. Furthermore, physiological concentrations of SA synergistically enhanced the induction of H_2O_2 accumulation by the protein phosphatase 2A inhibitor cantharidin in the absence of a pathogen, and this rapid response was not inhibited by the protein synthesis inhibitor cycloheximide. Thus, SA appears to act through an agonist-dependent fine control in contrast to the time-dependent, inductive controls previously described for SA action in the absence of a pathogen. Induction of H_2O_2 accumulation and cell death by an avirulent strain of *P. s. glycinea* was blocked by the phenylpropanoid biosynthesis inhibitor α-aminooxy-β-phenylpropionic acid (AOPP), and the HR was rescued by low concentrations of exogenous SA. Because SA action at the fine control level is operative at physiological concentrations, we conclude that rapid activation of a preexisting, highly geared gain control mechanism for amplification of pathogen signals is a key component of SA function in the expression of induced resistance.

RESULTS

Physiological Concentrations of SA Potentiate the Oxidative Burst

P. s. glycinea carrying the avirulence gene *avrC* is not recognized by soybean cultivar Williams 82, which lacks the corresponding *Rpg3* resistance gene (Keen and Buzzell, 1991). In contrast, there is a rapid, *Rpg2*-mediated recognition of *P. s. glycinea* carrying *avrA*, inducing an HR. Inoculation of soybean cultivar Williams 82 cell suspensions with *P. s. glycinea* carrying either *avrA* or *avrC* initially evokes a rapid but weak transient burst of H_2O_2 production (Levine et al., 1994). No further H_2O_2 production has been observed in cells inoculated with virulent *P. s. glycinea* carrying *avrC*; however, ~3 hr after inoculation with avirulent *P. s. glycinea* carrying *avrA*, there is a second and massive oxidative burst (Levine et al., 1994).

Treatment of soybean cells with SA at concentrations in the range of 10 to 100 μM stimulated only weak accumulation of H_2O_2 in the absence of a pathogen. However, the addition of SA simultaneously with *P. s. glycinea* carrying *avrA* caused a dramatic enhancement of the bacterially induced oxidative burst, resulting in a rapid accumulation of H_2O_2 to high levels (Figures 1A and 1B). This accumulation was sustained for several hours. Stimulation of the rapid accumulation of H_2O_2 in response to *P. s. glycinea* carrying *avrA* was observed with concentrations of exogenous SA as low as 10 μM and was optimal at 50 μM. In contrast, when added alone, concentrations of SA >200 μM were required to give a modest stimulation of H_2O_2 accumulation (Figure 1A).

SA at a concentration of 50 μM also potentiated the rapid accumulation of H_2O_2 induced by virulent *P. s. glycinea* carrying *avrC* (Figure 1C). However, the response to *P. s. glycinea* carrying *avrC* in the presence of SA remained transient, with H_2O_2 levels rapidly declining from a maximum after 1 hr. Thus, the massive potentiation by exogenous SA of the response to avirulent *P. s. glycinea* carrying *avrA* cannot be accounted for simply by enhancement of the initial, nonspecific induction of H_2O_2 accumulation but also appears to involve an acceleration of the subsequent avirulence gene–dependent oxidative burst.

Enhancement of Events Downstream of the Oxidative Burst

The initial nonspecific induction of H_2O_2 is too weak to induce hypersensitive cell death, which is only triggered by

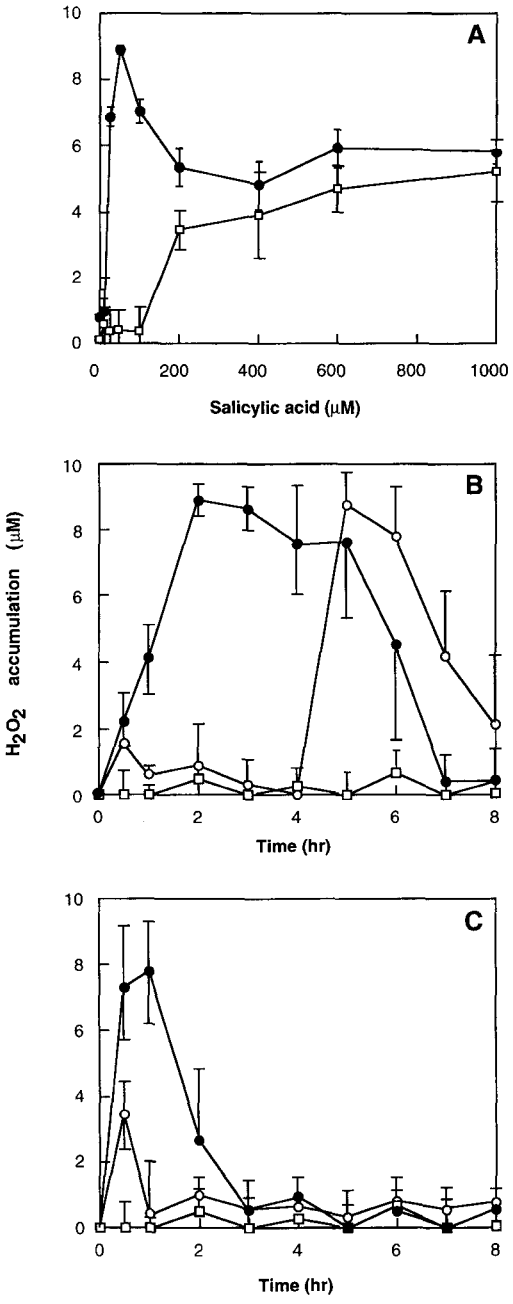

Figure 1. Potentiation of *P. s. glycinea* Induction of H_2O_2 Accumulation by Exogenous SA.

(A) Dose–response for SA stimulation of H_2O_2 accumulation. H_2O_2 levels were determined 2 hr after treatment of soybean cells with the indicated concentrations of SA either alone (□) or with *P. s. glycinea* carrying *avrA* (●).

(B) Effects of 50 μM SA on the kinetics of H_2O_2 accumulation in response to *P. s. glycinea* carrying *avrA*: SA (□), *P. s. glycinea* carrying *avrA* (○), and SA plus *P. s. glycinea* carrying *avrA* (●).

(C) Effects of 50 μM SA on the kinetics of H_2O_2 accumulation in response to *P. s. glycinea* carrying *avrC*: SA (□), *P. s. glycinea* carrying *avrC* (○), and SA plus *P. s. glycinea* carrying *avrC* (●).

the subsequent avirulence gene–dependent oxidative burst (Levine et al., 1994). The addition of 50 μM SA simultaneously with *P. s. glycinea* carrying *avrA* reduced the lag time for the onset of cell death from 8 to 4 hr, which is in line with the accelerated activation of a sustained burst (Figure 2A). Potentiation of cell death was optimal at 25 μM SA, whereas SA alone, at all concentrations tested from 10 μM to 1 mM, did not cause appreciable cell death (Figure 2B). Moreover, SA in this concentration range did not promote cell death in response to virulent *P. s. glycinea* carrying *avrC* (Figure 2B). This is consistent with the dependence of hypersensitive cell

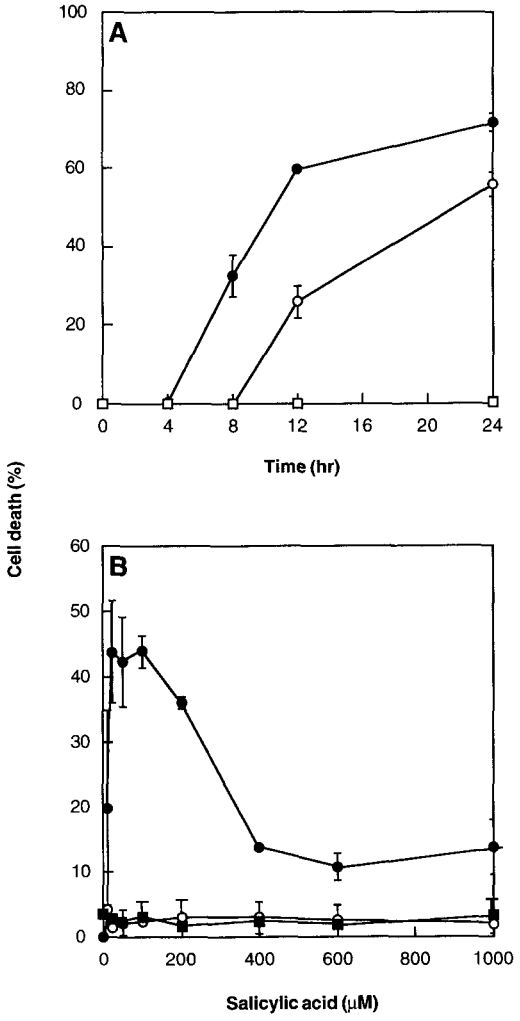

Figure 2. SA Potentiation of the Induction of Cell Death by *P. s. glycinea* Carrying *avrA*.

(A) Kinetics of cell death in response to 50 μM SA (□), *P. s. glycinea* carrying *avrA* (○), and *P. s. glycinea* carrying *avrA* plus 50 μM SA (●).

(B) Dose–response for SA stimulation of cell death. Cell death was determined 8 hr after treatment of soybean cells with the indicated concentrations of SA alone (○), SA together with *P. s. glycinea* carrying *avrA* (●), or SA together with *P. s. glycinea* carrying *avrC* (■).

death in this system on the second *avrA*-mediated oxidative burst (Levine et al., 1994). The observation of an acceleration of avirulence gene–dependent cell death further supports the conclusion that exogenous SA not only enhances the initial, nonspecific stimulation of H_2O_2 accumulation but also accelerates the subsequent massive *avrA*-dependent oxidative burst. Concentrations of SA >200 μM gave a relatively weak potentiation of the response to *P. s. glycinea* carrying *avrA*. This weak effect at supraoptimal concentrations likely reflects the antioxidant properties of SA.

These experiments revealed a powerful synergy with the pathogen such that SA, at physiological concentrations having negligible effects when administered alone, dramatically potentiated the HR to *P. s. glycinea* carrying *avrA*. The marked increase in potency when tested in the presence of the avirulent pathogen suggests that SA does not act as a linear, inductive signal in this system. SA had no effect on the induction of cell death by exogenous H_2O_2, and the catalase inhibitor 3-aminotriazole (Chen et al., 1995) failed to mimic its effects on the response to *P. s. glycinea* carrying *avrA* (Table 1), placing SA action upstream of H_2O_2 accumulation. Moreover, SA potentiated the induction of transcripts encoding PAL, which catalyzes the first step in SA synthesis as well as the antioxidant enzyme GST by *P. s. glycinea* carrying *avrA* (Figure 3). Unlike cellular protectant genes such as *gst*, induction of *pal* in soybean cells is independent of the oxidative burst, and *pal* is not induced by exogenous H_2O_2 (Levine et al., 1994). Thus, SA acts at an early step in the response to *P. s. glycinea* carrying *avrA*, either at or upstream of the point where the signal pathways for activation of the oxidative burst and early defense genes diverge.

Table 1. Effect of SA Analogs and 3-Aminotriazole on the Induction of H_2O_2 Accumulation and Cell Death by *P. s. glycinea* Carrying *avrA*[a]

Compound	H_2O_2 Accumulation (μM) (2 hr)		Cell Death (%) (8 hr)	
Control	0.91 ± 0.83	(1.00)	6.5 ± 2.40	(1.00)
SA	8.88 ± 0.13	(9.75)	42.6 ± 3.92	(6.55)
Acetylsalicylic acid	8.77 ± 0.26	(9.63)	36.6 ± 2.80	(5.63)
2,6-Dichloroisonicotinic acid	5.77 ± 0.05	(6.34)	14.9 ± 1.27	(2.29)
4-Hydroxybenzoic acid	0.66 ± 1.17	(0.72)	6.3 ± 1.65	(0.97)
2,4-Dihydroxybenzoic acid	0.25 ± 0.05	(0.27)	2.1 ± 2.50	(0.32)
2,6-Dihydroxybenzoic acid	0.01 ± 0.91	(0.01)	5.9 ± 1.04	(0.91)
3,4-Dihydroxybenzoic acid	0.00 ± 0.12	(0.00)	4.5 ± 3.35	(0.69)
3-Aminotriazole (10 μM)	0.80 ± 0.74	(0.88)	4.1 ± 2.26	(0.63)
3-Aminotriazole (100 μM)	0.64 ± 0.26	(0.70)	3.1 ± 2.31	(0.48)
3-Aminotriazole (1 mM)	0.37 ± 0.34	(0.41)	3.8 ± 0.90	(0.58)

[a] SA and analogs were added simultaneously with bacteria to a final concentration of 50 μM, 3-aminotriazole at the indicated final concentrations. Numbers within parentheses are the relative inductions of H_2O_2 accumulation and cell death compared with the respective controls with *P. s. glycinea* carrying *avrA* alone.

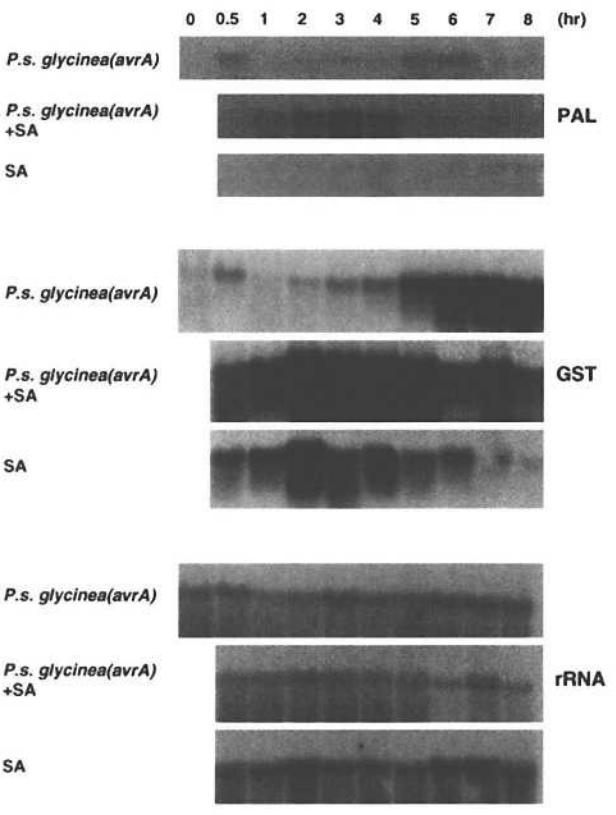

Figure 3. SA Potentiation of Defense Gene Induction by *P. s. glycinea* Carrying *avrA*.

The accumulation of *pal* and *gst* transcripts was monitored by RNA gel blot analysis of total cellular RNA isolated at the indicated times after treatment of soybean cells with *P. s. glycinea* carrying *avrA* (*P.s. glycinea(avrA)*), 50 μM SA, or *P. s. glycinea* carrying *avrA* plus 50 μM SA (*P.s. glycinea(avrA)* + SA). RNA loading was checked by hybridization with an rRNA gene sequence.

Activity of SA Analogs

We next examined the activity of several SA analogs to determine whether structure–activity relationships similar to those previously established for long-term induction of PR protein gene expression and acquired resistance in plant tissues before pathogen challenge (Ward et al., 1991; Chen et al., 1993) also govern potentiation of the response of soybean cells to *P. s. glycinea* carrying *avrA*. The potentiation assay was based on the enhancement of H_2O_2 accumulation at 2 hr and cell death at 8 hr relative to the responses of equivalent cells to *P. s. glycinea* carrying *avrA* alone. 4-Hydroxybenzoic acid and various dihydroxybenzoic acid isomers,

including 2,6-dihydroxybenzoic acid, a well-characterized inducer of PR proteins and SAR (Ward et al., 1991; Chen et al., 1993), were inactive in these assays, whereas acetylsalicylic acid potentiated both H_2O_2 accumulation and cell death almost as effectively as SA (Table 1). Moreover, 2,6-dichloroisonicotinic acid, a plant protectant agrichemical that mimics SA function (Ward et al., 1991), also potentiated these responses to *P. s. glycinea* carrying *avrA* but less strongly than SA (Table 1).

Potentiation Mechanism

A protein kinase cascade is activated after the perception of pathogen avirulence signals (Dietrich et al., 1990; Felix et al., 1994; Zhou et al., 1996), and the protein phosphatase type 2A inhibitor cantharidin enhances the response to *P. s. glycinea* carrying *avrA* and stimulates H_2O_2 accumulation in the absence of bacteria (Levine et al., 1994; Tenhaken et al., 1995). We exploited cantharidin perturbation of this phosphorylation/dephosphorylation regulatory poise to investigate the properties of the SA-mediated gain control in a simplified experimental system. A 50-μM concentration of SA stimulated rapid accumulation of H_2O_2 at a cantharidin concentration of 1 μM that was ineffective alone and potentiated a massive response at higher cantharidin concentrations (Figure 4A). 4-Hydroxybenzoic acid, which did not potentiate H_2O_2 accumulation and cell death in response to *P. s. glycinea* carrying *avrA*, also failed to potentiate the response to cantharidin (data not shown). SA was effective in the physiological concentration range of 10 to 100 μM, with half-maximal potentiation at ~50 μM (Figure 4B). Activation of the oxidative burst by SA in combination with cantharidin was not inhibited by AOPP, which is a specific and potent inhibitor of PAL (Amrhein and Gödeke, 1977), or by cycloheximide at a concentration that almost completely blocked protein synthesis (Figure 4C).

Operation of this agonist-dependent gain control by endogenous SA after activation of phenylpropanoid biosynthesis in the HR to *P. s. glycinea* carrying *avrA* was examined by analysis of the effects of AOPP. AOPP had no effect on the initial weak oxidative burst in response to *P. s. glycinea* carrying either *avrA* or *avrC* (data not shown). However, addition of AOPP simultaneously with *P. s. glycinea* carrying *avrA* blocked the subsequent avirulence gene–dependent induction of H_2O_2 accumulation and cell death, and these responses could be restored by exogenous SA (Figures 5A and 5C). A 50-μM concentration of SA only partially restored H_2O_2 accumulation and cell death, and 150 μM SA was required for full restoration of the HR to *P. s. glycinea* carrying *avrA* when endogenous phenylpropanoid biosynthesis was blocked by AOPP (data not shown). If the addition of AOPP was delayed until 2 or 3 hr after inoculation with *P. s. glycinea* carrying *avrA*, only partial inhibition of H_2O_2 accumulation was observed, indicating that phenylpropanoid synthesis is required before as well as during the avirulence gene–dependent oxidative burst (Figure 5B). However, delayed addition of AOPP during the avirulence gene–dependent oxidative burst inhibited H_2O_2 accumulation within 1 to 2 hr of AOPP treatment (Figure 5B), which is consistent with the operation of a direct,

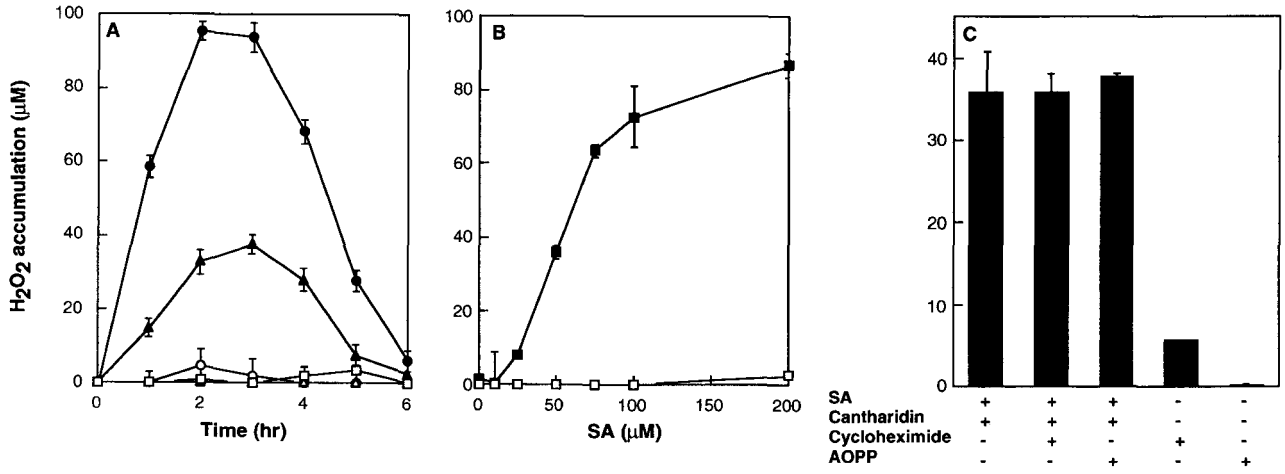

Figure 4. Operation of an Agonist-Dependent, Intrinsic Gain Control Mediated by SA.

(A) Synergistic induction of H_2O_2 accumulation by SA and cantharidin in the absence of a pathogen. SA was added at a concentration of 50 μM; SA (□), 1 μM cantharidin (△), 5 μM cantharidin (○), SA together with 1 μM cantharidin (▲), or SA together with 5 μM cantharidin (●).

(B) Dose–response for SA enhancement of the induction of H_2O_2 accumulation by 1 μM cantharidin. H_2O_2 was measured 2 hr after the addition of SA at the indicated concentrations either alone (□) or with cantharidin (■).

(C) Effects of cycloheximide and AOPP on the synergy between SA and cantharidin. (+) and (−) denote the presence or absence, respectively, of the indicated reagent.

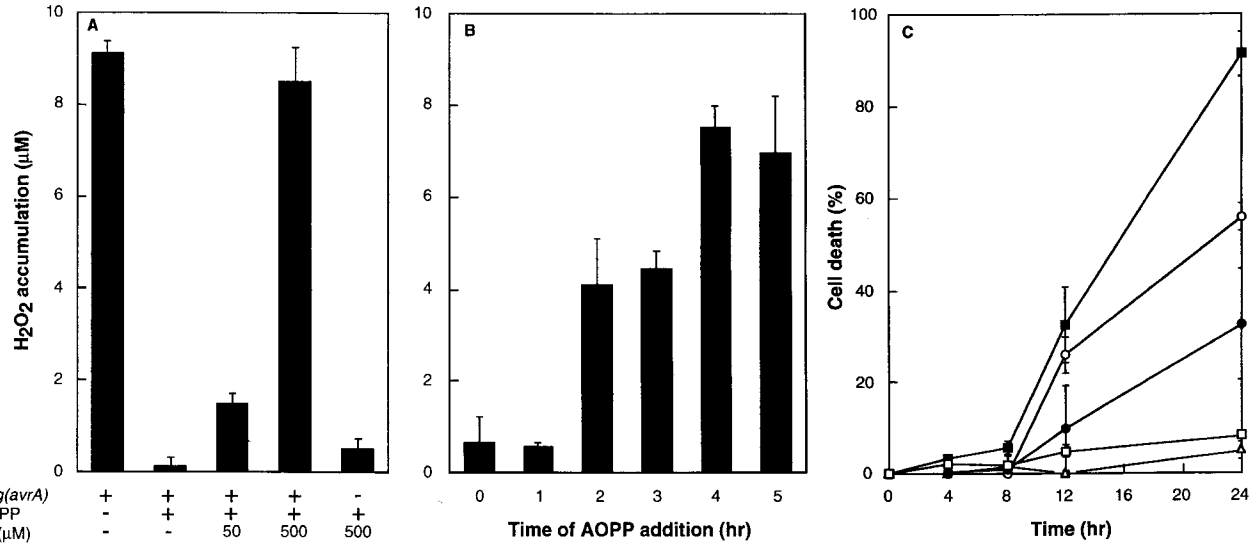

Figure 5. Effects of AOPP on Avirulence Gene-Dependent Induction of H_2O_2 Accumulation and Cell Death.

(A) Effects of AOPP on H_2O_2 accumulation in response to *P. s. glycinea* carrying *avrA* (*Psg(avrA)*). AOPP (100 μM) and SA (50 or 500 μM, as indicated) were administered at the time of bacterial inoculation or to equivalent uninoculated cells, and H_2O_2 accumulation was measured 5 hr after inoculation with *P. s. glycinea* carrying *avrA*. (+) and (−) denote the presence or absence, respectively, of the indicated reagent.

(B) Escape kinetics for AOPP inhibition of the response to *P. s. glycinea* carrying *avrA*. H_2O_2 accumulation was measured 5 hr after inoculation with *P. s. glycinea* carrying *avrA*. AOPP was administered at the indicated times after bacterial inoculation.

(C) Effect of AOPP on the kinetics of induction of cell death by *P. s. glycinea* carrying *avrA*: *P. s. glycinea* carrying *avrA* (○), *P. s. glycinea* carrying *avrA* plus 100 μM AOPP (△), *P. s. glycinea* carrying *avrA* plus 100 μM AOPP plus 50 μM SA (●), *P. s. glycinea* carrying *avrA* plus 100 μM AOPP plus 500 μM SA (■), and 100 μM AOPP plus 500 μM SA (□).

fine control signal amplification mechanism after the accumulation of endogenous SA.

DISCUSSION

Many studies of SA action have been predicated on its function in SAR and hence involved experiments designed to monitor the induction of defense-related responses in tissues or cells treated with SA in the absence of a pathogen. The demonstration that SA also functions in the initial, genetically determined localized resistance (Delaney et al., 1994) prompted us to examine the effects of SA when added to cells simultaneously with a pathogen. These experiments revealed a powerful synergy between the pathogen and signal molecule such that exogenous SA at concentrations having little effect when administered to soybean cells in the absence of a pathogen markedly potentiated H_2O_2 accumulation, defense gene induction, and cell death in response to *P. s. glycinea* carrying *avrA*.

Signal potentiation was observed with SA concentrations as low as 10 μM and was optimal at 50 μM, whereas induction of SAR, PR proteins, and other defense-related responses in healthy leaf tissue typically requires administration of SA at concentrations ranging from 0.5 to 5 mM (Raskin, 1992). Although less efficient uptake of SA by intact tissues very likely contributes to this difference, SA even at concentrations >200 μM was, in the absence of a pathogen, only a weak inducer of H_2O_2 accumulation, defense gene transcripts, and cell death in the soybean cell suspensions. SA accumulates to levels of ~6 μg/g fresh weight in tissues immediately adjacent to the developing hypersensitive lesion (Enyedi et al., 1992), corresponding to a cytosolic concentration of ~70 μM, assuming that there is no compartmentalization (Bi et al., 1995). This result agrees with our observation that 10 to 50 μM exogenous SA potentiates the response of soybean cells to *P. s. glycinea*.

Signal potentiation by SA has been reported previously in several different systems. In parsley suspension cells, pretreatment with SA enhances the subsequent defense responses to a fungal elicitor (Kauss et al., 1992; Kauss and Jeblick, 1995). The optimal effects were obtained at >500 μM SA after 24 hr of preincubation. Although it is difficult to extrapolate between cell cultures and intact plants with respect to uptake and metabolism of exogenous SA, similar effects have also been observed in intact plant tissues. For example, in experiments using cucumber hypocotyl segments, the abraded segments were not competent for elicitation of H_2O_2 immediately after treatment with SA or 2,6-dichloroisonicotinic acid, and the gradual development of competence required protein synthesis (Siegrist et al., 1994; Fauth et al., 1996;

Kauss and Jeblick, 1996). Hence, the presence of SA or 2,6-dichloroisonicotinic acid in the tissue was not itself sufficient for potentiation, and induction of competence factors was required (Kauss, 1994; Fauth et al., 1996; Kauss and Jeblick, 1996). Likewise, hydroponically feeding tobacco plants with 1 to 2 mM SA for 1 to 7 days potentiates expression of local defense genes (Mur et al., 1996), and this effect slowly develops over time.

A different picture emerges when SA action is examined in the presence of a pathogen. First, the optimal concentration for SA stimulation of the agonist-dependent gain control was ~50 μM and hence within the physiological concentration range of SA in the HR; however, in the absence of a pathogen, >500 μM SA caused only modest accumulation of H_2O_2 and almost no cell death. Thus, in the soybean cell suspensions, SA was markedly more potent when interacting synergistically with an appropriate agonist than when tested on naive cells in the absence of a pathogen. Second, preincubation was not required, and SA in the presence of an appropriate agonist evoked a direct, rapid response as determined by the kinetics of H_2O_2 accumulation in response to SA and cantharidin. Third, de novo protein synthesis was not required. Thus, our experiments reveal a rapid, noninductive fine control mechanism for SA potentiation of defense responses in contrast to the time-dependent inductive mechanisms previously described for SA action in the absence of a pathogen. The relative activities of a set of SA analogs in the potentiation assay are similar but not identical to the structure–activity relationships observed for induction of PR protein genes and SAR in the absence of a pathogen. In particular, 2,6-dihydroxybenzoic acid is inactive in the potentiation assay, and 2,6-dichloroisonicotinic acid is markedly less active than SA. Therefore, the agonist-dependent potentiation mechanism may involve a different SA receptor system than that underlying direct inductive effects.

The involvement of protein phosphorylation in the early stages of the HR, inferred from physiological and pharmacological experiments, has been confirmed by recent reports that the tomato *Pto* and rice *Xa21* disease resistance genes encode protein serine/threonine kinases (Martin et al., 1993; Song et al., 1995). Moreover, the Pto kinase phosphorylates a second protein serine kinase, Pti1, that is also involved in the induction of the HR, implying the operation of a protein kinase signal cascade (Zhou et al., 1996). The acute activation of the oxidative burst by SA in combination with the protein phosphatase type 2A inhibitor cantharidin demonstrates SA potentiation of a protein phosphorylation–mediated signal pathway. The oxidative burst appears to be regulated by a poise between phosphorylation and dephosphorylation of one or more components of the signal pathway (Levine et al., 1994; Tenhaken et al., 1995). SA acting synergistically with an appropriate agonist, such as cantharidin or *P. s. glycinea* carrying *avrA*, thus switches this regulatory poise to pathway activation. The massive synergistic enhancement of the oxidative burst by cantharidin and SA acting in concert reveals a highly geared gain control. Because the response was not blocked by AOPP or cycloheximide, this gain control is intrinsic to the proximal signal pathway and does not require feedback autoamplification of SA synthesis or induction of SA biosynthetic enzymes. These rapid, direct, agonist-dependent effects are reminiscent of forskolin potentiation of G protein activation of adenyl cyclase (Whisnant et al., 1996). By analogy, it is possible that SA potentiates the physical interaction between two proteins involved in the HR signal transduction pathway, thereby increasing the sensitivity of the system to upstream agonists to give an intrinsic gain control mechanism providing rapid, noninductive amplification of pathogen signals.

In soybean cells, the fine control mechanism operates at physiological concentrations of SA, whereas time-dependent, inductive effects in the absence of a pathogen require substantially higher concentrations. Hence, the rapid, agonist-dependent gain control for amplification of pathogen signals may make an important contribution to the function of SA in mediating the effective expression of inducible defense mechanisms. Consistent with this conclusion, AOPP blocks the induction of H_2O_2 accumulation and cell death by *P. s. glycinea* carrying *avrA*, and these responses can be efficiently rescued by exogenous SA. Moreover, the rapid inhibition of H_2O_2 accumulation after delayed addition of AOPP during the *avrA*-dependent oxidative burst, taken together with the potency of SA in the presence of pathogen, indicates that endogenous SA potentiates the agonist-dependent gain control in the HR of soybean cells to *P. s. glycinea* carrying *avrA*.

PAL activity is the overall rate-determining step in phenylpropanoid biosynthesis (Bate et al., 1994), and SA potentiation of an intrinsic gain control acting upstream of *pal* induction subsumes further, extrinsic amplification by creating a positive feedback loop regulating SA synthesis. Such a positive feedback loop of SA synthesis mediated by stimulation of *pal* induction would further enhance signal amplification. Consistent with this conclusion, AOPP delays the initial induction of *pal* and chalcone synthase genes (Bolwell et al., 1988), and low concentrations of pathway intermediates stimulate expression of these genes (Mavandad et al., 1990; Loake et al., 1992). Another PAL inhibitor, 2-aminoindan-2-phosphonic acid, suppresses *pal* promoter activity, and SA is able to restore *pal* expression in Arabidopsis challenged with *Peronospora parasitica* (Mauch-Mani and Slusarenko, 1996). An SA-dependent rheostat amplification loop has also been inferred from studies of the function of SA in the phenotypic expression of lesion mimic mutants (Weymann et al., 1995). AOPP inhibition of the endogenous phenylpropanoid biosynthesis required for operation of an autoamplification cycle would explain why concentrations of SA greater than the optimal for potentiation in the absence of AOPP are required for full restoration of induction of H_2O_2 accumulation and cell death by *P. s. glycinea* carrying *avrA*.

Bacterial avirulence genes are first expressed ~2 to 3 hr after the pathogen comes in contact with plant cells (Huynh et al., 1989; Innes et al., 1993), and the effects of delayed

addition of AOPP on induction of H_2O_2 accumulation by *P. s. glycinea* carrying *avrA* indicate that phenylpropanoid synthesis is required before as well as during the avirulence gene–dependent oxidative burst. Therefore, we propose a model in which SA, accumulating as a result of phenylpropanoid synthesis during the weak initial response, is available to prime the signal pathway activated after avirulence gene expression ~2 hr after the pathogen comes in contact with plant cells. Priming and subsequent autoamplification of a signal that potentiates a highly geared intrinsic gain control would account for the abrupt activation of a sustained oxidative burst sufficient to trigger cell death and also help to integrate the activation of H_2O_2-dependent responses with early defense gene transcription (León et al., 1995). Moreover, after localized inoculation with an avirulent pathogen, SA accumulates throughout the plant, albeit to lower levels than in tissues immediately adjacent to the hypersensitive lesion (Raskin, 1992; Vernooij et al., 1994). SA is therefore available to potentiate the activation of inducible defenses in immunized plants after subsequent challenge inoculation with a normally virulent pathogen, in a fashion similar to potentiation of the weak response of naive soybean cells to *P. s. glycinea* carrying *avrC* by simultaneous treatment with low concentrations of SA. This model also provides a plausible function for the SA glucoside that accumulates in immunized plants (Enyedi et al., 1992; Chen et al., 1995), because SA released from this inactive conjugate would be immediately effective at the fine control level in agonist-dependent amplification of weak nonspecific signals to potentiate rapid defense responses such as oxidative cross-linking of the cell wall (Bradley et al., 1992; Tenhaken et al., 1995) after pathogen challenge.

METHODS

Inoculation of Soybean Cell Cultures

Soybean (*Glycine max* cv Williams 82) cell suspensions were subcultured every 7 days by 1-to-5 dilution in fresh Murashige and Skoog (1962) medium containing 3% sucrose, 0.5 mg/L 2,4-dichlorophenoxyacetic acid, and 0.5 mg/L 6-benzylaminopurine, pH 5.5, and physiological experiments were performed on cell suspensions 3 days after subculture (Levine et al., 1994). *Pseudomonas syringae* pv *glycinea* race 4 with plasmid pLAFR1 carrying the *avrA* or *avrC* avirulence genes (Keen and Buzzell, 1991) were provided by N.T. Keen (University of California, Riverside). Bacteria were grown overnight in King's B medium supplemented with the appropriate antibiotics, centrifuged, and resuspended in sterile H_2O before addition to soybean cell suspensions at a final inoculum of 10^8 colony-forming units/mL (Levine et al., 1994). Except where otherwise noted, salicylic acid (SA) or other reagents were added simultaneously with bacteria at the indicated final concentrations. Cycloheximide (0.5 μg/mL) was added 4 hr before SA (50 μM) and cantharidin (1 μM), and H_2O_2 accumulation was measured 2 hr after stimulation. Protein synthesis was inhibited 97% by the cycloheximide treatment. α-Aminooxy-β-phenylpropionic acid (AOPP) was added as indicated to a final concentration of 100 μM.

H_2O_2 Accumulation and Cell Death

H_2O_2 accumulation in soybean cell suspension cultures was assayed by scopoletin destruction monitored by the loss of fluorescence at 460 nm after excitation at 350 nm (Levine et al., 1994). The relationship between loss of fluorescence and increasing H_2O_2 concentration is linear (Root et al., 1975). For the assay of cell death, soybean cell suspensions were incubated for 15 min with 0.05% Evans Blue (Sigma), and unbound dye was removed by washing. Dye bound to dead cells was solubilized in 50% methanol with 1% SDS for 30 min at 50°C and quantified by absorbance at 600 nm (Levine et al., 1994). Data are presented as means with standard deviations of three or more replicates.

RNA Analysis

Total cellular RNA was isolated, and transcript accumulation was monitored by using RNA gel blot hybridization (Levine et al., 1994) with the following probes: bean chalcone synthase *chs1* cDNA (Ryder et al., 1984), *Gmhsp-26 gst* cDNA (Czarnecka et al., 1988), and a 1046-bp phenylalanine ammonia–lyase *pal1* sequence generated by polymerase chain reaction amplification of soybean genomic DNA, using the primers 5′-ACCACCAGTGGCTTGGTCCTC-3′ and 5′-AGGCACTCCATAAGAGGATC-3′ (Frank and Vodkin, 1991). Equal loading of RNA in each lane was confirmed by hybridization with an rRNA gene sequence (Pepper et al., 1994).

Chemicals

AOPP was obtained from Genosys Biotechnologies Inc. (The Woodlands, TX); 3-aminotriazole, scopoletin, SA, and its derivatives from Sigma; and 2,6-dichloroisonicotinic acid from Sumitomo Chemical Co. (Takarazuka, Japan).

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