Salicylic Acid Induced Resistance to *Erwinia carotovora* subsp. *carotovora* in Tobacco

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Salicylic acid (SA) has been inferred to be an endogenous signal in the systemic acquired resistance response of plants. In this study, we demonstrated that exogenously added SA can enhance plant resistance to the phytopathogenic enterobacterium *Erwinia carotovora*. Addition of SA to the growth medium of axenically growing tobacco seedlings made them almost fully resistant to subsequent infection by the soft rot pathogen *E. c.* subsp. *carotovora*. Both the development of soft rot symptoms (tissue maceration) and the *in planta* proliferation of *E. c.* subsp. *carotovora* were inhibited in SA-treated plants. The observed effect was not caused by direct action of SA on growth, nor on extracellular enzyme production or activity of the pathogen at the physiological SA concentrations, but was rather a consequence of induction of plant defense response. This was suggested by the development of hypersensitive-like reactions in SA-treated *Erwinia*-infected plants, by the temporal pattern of resistance development, and by the parallel increase in pathogenesis-related proteins. The plants reacting hypersensitively to *E. c.* subsp. *carotovora* showed a further increase in endogenous SA levels, indicating that SA and SA-controlled processes such as systemic acquired resistance are involved in *Erwinia*-plant interaction. The molecular mechanism of the SA-induced resistance to *E. c.* subsp. *carotovora* is not clear but appears to involve inhibition of plant cell wall–degrading enzymes secreted by this pathogen.

Additional keywords: bacterial soft rot, hypersensitive reaction.

A plant's ability to survive pathogen attack depends both on preformed barriers and on induced active defense mechanisms. Induced resistance or systemic acquired resistance (SAR) has been characterized in a variety of plant species (Chester 1933; Ross 1961; Kuc 1982; Metraux *et al.* 1991; Uknes *et al.* 1992). The SAR response leads to enhanced resistance to a variety of fungal, viral, and bacterial pathogens and can be triggered either by localized infection with necrotrophic pathogens or by treatment with certain chemicals such as salicylic acid (SA) or 2,6-dichloroisonicotinic acid (Ross 1961; White 1979; Kuc 1982, 1983; van Loon and Antoniw 1982; Metraux *et al.* 1991; Ward *et al.* 1991). The localized necrosis in tobacco plants reacting hypersensitively to tobacco mosaic virus (TMV) was early on associated with SAR induction (Ross 1961), and similar SAR has since been observed in other incompatible plant-pathogen interactions (Kuc 1982, 1983; Ye *et al.* 1989; Smith *et al.* 1991).

The involvement of SA in SAR induction has been clearly established: exogenous application of SA or its derivatives can induce resistance to several viruses, some fungi, and some bacteria (White 1979; van Loon and Antoniw 1982; Ye *et al.* 1989; Malamy and Klessig 1992). Increased levels of endogenous SA in plants exhibiting induced resistance to viral or fungal infections has identified SA as a putative endogenous signal for the SAR response (Malamy *et al.* 1990, 1992; Metraux *et al.* 1990; Yalpani *et al.* 1991; Enyedi *et al.* 1992a,b), although the presence of other signals cannot be excluded (Rasmussen *et al.* 1991). A requirement for SA in the SAR response was recently demonstrated with transgenic plants expressing a bacterial gene for salicylate hydroxylase. These plants did not accumulate SA and were defective in the development of SAR (Gaffney *et al.* 1993).

The mechanism of SAR is not well understood, but the broad resistance achieved suggests the participation of several resistance mechanisms. Characterization of the biochemical changes associated with SAR has demonstrated a close correlation between development of resistance and induction of pathogenesis-related (PR) proteins, which are thought to play a role in the defense response of plants (van Loon 1985; Kauffman *et al.* 1987; Legrand *et al.* 1987; Bol *et al.* 1990). Expression of the corresponding genes as well as other recently described SAR-associated genes has been shown to be coordinately induced during onset of this response (Metraux *et al.* 1991; Ward *et al.* 1991; Uknes *et al.* 1992).

Soft rot erwinias constitute one of the most important groups of bacterial phytopathogens that are destructive to a wide variety of plants both in culture and in storage (Pérombelon and Kellman 1980). Members of this group such as *Erwinia carotovora* subsp. *carotovora* can infect a multitude of plant species under the right environmental conditions, particularly high humidity (Pérombelon and Kelman 1980; Kotoujansky 1987). For example, *E. c.* subsp. *carotovora* is the causal agent of potato soft rot, a disease characterized by extensive maceration of the infected plant tissue by a variety of plant cell wall–degrading enzymes secreted by the bacteria (Collmer and Keen 1986). These extracellular enzymes constitute the main pathogenicity factors of

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the soft rot erwinias, and their crucial role in virulence is demonstrated by isolation of avirulent mutants that are defective in production or secretion of the enzymes (Boccara *et al.* 1988; Ried and Collmer 1988; Pirhonen *et al.* 1991, 1993). The production of the enzymes is a tightly regulated process that is controlled by several regulatory genes (Murata *et al.* 1991; Pirhonen *et al.* 1991, 1993; Saarilahti *et al.* 1992). Global control of both extracellular enzyme production and virulence is by autoinducer, a small, diffusible signal molecule produced in a cell-density-dependent fashion (Pirhonen *et al.* 1993). Accumulation of this autoinducer, whose synthesis is directed by the *expI* gene, is required for coordinate expression of the extracellular enzyme encoding genes (Pirhonen *et al.* 1993).

E. c. subsp. *carotovora* does not contain avirulence genes nor appear to cause a hypersensitive reaction in plants, nor has any genetically defined resistance to *E. c.* subsp. *carotovora* been described (Collmer and Keen 1986; Kotoujansky 1987; Keen 1990). Resistance to this pathogen has been observed in *Solanum brevidens*, a wild, nontuber-bearing potato species (Austin *et al.* 1988), but the genetic basis for this resistance is not clear.

We demonstrated previously that the extracellular enzymes produced by *E. c.* subsp. *carotovora* appear to release endogenous elicitors of plant defense responses including the expression of a PR β-1,3-glucanase gene (Palva *et al.* 1993). Induction of plant defense by treatment with acellular preparations of pectic enzymes was shown to induce resistance to subsequent infections by *E. c.* subsp. *carotovora* (Palva *et al.* 1993) and may be indicative of a SAR-like response. To address the question of whether SAR is involved in plant-*Erwinia* interaction, we characterized the effect of SA on plant resistance to *E. c.* subsp. *carotovora*. In this study, we demonstrated that exogenous SA could effectively induce resistance to *E. c.* subsp. *carotovora* in tobacco. Our results indicate that although SA shows some antibacterial activity *in vitro*, it does not accumulate in plant tissues to a level sufficient to account for the cessation of bacterial growth *in planta*. The resistance induced by SA treatment is correlated with PR mRNA accumulation and appears to involve inhibition of plant tissue maceration.

RESULTS AND DISCUSSION

Salicylic acid prevents development of soft rot symptoms in tobacco plants infected with *E. c.* subsp. *carotovora*.

To study the effect of exogenously applied SA on *E. c.* subsp. *carotovora*–plant interaction, we employed axenic tobacco seedlings grown in 24-well tissue culture plates. This test system has been successfully used for large-scale virulence screening as well as for monitoring defense gene activation (Pirhonen *et al.* 1991; Palva *et al.* 1993), and it provided a controlled way of SA application. The chemical could be directly added to the plant culture medium, and this application method gave more reproducible results than did foliar sprays with SA. SA was rapidly taken up by the plant root system as monitored with radioactively labeled SA (data not shown). SA was not found to have any visual phytotoxic effect on plant growth at concentrations up to 1 mM (the concentration used), whereas 2 mM SA had a slight inhibitory effect (data not shown).

SA application drastically altered the *Erwinia*-plant interaction from susceptibility to resistance. Figure 1 illustrates a typical SA-induced resistance response in tobacco leaf inoculated with *E. c.* subsp. *carotovora*. Clear maceration of control (nontreated) tissues was visible within 4 hr of inoculation, and the inoculated leaf was normally macerated within 24 hr (Fig. 1A). The infection subsequently spread throughout the plants, and all the plant material in the well was normally macerated within 2 days. In contrast, treatment of the seedlings with SA induced significant resistance to *E. c.* subsp. *carotovora* infection. No extensive maceration of the inoculated leaf was observed in SA-treated plants (Fig. 1B). Limited maceration of plant tissue was observed at the site of inoculation, but this was rapidly surrounded by a zone of necrotic tissue, and prolonged incubation of the plantlets for 2 days resulted in what appeared to be a hypersensitive reac-

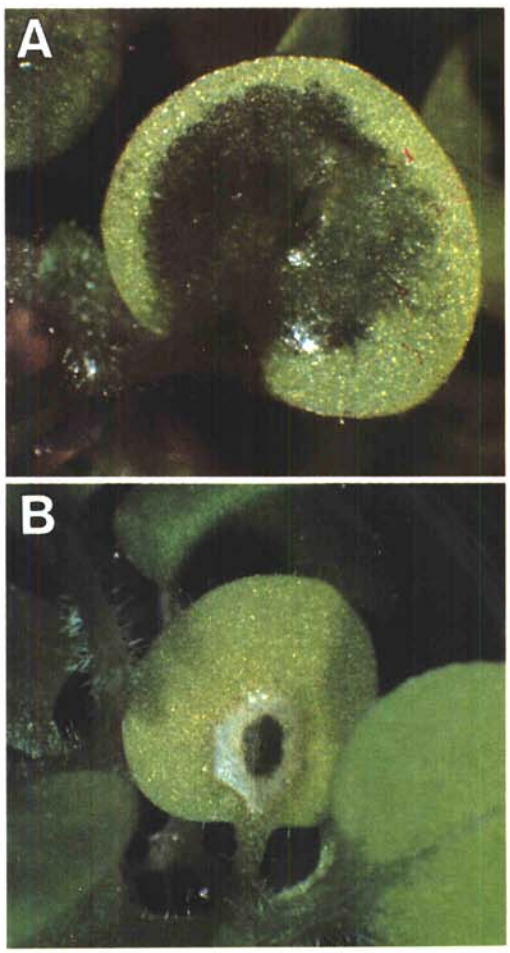

Fig. 1. Inhibition of soft-rot symptom development by salicylic acid. Axenic seedlings of *Nicotiana tabacum* L. 'Samsun' were propagated on MS-2 medium in 24-well tissue culture plates. After 2 wk of growth, **A,** 100 µl of H_2O or **B,** 100 µl of 10 mM K-salicylate (SA, 1 mM final concentration) was added to each well. The plantlets were further propagated for 4 days, after which they were locally inoculated by infiltration of 5×10^5 CFU of *Erwinia carotovora* subsp. *carotovora* strain SCC3193. The bacteria used for the inoculum were grown overnight in L medium to early stationary phase. The inoculated plantlets were incubated for 8 hr by which time the nontreated leaves were almost fully macerated (A). The SA-treated plants were further incubated for 2 days (B). The infected plants were documented by photography using a Wild M8 stereo microscope and a Wild MPS46 camera system.

tion. The localized cell death around the infection site seemed to inhibit the spreading of the infection (Fig. 1B).

To characterize the temporal pattern of resistance development, plants were inoculated with *E. c.* subsp. *carotovora* at different times after SA application, and the appearance of disease symptoms was documented (Table 1). We used an inoculum size that under the assay conditions resulted in a successful infection in about half of the control plants. Most of the successfully infected leaves were completely macerated within 24 hr, and the infection spread rapidly into other parts of the plants. The first signs of SA-induced resistance were detectable within 1 day, but the effect was most dramatic after 4–5 days of SA treatment. This is in accordance with the SAR observed in other systems (Ward *et al.* 1991; Uknes *et al.* 1992) and is preceded by the accumulation of SA in the tissue (see below and Fig. 3). In conclusion, the SA treatment dramatically altered the response of tobacco plants to *E. c.* subsp. *carotovora* infection. The visual demonstration of a necrotic response reminiscent of the hypersensitive reaction (HR) in SA-treated infected plants (Fig. 1) is of particular interest, because this type of response has not been previously characterized in *E. c.* subsp. *carotovora*–plant interactions. HR is usually associated with incompatibility in more specific race-cultivar types of interactions (Flor 1946; Keen 1990) and is dependent on particular genes of the pathogen such as *avr* and *hrp* genes. The *hrp* genes are required for both elicitation of HR and pathogenicity. Such genes have not been previously characterized from *E. c.* subsp. *carotovora* but their identification from *Erwinia amylovora* (Wei *et al.* 1992) and possibly even from *E. chrysanthemi* (A. Collmer, personal communication; compare He *et al.* 1993), another soft rot *Erwinia* species, is very intriguing.

Table 1. Inhibition of *Erwinia carotovora* subsp. *carotovora* infection by salicylic acid

Duration of treatment (days)	Disease development[a]	
	Infected/ inoculated	Percent infected
Salicylic acid		
0	19/48	40
1	14/48	29
2	8/48	17
3	5/44	11
4	0/44	0
5	0/40	0
6	2/40	5
Untreated control		
6	16/40	40

[a] Salicylic acid (SA, 1 mM final concentration) was added to the growth medium of axenically grown 2-wk-old seedlings of tobacco (*Nicotiana tabacum* L. 'Samsun'). At times indicated after SA addition, the plantlets were locally inoculated with 5×10^5 colony-forming units of overnight suspension of *E. c.* subsp. *carotovora* strain SCC3193. The development of soft rot symptoms (tissue maceration) was followed for 2 days; the number of plants successfully infected (showing clear tissue maceration) and the number inoculated are shown.

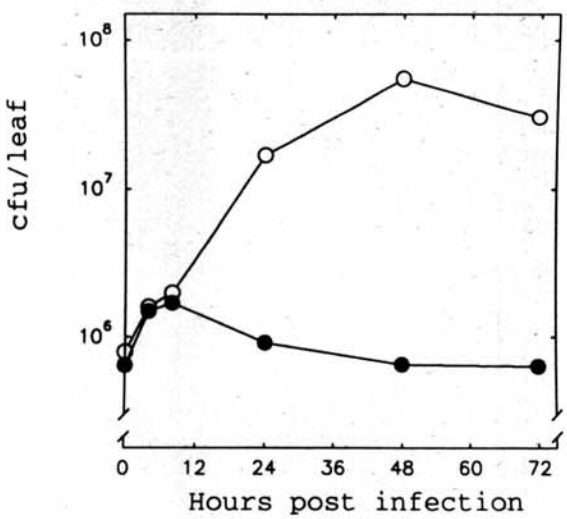

Fig. 2. Inhibition of *in planta* growth of *Erwinia carotovora* subsp. *carotovora* by salicylic acid. Axenic tobacco seedlings were propagated and treated with SA as described in the caption to Figure 1. The 2-week old plantlets were further propagated with 1 mM SA for 6 days prior to inoculation with *E. c.* subsp. *carotovora* strain SCC3193. The local inoculum with 5×10^5 CFU of an overnight culture of SCC3193 was by infiltration. At times indicated, inoculated leaves were collected, homogenized individually in 50 µl of 10 mM $MgSO_4$, and the number of viable bacteria in the samples was determined by plating serial dilutions of these leaf extracts onto L medium. The open symbols indicate the growth of bacteria in control plants treated with H_2O, and the solid symbols that in SA-treated plants. The control leaves were nearly macerated 12 hr after inoculation.

SA-treated tobacco plants do not sustain growth of *E. c.* subsp. *carotovora*.

As demonstrated in Figure 1, SA effectively blocks the tissue maceration associated with the soft rot disease. This could be explained by several reasons, e.g., SA could inhibit the extracellular enzymes of *E. c.* subsp. *carotovora* or the propagation of the bacterium. To address this question we followed the *in planta* growth of the bacterium in SA-treated plants (Fig. 2 and Table 2). This growth was measured by determining the number of viable cells in inoculated plants, and the results clearly demonstrate that SA treatment leads to inhibition of the *in planta* multiplication of the bacteria. As with inhibition of the soft rot symptoms (Table 1), at least 4 days of SA treatment was required for optimal inhibitory effect on bacterial multiplication (Table 2). Inhibition of the *in planta* growth of bacteria by SA was not immediate (Fig.

Table 2. Inhibition of growth of *Erwinia carotovora* subsp. *carotovora* in tobacco plants treated with salicylic acid

Duration of treatment (days)	Bacterial growth[a] (CFU) at different times postinoculation		
	0 hr	24 hr	48 hr
Salicylic acid			
0	0.5	55.0	170.0
2	0.8	26.0	83.0
4	1.7	31.0	1.0
6	0.7	0.9	0.7
Untreated control			
6	0.7	42.0	82.0

[a] Axenic tobacco seedlings were propagated and treated with salicylic acid (SA, 1 mM final concentration) as described in detail in the legend to Table 1. At times indicated after SA addition, the plants were inoculated with *E. c. subsp. carotovora* strain SCC3193. At 0, 24, and 48 hr after inoculation, the inoculated leaves were collected and homogenized, and the number of viable bacteria was determined by plating serial dilutions of leaf extracts onto L medium. The figures indicate the colony forming units (CFU)/leaf sample $\times 10^{-6}$.

2). Bacteria seemed to multiply in SA-treated plants as in control plants for the first 8 hr after inoculation (Fig. 2). After this, there was a sharp growth cessation in SA-treated plants, but the bacteria in control plants continued to multiply normally until the inoculated leaf was totally macerated. The initial multiplication of bacteria (three to four cell divisions) shows that the inhibitory effect of SA treatment on the bacteria is not immediate and could indicate, for example, a gradual depletion of nutrients at the inoculation site. In conclusion, the results demonstrated that SA can effectively inhibit the growth of *E. c.* subsp. *carotovora in planta*, an inhibition that is subsequently manifested in a localized necrosis reminiscent of HR around the infection site (Fig. 1).

Extracellular effect of SA on growth and enzyme production of *E. c.* subsp. *carotovora in vitro*.

There are several possible ways SA could inhibit the *in planta* growth of *E. c.* subsp. *carotovora*: 1) SA could directly affect the bacteria, e.g., as a chelating agent; 2) SA could act as an inducer of plant defense compounds, which in turn would inhibit bacterial growth; or 3) the inhibition could be a combination of both effects. There is previous data to support both possibilities. SA has been established as a putative signal molecule that induces plant defense and SAR (Malamy *et al.* 1990; Metraux *et al.* 1990; Yalpani *et al.* 1991; Enyedi *et al.* 1992a,b). On the other hand, SA at millimolar concentrations has been shown to affect production of bacterial virulence factors (Domenico *et al.* 1989) and expression of genes encoding outer membrane porins, which control permeation of nutrients (Rosner *et al.* 1991). To distinguish between these possibilities, we examined the effects of SA on the *in vitro* growth and enzyme production of *E. c.* subsp. *carotovora*. Addition of increasing concentrations of SA into the growth medium had a marginal effect on bacterial growth (Fig. 3). Only at higher SA concentrations (over 5 mM) could a clear decrease in the growth rate be observed. Similar growth inhibition was observed both in liquid (Fig. 3) and on solid growth media (data not shown). As plant cell wall–degrading enzymes constitute the main virulence factors in *E. c.* subsp. *carotovora* (Pirhonen *et al.* 1991, 1993), we wanted to determine whether SA could affect their production. The results of this analysis (Table 3) show a partial reduction in the amount of extracellular enzymes produced by cells grown in the presence of SA. This reduction, which seems to affect the different categories of enzymes (polygalacturonase, pectate lyase, or cellulase) similarly, is only evident at higher SA concentrations. Furthermore, only a limited decrease in extracellular enzyme production was observed, even at 5 mM SA there was 30 to 40% residual enzyme activity. The observed effect of SA was on enzyme production; the

Table 3. Effect of salicylic acid on *in vitro* extracellular enzyme production of *Erwinia carotovora* subsp. *carotovora*

Salicylic acid addition (mM)	Enzyme activity[a]		
	Peh	Pel	Cel
0.5	13.1	0.89	0.71
1.0	7.9	0.81	0.67
5.0	5.0	0.31	0.35
Untreated control	12.3	1.0	0.92

[a] Peh = polygalacturonase, Pel = pectate lyase, and Cel = cellulase. For assays, the bacteria were propagated in L medium supplemented with 0.4% glycerol and increasing concentrations of salicyic acid. After 8 hr of growth, the culture supernatants were collected and assayed for extracellular enzymes as described by Pirhonen *et al.* (1991). Pel activity was assayed by the thiobarbituric acid method, and the results were expressed as A_{548}. Peh and Cel activities were determined by measuring the release of reducing groups.

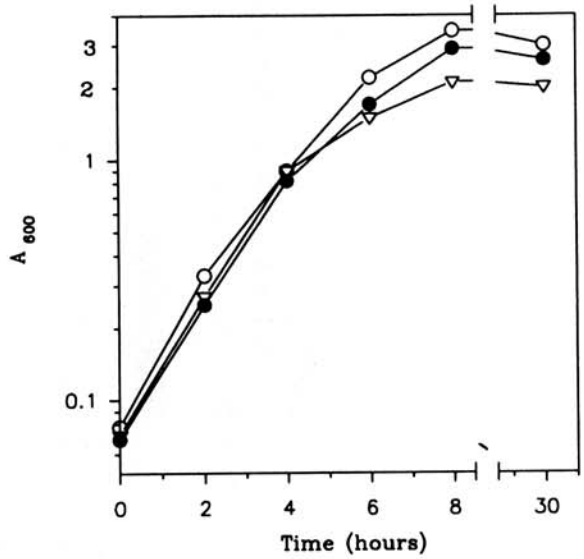

Fig. 3. Effect of salicylic acid on *in vitro* growth of *Erwinia carotovora* subsp. *carotovora*. The *E. c.* subsp. *carotovora* strain SCC3193 was propagated in liquid L medium (Miller 1972) on a rotary shaker (200 rpm), and the growth of the bacteria was followed by determination of the turbidity of the bacterial suspension (A_{600}). Salicylic acid (SA) was added to exponentially growing cultures at time 0, and the growth was followed for times indicated. The final concentrations of SA in the medium were 0 (open circles), 1 mM (solid circles), and 5 mM (open triangles).

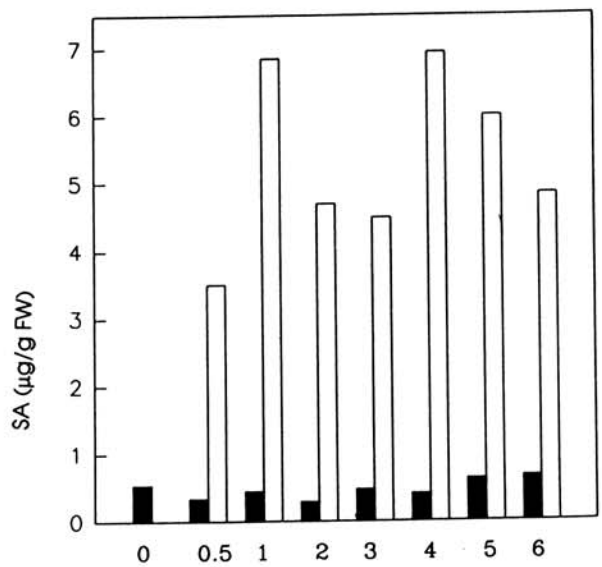

Fig. 4. Accumulation of exogenously added salicylic acid in tobacco seedlings. Salicylic acid (SA) was added to axenically grown *Nicotiana tabacum* L. 'Samsun' seedlings to 1 mM final concentration. The plants were further propagated for times indicated (in days), after which leaf samples (0.5 g fresh weight, FW) were collected and SA was extracted and analyzed as described in detail in Materials and Methods. The open bars represent the amount of free SA in SA-treated samples and the solid bars that in nontreated control samples.

addition of SA did not affect the activity of the enzymes (data not shown).

Is the observed growth inhibition and reduction in extracellular enzyme production in millimolar SA concentrations biologically relevant during plant infection? To address this question, we measured the accumulation of free SA in the SA-treated plants (Fig. 4). The result of this analysis showed that the maximal SA concentration obtained was less than 100 μM (7 μg/g fresh weight), which is well below those concentrations that inhibit bacterial growth and extracellular enzyme production *in vitro* (Fig. 3). Furthermore, this maximum free SA content was already reached 2 days after SA application, yet complete inhibition of bacterial multiplication required 6 days of SA treatment (Table 2). Therefore, a direct effect of SA on bacterial multiplication is not a likely explanation for the observed *in planta* inhibition. Neither could the effect of SA on the *in vitro* production of pectolytic and cellulolytic enzymes explain the growth inhibition at physiological SA concentrations. Consequently, we favor the interpretation that SA prevents *in planta* propagation of *E. c.* subsp. *carotovora* by inducing plant substances inhibitory to this pathogen. This interpretation is in accordance with the proposed role of SA as a signal molecule in SAR response (Malamy *et al.* 1990; Metraux *et al.* 1990; Yalpani *et al.* 1991; Enyedi *et al.* 1992a,b).

SA-induced resistance to *E. c.* subsp. *carotovora* is related to SAR.

The most likely explanation for SA-induced resistance to *E. c.* subsp. *carotovora* is the elicitation of plant defense. PR proteins are thought to constitute part of such induced defense responses (SAR) of plants; therefore, we characterized the induction of a representative of the PR-proteins (PR-1) by the same SA treatment. Figure 5 illustrates the time course of accumulation of PR-1–related mRNA in response to SA-treatment. The level of transcript started to increase within 12 hr of SA application, in parallel to the increase in SA concentration in the plant tissue. Although the free SA concentration seemed to level off already after 1 day, the accumulation of the PR gene–related mRNAs continued to increase for several days (Fig. 5). Quantification of the relative steady-state levels of the PR-1 transcript in control and SA-treated plants showed approximately 9,000-fold induction by SA.

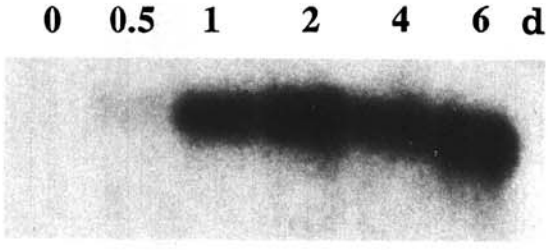

Fig. 5. Accumulation of PR-1 mRNA in tobacco treated with salicylic acid (SA). Axenic seedlings of *Nicotiana tabacum* L. 'Samsun' were propagated and treated with SA as described in the caption to Figure 1. Plant tissues were harvested at times indicated (in days) after SA treatment; the total RNA was isolated, and 10-μg RNA samples were subjected to Northern analysis. The RNA was separated by electrophoresis through formaldehyde agarose gels, blotted onto nitrocellulose filters, and hybridized to ^{32}P-labeled tobacco PR-1 cDNA.

This is in accordance with the 1,000- to 10,000-fold induction of PR-1a seen previously in TMV-infected or SA-treated tobacco (Ward *et al.* 1991; Uknes *et al.* 1993). The observed accumulation of PR-1 transcript was followed by accumulation of the corresponding protein (data not shown). The increase in the steady-state mRNA and protein level seemed to parallel the observed increase in resistance against *E. c.* subsp. *carotovora* infection (Table 1). These results are in accordance with the hypothesis that some of the SAR-associated defense mechanisms, such as induction of PR proteins, could be responsible for resistance to *E. c.* subsp. *carotovora*.

We demonstrated earlier that the extracellular enzymes of *E. c.* subsp. *carotovora* elicit the plant defense response and can result in enhanced tolerance to subsequent infections by the same pathogen (Palva *et al.* 1993). This SAR-like response is apparently due to endogenous elicitors released by the action of the enzymes but is not normally observed during *E. c.* subsp. *carotovora* infections, as the pathogen appears to suppress and overcome the plant defense. The possibility of altering the normally compatible *E. c.* subsp. *carotovora*–plant interaction to an incompatible one by SA addition allowed us to test whether SA is also a signal molecule in this latter interaction. To this aim we analyzed the effect of *E. c.* subsp. *carotovora* infection on endogenous SA levels. The results of this analysis showed that both free and conjugated SA were drastically increased in response to bacterial infection, both in the inoculated and in the systemic leaves (Fig. 6). These data suggest that the tissue necrosis (HR) observed in this incompatible interaction could lead to a further SAR response as indicated by the elevated SA levels. Whether this increase in endogenous SA also leads to a fur-

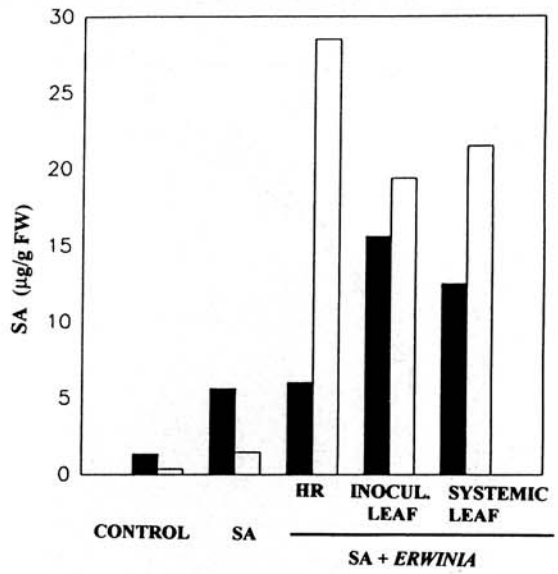

Fig. 6. Effect of *Erwinia carotovora* subsp. *carotovora* infection on salicylic acid (SA) accumulation. Tobacco seedlings were propagated and treated with SA for 6 days as described in the caption to Figure 1. The SA+Erwinia samples came from plants which were then locally inoculated with 5×10^5 CFU of *E. c.* subsp. *carotovora*. After a further 48 hr of incubation, SA was extracted from leaf samples and the amount of free (solid bars) and conjugated (open bars) SA was determined. From the inoculated samples, separate extracts were made within the necrotic lesions (hypersensitive response), from the rest of the inoculated leaf, and from a systemic leaf opposite the inoculated leaf.

ther increase in resistance to *E. c.* subsp. *carotovora* could not be resolved in these studies because of the high degree of resistance obtained by SA treatment only.

What is the basis of SA-induced resistance to *E. c.* subsp. *carotovora*? So far no PR protein that would inhibit growth of bacteria has been characterized. Neither could we observe any antibacterial activity in apoplastic fluids of SA-treated plants. Because the bacterial growth in plants is dependent on the production of the tissue-macerating extracellular enzymes (Pirhonen *et al.* 1991, 1993), it is a distinct possibility that plants would inhibit or become resistant to the action of these enzymes. To test this, we used acellular enzyme preparations to inoculate the plant tissue. These preparations can effectively macerate the leaf tissue of the control plant but are not effective on SA-treated plants (Fig. 7). This suggests that the SAR to *E. c.* subsp. *carotovora* may function by neutralizing the primary weapons of this pathogen, the plant cell wall–degrading enzymes. Whether the mechanism of this resistance is by changing the substrate to a less degradable form or by producing inhibitors to these enzymes, as has been found with fungal pathogens (Toubart *et al.* 1992), remains to be determined. The SA-induced resistance to *E. c.* subsp. *carotovora* described here provides us with an experimental system for such studies.

MATERIALS AND METHODS

Plant material.

The plant material was propagated as described previously (Pirhonen *et al.* 1991; Palva *et al.* 1993). Axenic tobacco seedlings (*Nicotiana tabacum* L. 'Samsun') were propagated in 24-well tissue culture plates on MS-2 (Murashige and Skoog 1962) medium (about five plantlets per well). The seedlings were grown in a Conviron growth chamber at 26° C with a 16-hr light regime (2 W/m^2) approximately 2 to 3 wk before bacterial inoculation or SA treatment.

Bacterial growth and inoculation of plants.

The *E. c.* subsp. *carotovora* strain SCC3193 (Pirhonen *et al.* 1988) was cultured overnight at 28° C in L medium (Miller 1972). The culture was harvested by centrifugation, and the pellet was resuspended in 10 mM $MgSO_4$ at approximately 5×10^8 colony-forming units (CFU) per milliliter. A single site on each leaf was inoculated with 1 µl (5×10^8 CFU/ml) of a bacterial suspension by gently pressing the tip of an automatic pipette against the leaf surface. The progress of the infection was evaluated both visually and by counting the number of viable bacteria in the infected leaf tissue. These counts were done from leaves infected with *E. c.* subsp. *carotovora* at the times indicated and in the following manner: The infected leaf was detached and placed into a microcentrifuge tube containing 50 µl of 10 mM $MgSO_4$. The leaf was then homogenized with a sterile disposable pestle, and 500 µl of L medium was added. The suspension was filtered through sterile Miracloth (Calbiochem, La Jolla, CA) to remove the plant debris. The amount of bacteria in these suspensions was then determined by planting serial dilutions onto L-agar plates.

Application of SA to the plants.

SA was applied directly to the growth medium of 2- to 3-wk-old plantlets to give a 1 mM final concentration. This was done by pipetting 100 µl of a K^+-salicylate solution, pH 6.5, into each well of 24-well tissue culture plates. Control plants received 100 µl of sterile water. The SA-treated plants were further propagated as described above.

Determination of SA.

Leaf tissues were harvested at various times after SA treatment. Tissue samples (0.5 g) were ground in 1 ml of precooled 80% ethanol. The extract was centrifuged at 13,000 × *g* for 20 min. The pellet was resuspended in 1 ml of 80% ethanol and reextracted at 13,000 × *g* for another 20 min. Su-

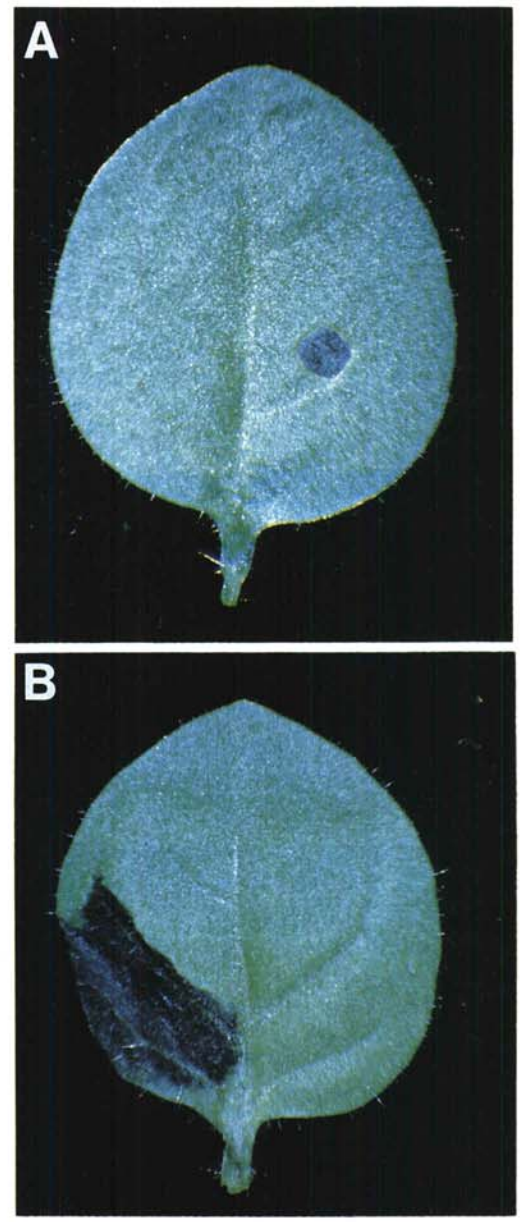

Fig. 7. Inhibition of plant tissue maceration by salicylic acid (SA). Axenic tobacco seedlings, propagated and exposed to SA for 6 days as described in the caption to Figure 1, were treated with acellular extracellular enzyme preparations (culture filtrates) from *Erwinia carotovora* subsp. *carotovora*. Local inoculation of these enzyme preparations was by infiltration of 1 µl of the culture filtrate per leaf, and symptom development was followed for up to 48 hr. The documentation was described in the caption to Figure 1. The plant shown in **A** was treated with SA; the plant in **B** is an untreated control.

pernatants from both extractions were combined, incubated at −20° C for 1 hr and then centrifuged at 13,000 × *g* for 10 min to remove insoluble material. The supernatant was evaporated to one-tenth of the original volume under reduced pressure at 45° C. The volume was then adjusted to 4 ml with water, and metaphosphoric acid was added to give a final concentration of 2% (w/v). The aqueous phase was then partitioned three times with ethyl acetate (v/v). The top organic phases were pooled and evaporated to dryness *in vacuo*. The remaining aqueous phases were adjusted to 1 M HCl and heated at 80° C in a sealed tube for 1 hr. After cooling, the partitioning was done as described above. Extracts were then redissolved in 70% methanol and passed through a C18 Sep-Pak cartridge (Millipore, Bedford, MA) that had been equilibrated with 5 ml of 70% methanol. Salicylic acid was eluted with 6 ml of 70% methanol. The eluted fraction was dried under reduced pressure at 45° C and redissolved in 400 µl of HPLC mobile phase (2% acetonitrile, 5% methanol, and 93% of a 2% acetic acid solution). Samples were analyzed on a Merck-Hitachi system liquid chromatograph (spectrofluorescence detection: excitation 313 nm; emission 405 nm; Raskin *et al.* 1989, 1990). A 100-µl aliquot was injected onto a 5-µm Licrospher 60, RP-select B column (125 × 4 mm i.d.). After 2 min isocratic flow at 2 ml/min, a 12-min linear gradient was used to alter the solvent composition to 10% acetonitrile, 20% methanol, and 70% acetic acid. The SA concentration was determined by comparison of integrated peak area (t_R = 10.5) to peak areas representative of known concentrations of the standards. All data were corrected for SA recovery (49%). Thin-layer chromatography was used to confirm the identity of SA on Merck silica gel 60F254 plates developed with benzene-acetic acid-eau (6:7:3, v/v), upper phase (Saindrenan and Bompeix 1982). The R_f value for SA was 0.55.

Extracellular enzyme preparations and enzyme assays.

The acellular preparations of *E. c.* subsp. *carotovora* extracellular enzymes were done as described earlier (Palva *et al.* 1993). Polygalacturonase, pectate lyase, and cellulase activities were measured and the units defined as described previously by Pirhonen *et al.* (1991).

DNA and RNA preparations and Northern analysis.

Standard techniques for DNA analysis were conducted according to Maniatis *et al.* (1982). Total RNA from plant leaf tissue was isolated as described by Verwoerd *et al.* (1989), and 10-mg samples of total RNA were separated by electrophoresis through formaldehyde agarose gels and blotted to nitrocellulose filters (Ausubel *et al.* 1987). Nick-translated probes were prepared as described by Maniatis *et al.* (1982) from PR-1a cDNA fragment. The hybridization and washing conditions were according to Maniatis *et al.* (1982). The quantification of mRNA levels was by densitometric scanning of the resulting autoradiograms.

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