Cell Wall–Degrading Enzymes from *Erwinia carotovora* Cooperate in the Salicylic Acid–Independent Induction of a Plant Defense Response

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The virulence of the plant pathogen *Erwinia carotovora* subsp. *carotovora* is dependent on the production and secretion of a large variety of plant cell wall–degrading enzymes, including several pectinases and cellulases. Treatment of tobacco plants with culture filtrates (CFs) from the pathogen (containing the secreted cell wall–degrading enzymes) induces both local and systemic expression of genes involved in plant defense response. We have characterized the contribution of individually produced cell wall–degrading enzymes from *E. carotovora* subsp. *carotovora* in their ability to trigger local and systemic induction of a defense-related gene (coding for a basic β -1,3-glucanase) as well as resistance to this pathogen in tobacco. Our results show that mainly pectic enzymes and to some extent one cellulase induce expression of the β -1,3-glucanase gene. Cellulases appear to act cooperatively in the defense gene induction when added in combination with pectic enzymes. Similarly, pectinases and cellulases cooperate in triggering systemic resistance to the pathogen. Salicylic acid (SA) does not appear to be involved in this process, as systemic resistance was induced similarly in transgenic NahG plants that overproduce a salicylate hydroxylase and cannot accumulate SA and in nontransformed control plants. The lack of SA requirement for the induced resistance against *E. carotovora* subsp. *carotovora* suggests the presence of a different signal transduction pathway involved in this plant–pathogen interaction.

Additional keywords: elicitors, pathogenesis-related (PR).

Plants have the ability to perceive and respond to signals resulting from pathogen attack. This recognition triggers the induction of a large arsenal of defense measures that plants em-

ploy to protect themselves from the invading pathogen. The defense responses may be induced specifically, as in a gene-for-gene type of interaction (Flor 1971; Keen 1990; Martin et al. 1993; Staskawicz et al. 1995), or nonspecifically by a range of biotic and abiotic elicitors (Darvill and Albersheim 1984; Darvill et al. 1992; Rohwer et al. 1987, reviewed by Templeton and Lamb 1988). Many fungal and bacterial pathogens secrete a large arsenal of hydrolytic enzymes that digest the plant cell wall, allowing the pathogen to have access to nutrients (Salmond 1994; Walton 1994). Such enzymes can release plant cell wall–derived elicitors that activate plant defense responses (Davis et al. 1984; Hahn et al. 1981; Nothnagel et al. 1983). How these molecules interact with plant cells to induce defense mechanisms is not clear, but a number of studies have identified putative elicitor-binding receptors in the plasma membrane from different plant cells (Cosio et al. 1992; Ebel et al. 1993, 1995; Farmer et al. 1991).

Most of the inducible plant defense mechanisms include both local and systemic transcriptional activation of defense genes. Local defense responses are thought to have a direct effect on the pathogens and they involve changes in ion fluxes across the plasma membrane, generation of active oxygen species (oxidative burst) (reviewed in Atkinson 1993; Mehdy 1994), the hypersensitive response (HR) (Klement 1982), the synthesis of several proteins including enzymes involved in the phenylpropanoid metabolism, the biosynthesis of phytoalexins, and pathogenesis-related (PR) proteins. PR proteins (reviewed by Linthorst 1991; Stintzi et al. 1993; van Loon 1985; White and Antoniw 1991) accumulate as a result of infection by pathogens or after treatment with elicitors. Some PR proteins appear to have protective properties against pathogens (Alexander et al. 1993; Broglie et al. 1991; Mauch et al. 1988; Sela-Buurlage et al. 1993) but in many cases no direct function has been assigned (Neuhaus et al. 1992). Depending on the pathogen, a local infection can also lead to the activation of defense responses in uninfected parts of the plants. An example of this is the systemic acquired resistance (SAR), which is characterized by a general, induced, disease resistance in uninfected parts of the plant and is effective against a broad range of pathogens (reviewed by Malamy and Klessig 1992; Ryals et al. 1994). SAR is associated with the systemic coordinated synthesis of a large number of PR proteins, especially the acidic (extracellular) isoforms (Uknes et

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al. 1992; Ward et al. 1991). The correlation between PR protein synthesis and the acquired resistance suggests a direct involvement of these proteins in plant defense against pathogens (Bol et al. 1990).

Salicylic acid (SA) has been shown to have a central role in SAR. SA concentration rises dramatically after pathogen infection (Enyedi et al. 1992; Malamy et al. 1990; Métraux et al. 1990) and exogenously applied SA leads to the induction of the same set of PR genes induced during SAR (Ward et al. 1991) as well as resistance to pathogen infection (reviewed by Malamy and Klessig 1992). However, the most direct evidence for the role of SA in SAR comes from experiments with transgenic tobacco plants expressing a salicylate hydroxylase (*nahG*) gene from *Pseudomonas putida* (Gaffney et al. 1993). These plants cannot accumulate SA and are unable to manifest an SAR response (Delaney et al. 1994; Gaffney et al. 1993), nor can they induce SAR protein synthesis in response to SA. However, the presence of other signal transduction pathways leading to the induction of genes encoding PR proteins has been shown (Xu et al. 1994).

We are studying the interaction between the nonspecific bacterial phytopathogen *Erwinia carotovora* subsp. *carotovora* and plants. *Erwinias* are gram-negative enterobacteria that cause soft rot in many plant species (Pérombelon and Kelman 1980). They have a very wide host range, attacking a number of plant species if the conditions are favorable. Genetically defined resistance to *E. carotovora* subsp. *carotovora* has not been described; neither does *E. carotovora* subsp. *carotovora* appear to contain typical avirulence genes. Therefore, it provides a good model system to study a nonspecific plant-pathogen interaction. The virulence of this pathogen is dependent on the production and secretion of a large arsenal of plant cell wall-degrading enzymes, including pectinases, cellulases, and proteases, that macerate the plant tissue and release the nutrients for the pathogen (Collmer and Keen 1986; Kotoujansky 1987; Pirhonen et al. 1991). Many of the cell wall-degrading enzymes have been shown to trigger defense mechanisms in plants, probably by releasing cell wall fragments active as elicitors of host defense responses (Davis and Ausubel 1989; Davis et al. 1984; Palva et al. 1993; Yang et al. 1992). We have previously demonstrated that acellular preparations of cell wall-degrading enzymes of *E. carotovora* subsp. *carotovora* induce the expression of genes encoding PR proteins (Palva et al. 1993; Vidal et al. 1997). We have also shown that treatment of plants with pectic enzymes of *E. carotovora* subsp. *carotovora* as well as with SA can induce resistance against this pathogen, and this resistance appears to involve inhibition of plant tissue maceration (Palva et al. 1993, 1994).

In this study, we have analyzed the contribution of individually produced cell wall-degrading enzymes of *E. carotovora* subsp. *carotovora* in the induction of plant defense genes, and the ability of these enzymes to trigger a systemic response in the host. Our results indicate that pectic enzymes and cellulases cooperatively elicit plant defense gene induction. Moreover, our data suggest that cell wall-degrading enzymes of *E. carotovora* subsp. *carotovora* induce systemic resistance to further bacterial infections by affecting the growth of bacteria in planta and that this induced resistance does not require SA accumulation but must rely on a different signal transduction pathway.

RESULTS

Pectic enzymes and cellulases are needed to elicit a full plant defense response.

We have previously shown that acellular culture filtrates (CFs) that contain the secreted plant cell wall-degrading enzymes of *E. carotovora* subsp. *carotovora* induce the expression of several genes encoding PR proteins in tobacco (Palva et al. 1993; Vidal et al. 1997). The induction of these genes was shown to be similar in response to the CF treatment, as in response to infection by *E. carotovora* subsp. *carotovora* itself, suggesting that the CF contains the components necessary to elicit the plant response to this pathogen (Palva et al. 1993; Vidal et al. 1997). One of the genes strongly induced by CF is a gene encoding a basic glucanase in tobacco (Castresana et al. 1990), whose expression has been shown to be regulated by a variety of stress conditions. To monitor the plant defense gene activation in response to elicitors derived from *E. carotovora* subsp. *carotovora*, we utilized transgenic tobacco plants containing a chimeric gene (*gnI-uidA*) coding for the reporter enzyme β -glucuronidase (GUS) under the control of a basic glucanase promoter from *Nicotiana plumbaginifolia* (Castresana et al. 1990). The expression from the *gnI* promoter in response to cell wall-degrading enzymes of *E. carotovora* subsp. *carotovora* was thus analyzed by the measurement of GUS activity in the transgenic plants. In addition, we characterized the accumulation of the endogenous transcripts for this gene by Northern (RNA) analysis in wild-type tobacco.

In order to characterize the contribution of different cell wall-degrading enzymes in eliciting defense gene activation, we employed enzymes individually produced in *Escherichia coli* from cloned pectinase and cellulase genes of *E. carotovora* subsp. *carotovora*. This ensured the controlled application of a defined enzyme without contaminating enzymes with similar activity. An endopolygalacturonase (PehA; Saarilahti et al. 1990a), two pectate lyases (PelA and PelD, produced from the same clone; Heikinheimo et al. 1995) and two cellulases (CelS and CelV1; Saarilahti et al. 1990b; Mäe et al. 1995) were produced in *E. coli* and extracted from the periplasm by osmotic shock. Activities of the isolated enzymes were related to the amount of enzyme activity present in the CF by performing assays for pectic and cellulolytic enzymes as described in Materials and Methods. Different enzymes and enzyme combinations were applied to plants as described in Materials and Methods; GUS activity and accumulation of the endogenous glucanase transcript (Castresana et al. 1990) were analyzed 14 h after the treatment (Fig. 1). The results show that mainly the pectic enzymes (PehA and PelA/D) can induce GUS activity in transgenic tobacco plants (Fig. 1A) as well as trigger the accumulation of the endogenous transcript for the basic glucanase (Fig. 1B). One of the cellulases (CelV1) does also induce the target gene, albeit marginally. However, none of the individual enzymes is able to cause induction as high as that obtained by treatment with a corresponding amount of CF. It is possible that CF contains other unidentified elicitors, and/or that the different enzymes act in concert. To test this possibility, plants were treated with a combination of all the tested enzymes together. Interestingly, this treatment resulted in an induction level of the basic glucanase gene similar to that obtained after treatment with CF. Treatment of plants with

combinations of one pectic enzyme and one cellulase induced target gene expression to a higher extent than treatments with individual enzymes (data not shown). However, combinations of different pectic enzymes (PehA + PelA/D) as well as combinations of cellulases (CelV1 + CelS) did not result in such an additive effect, suggesting that pectic enzymes and cellulases cooperate in eliciting the induction of defense genes in response to *E. carotovora* subsp. *carotovora*.

Fig. 1. Pectic enzymes and cellulases of *Erwinia carotovora* subsp. *carotovora* cooperate in inducing expression of a basic glucanase gene. **A**, Analysis of β -glucuronidase (GUS) activity in transgenic tobacco plants containing a basic glucanase promoter-GUS construct (*gn1-uidA*). Data represent average GUS activities determined for three independent experiments. Transgenic tobacco GN1-GUS plants grown axenically for 3 weeks were treated with individually produced extracellular glucanases of *E. carotovora* subsp. *carotovora* (with an enzyme activity similar to that present in the culture filtrate [CF]) as described in Materials and Methods. Plants were treated with the following: 20 mM $MgCl_2$ (1); CF (2); PehA (3); PelA/D (4); CelS (5); CelV1 (6); PehA + PelA/D + CelS + CelV1 (7); PehA + PelA/D (8); CelS + CelV1 (9). Plant samples were harvested 14 h after the different treatments and the specific GUS activity was determined as described in Materials and Methods. Error bars represent standard deviations. **B**, Accumulation of a basic glucanase mRNA in tobacco SR1 plants. Transcript accumulation for a basic glucanase gene was analyzed by Northern (RNA) blot experiments in *Nicotiana tabacum* SR1 plants grown axenically for 3 weeks. Plants were treated with the following: 20 mM $MgCl_2$ (1); CF (2); PehA (3); PelA/D (4); CelS (5); CelV1 (6); PehA + PelA/D + CelS + CelV1 (7); PehA + PelA/D (8); CelS + CelV1 (9). Plant samples were harvested 14 h after the different treatments; total RNA was isolated and analyzed in Northern blot experiments and hybridized with a probe against the tobacco basic glucanase gene *gn1*. Lower panel indicates the amount of the corresponding RNA samples by hybridizing the same filters with a probe against ribosomal RNA from tobacco.

Local application of isolated cell wall-degrading enzymes of *E. carotovora* subsp. *carotovora* induces a systemic response in plants.

The above data indicate that the induction of a basic glucanase gene in response to *E. carotovora* subsp. *carotovora* is due to the action of cell wall-degrading enzymes. To assess whether this induction was only local or could also lead to a systemic response in tobacco, we characterized the expression of the basic glucanase gene after treatment of tobacco plants with CF as well as with different, isolated, cell wall-degrading enzymes both at the site of treatment and in untreated parts of the plant. The expression of this particular defense gene was analyzed in transgenic tobacco plants containing the *gn1-uidA* construct by measuring GUS activity in local and systemic tissue after treatment with CF (Fig. 2A) and by measuring accumulation of the corresponding endogenous transcript (Fig. 2B). The results show that CF application leads to a rapid systemic induction of the reporter gene with essentially the same timing as in the local induction (Fig. 2A). Similar accumulation of the endogenous transcript was achieved in local and systemic leaves. Application of PehA, PelA/D, CelV1, CelS, and a combination of these five enzymes containing the same activity as in the CF suggested a major role for the pectic enzymes in systemic activation of the plant response. Our results show that the pectic enzymes (PehA and PelA/D) trigger a very rapid, systemic, PR gene activation with a timing similar to that observed after treatment with total CF (Fig. 2B). The induction of the basic glucanase by PehA treatment appears transient and reached maximum levels 8 to 12 h after treatment, both locally and systemically. In contrast, PR gene induction by PelA/D treatment seems to continue even after 12 h. CelV1 induces a weak local expression of the glucanase gene and quite late (24 h after treatment), compared with the induction by the pectic enzymes. Moreover, CelV1 does not appear to induce a systemic response. CelS treatment does not seem to induce the expression of the glucanase gene above the level observed in the control (plants treated with *E. coli* periplasmic extract). When plants were treated with a combination of the five enzymes, the level of basic glucanase gene expression was as high as that observed with CF treatment. These results suggest that pectic enzymes and cellulases cooperate also in the induction of systemic gene expression.

Treatment of tobacco with extracellular enzymes of *E. carotovora* subsp. *carotovora* results in enhanced systemic resistance to bacterial infection.

Our results show that extracellular enzymes of *E. carotovora* subsp. *carotovora* can induce both local and systemic expression of a gene coding for a basic glucanase. A correlation between the induction of defense genes and the development of resistance has been shown in many plant-pathogen interactions. We wanted to investigate if the plant defense responses triggered by cell wall-degrading enzymes of *E. carotovora* subsp. *carotovora* can lead to resistance against this pathogen. Preliminary experiments had indicated that treatment of plants with cell wall-degrading enzymes of *E. carotovora* subsp. *carotovora* prior to bacterial inoculation reduces the maceration of the treated tissue (Palva et al. 1993). To study whether treatment of plants with cell wall-degrading enzymes of *E. carotovora* subsp. *carotovora* in-

Fig. 2. Local and systemic induction of a basic glucanase gene in response to extracellular enzymes of *Erwinia carotovora* subsp. *carotovora*. **A**, Time course of β -glucuronidase (GUS) activity accumulation (in hours) in primary (local) and secondary (systemic) leaf tissue of transgenic tobacco plants containing a basic glucanase promoter-GUS construct (*gnl-uidA*) is shown after treatment with culture filtrate (CF) and 20 mM $MgCl_2$ (control). GUS-specific activity was determined as described in Materials and Methods. Data represent average GUS activities determined for three independent experiments. Standard deviations are represented by the error bars. **B**, Transcript accumulation for a basic glucanase gene was analyzed in Northern (RNA) blot experiments both in primary (local) and secondary (systemic) tobacco SR1 leaves in response to individually produced extracellular enzymes of *E. carotovora* subsp. *carotovora* (with activities similar to that present in the CF). Plants were treated with the following: CF of *E. carotovora* subsp. *carotovora* (CF); PelA/D; PehA; CelV1; CelS; a combination of PelA/D; PehA; CelV1 and CelS (PelA/D; PehA; CelV1; CelS) and periplasmic extracts of *Escherichia coli* (*E. coli*). Plant samples were harvested at the indicated time points (hours), total RNA was isolated and 10 μ g of RNA was analyzed by Northern blots and hybridized with a ^{32}P -labeled probe against the tobacco basic glucanase gene *gnl*.

duces resistance to bacterial growth in planta and whether this resistance is exhibited also in systemic leaves, we treated in vitro-grown tobacco plants with CF and, 24 h after treatment, plants were inoculated in another leaf with a defined amount of bacterial suspensions, 2 to 3×10^7 CFU. The effect of CF treatment on bacterial growth in planta was monitored by extracting and determining the number of bacteria at times indicated after the inoculation (Fig. 3). Our data show that plants treated with CF show an enhanced resistance to bacterial infection. The difference in bacterial growth becomes particularly evident 48 h after inoculation, when bacterial concentration in nontreated plants is 5 to 10 times higher than that obtained in CF-treated plants. Our results show that CF treatment of plants enhances systemic resistance against *E. carotovora* subsp. *carotovora* infection by inhibiting bacterial growth. To study whether treatment of plants with individual cell wall-degrading enzymes of *E. carotovora* subsp. *carotovora* enhances systemic resistance to bacterial growth as well, tobacco plants were treated with PelA/D, PehA, CelV1, and a combination of PelA/D, PehA, CelV1 and CelS, adjusting the enzyme activities to levels present in the CF. Local application of the enzyme preparations was followed after 24 h by inoculation in a systemic leaf with 2 to 3×10^7 CFU and bacterial growth in planta was monitored 48 h after inoculation as described above. Our results show that treatment of plants with isolated enzymes of *E. carotovora* subsp. *carotovora* has an inhibitory effect on bacterial growth in planta (Table 1). Treatment with pectic enzymes PehA and PelA/D resulted in a more pronounced increase in resistance than treatment with the cellulase tested (CelV1). Bacterial growth in CelV1-treated plants was not significantly different from that in nontreated plants. However, the most effective treatment was a combination of pectic enzymes and cellulases, the resulting inhibitory effect being comparable to that achieved in CF-treated plants. These results suggest that cell wall-degrading enzymes of *E. carotovora* subsp. *carotovora* cooperate in the induction of the observed enhanced resistance. To study if the induced systemic resistance is specific only to *E. carotovora* subsp. *carotovora*, we analyzed whether treatment of plants with CF from *E. carotovora* subsp. *carotovora* leads to enhanced resistance to the bacterial pathogen *Xanthomonas campestris*. Our results show that treatment of plants with extracellular enzymes from *E. carotovora* subsp. *carotovora* has an inhibitory effect on the in planta growth of *X. campestris* (Table 2), suggesting that the induced systemic resistance is effective against other bacterial pathogens.

CF-induced resistance against *E. carotovora* subsp. *carotovora* is SA independent.

Our results showing that CF of *E. carotovora* subsp. *carotovora* induces resistance against a subsequent infection by the same bacteria may be indicative of an SAR-like response. SA has a central role in the SAR response and previous results show that treatment of plants with SA enhances plant resistance against this pathogen and that this resistance appears to involve inhibition of plant tissue maceration. (Palva et al. 1994). Thus, to assess the involvement of SA in the CF-induced resistance against *E. carotovora* subsp. *carotovora* we followed the in planta growth of *E. carotovora* subsp. *carotovora* in transgenic NahG tobacco, which cannot accumulate SA (Gaffney et al. 1993) (Fig. 4A). These plants were treated

with CF 24 h before bacterial inoculation (2 to 3×10^7 CFU) and the growth of *E. carotovora* subsp. *carotovora* was measured by determining the number of viable cells in inoculated plants, as described above. The data indicate clearly that treatment of transgenic NahG plants with CF leads to inhibition of the in planta multiplication of the bacteria, in the same way as in wild-type tobacco plants (Fig. 3). In order to rule out the possibility that NahG plants can still respond to SA, bacterial growth was evaluated in transgenic NahG tobacco plants (Fig. 4B) and in wild-type plants (Fig. 4D) 48 h after treatment with 1 mM SA. Figure 4B shows that treatment with SA of NahG plants does not have any effect on the in planta growth of *E. carotovora* subsp. *carotovora*, while SA treatment of wild-type plants induces a clear resistance to this pathogen (Fig. 4D), as previously shown (Palva et al. 1994). Furthermore, treatment of NahG plants with both SA and CF (Fig. 4C) leads to enhanced systemic resistance in the same way as treatment of NahG plants with only CF (Fig. 4A). Taken together, these results indicate that the CF-induced systemic resistance to *E. carotovora* subsp. *carotovora* is not mediated via SA, but must rely on a different signal transduction pathway.

DISCUSSION

Cellulases and pectinases cooperate in defense gene induction.

Treatment of tobacco plants with suspensions of *E. carotovora* subsp. *carotovora* or acellular CFs containing the secreted plant cell wall-degrading enzymes from this pathogen was shown to induce the expression of several genes involved in plant defense responses, both at the site of treatment (Palva et al. 1993) and in distant parts of the treated plants (Vidal et al. 1997). Previous work has indicated that pectic enzymes

from *E. carotovora* subsp. *carotovora* are efficient elicitors of the plant response (Palva et al. 1993). In this work, we have characterized in more detail the role of individual enzymes and combinations of isolated enzymes of *E. carotovora* subsp. *carotovora* in the induction of defense gene expression in tobacco plants. Our results indicate that pectic enzymes (PelA/D and PehA) and at least one of the cellulases (CelV1) of *E. carotovora* subsp. *carotovora* trigger the expression of a basic glucanase gene in tobacco. These results were obtained both in transgenic plants containing a chimeric gene (*gnl-uidA*) coding for the reporter enzyme GUS under the control of the basic glucanase promoter from *Nicotiana plumbaginifolia* (Castresana et al. 1990) and in wild-type tobacco by assessing the accumulation of the endogenous transcripts probed with *gnl*. Furthermore, we show that combinations of pectic enzymes and cellulases cooperate in the induction of this basic glucanase gene in tobacco. Although pectic enzymes alone are good elicitors of this gene, combination with cellulases resulted in a cooperative induction of defense gene expression. The mechanism by which the extracellular enzymes from *E. carotovora* subsp. *carotovora* elicit defense responses in plants is probably the release of plant cell wall fragments active as elicitors (Palva et al. 1993; Davis and Ausubel 1989; Davis et al. 1984). If this is the case, the results presented here suggest that either the release or the perception of the different types of carbohydrate elicitors is cooperative. Our experiments showed that pectic enzymes (PelA/D and PehA) and one cellulase (CelV1) of *E. carotovora* subsp. *carotovora* induced GUS activity in transgenic *gnl-uidA* plants and increased the accumulation of endogenous basic glucanase mRNA. If only one type of elicitor would

Fig. 3. Cell wall-degrading enzymes of *Erwinia carotovora* subsp. *carotovora* enhance systemic resistance to the pathogen. *Nicotiana tabacum* SR1 plants grown axenically for 3 weeks were treated with 20 μ l of culture filtrate (CF) in one leaf; 24 h later plants were inoculated with 2 to 3×10^7 CFU of *E. carotovora* subsp. *carotovora* suspensions in another leaf. At the indicated time points (hours) after inoculation, plant tissue was homogenized, bacteria were extracted, and the numbers of viable bacteria were determined as described in Materials and Methods. Growth of the bacteria in CF-treated plants was compared with nontreated controls (control). Six independent experiments were carried out. Standard deviation is indicated by the error bars.

Table 1. Effect of treatment of plants with extracellular enzymes of *Erwinia carotovora* subsp. *carotovora* on bacterial growth in planta^a

Treatment	CFU per plant
Control	$36.4 \pm 13.5 \times 10^8$
CF	$5.9 \pm 2.8 \times 10^8$
PelA/D	$15.5 \pm 4.6 \times 10^8$
PehA	$9.4 \pm 7.3 \times 10^8$
CelV1	$21.4 \pm 8.7 \times 10^8$
Combined	$5.8 \pm 4.6 \times 10^8$

^a Number of bacteria per plant 48 h after inoculation of tobacco SR1 plants with 1 to 2×10^7 CFU of *E. carotovora* subsp. *carotovora* treated 24 h before inoculation with 25 μ l of the following: 10 mM $MgSO_4$ (control); culture filtrate from *E. carotovora* subsp. *carotovora* (CF); pectate lyases A and D (PelA/D); endopolygalacturonase A (PehA); cellulase V1 (CelV1); and a combination of PelA/D, PehA, CelS, and CelV1 (combined).

Table 2. Effect of treatment of plants with culture filtrate (CF) from *Erwinia carotovora* subsp. *carotovora* on *Xanthomonas campestris* bacterial growth in planta^a

Treatment	CFU per plant	
	24 h	48 h
Control	$12.9 \pm 3.0 \times 10^7$	$25.9 \pm 9.6 \times 10^8$
CF	$7.8 \pm 1.7 \times 10^7$	$3.6 \pm 1.2 \times 10^8$

^a Tobacco SR1 plants were treated with 20 μ l of CF from *E. carotovora* subsp. *carotovora* 24 h before bacterial inoculation. Number of bacteria per plant 24 and 48 h after inoculation with 7 to 8×10^5 CFU of *X. campestris* was determined from both treated (CF) and nontreated (control) plants.

be recognized, the induction by the isolated enzymes should not be possible in all cases. Therefore, it is possible that different types of elicitors exist and can be recognized by different receptor systems. However, another possibility is that the release of one type of elicitor would be enhanced by a combined action of two different types of enzymes. Since cellulases alone are not efficient elicitors of the basic glucanase gene expression, the possibility that cellulase activity may lead to increased release of carbohydrate elicitors by "opening the way" for pectic enzymes cannot be ruled out at this stage. Other examples of elicitor synergism have been described (Chang et al. 1995; Davis et al. 1986; Davis and Hahlbrock 1987).

Cell wall-degrading enzymes of *E. carotovora* subsp. *carotovora* elicit systemic induction of defense gene expression and resistance to further infections by the pathogen.

Our results show that CF containing the secreted cell wall-degrading enzymes from *E. carotovora* subsp. *carotovora* can induce a rapid local and systemic activation of a gene encoding a basic glucanase from tobacco. We have recently shown that this induction is not limited to this gene, but includes a group of genes encoding mainly basic isoforms of PR proteins (Vidal et al. 1997). In this study, we have characterized in more detail what factor(s) in the CF elicits a systemic response in tobacco. For that purpose, we have analyzed the lo-

Fig. 4. Salicylic acid (SA) is not involved in the culture filtrate (CF)-induced resistance against *Erwinia carotovora* subsp. *carotovora*. **A**, Transgenic NahG tobacco plants grown axenically for 3 weeks were treated with 20 μ l of CF in one leaf; 24 h later plants were inoculated with 2 to 3×10^7 CFU of bacterial suspensions in another leaf. At indicated time points (hours) after inoculation, plant tissues were homogenized, bacteria were extracted from both treated (CF) and nontreated (control) plants, and the number of viable bacteria was counted as described in Materials and Methods. Six independent experiments were carried out. Standard deviations are indicated by the error bars. **B**, NahG *Nicotiana tabacum* plants grown in vitro for 3 weeks were treated with 1 mM SA, as described in Materials and Methods; 48 h later, plants were inoculated with 4 to 5×10^7 CFU of bacterial suspensions of *E. carotovora* subsp. *carotovora* in one leaf. Bacterial growth was followed as described above in both SA-treated (SA) and nontreated (control) plants. **C**, NahG *N. tabacum* plants were treated with 1 mM SA 48 h before bacterial inoculation, and with 20 μ l of CF in one leaf, 24 h before bacterial inoculation. After the treatments, plants were inoculated with 4 to 5×10^7 CFU of bacterial suspensions of *E. carotovora* subsp. *carotovora* in one leaf. Bacterial growth was followed as described above in both SA-treated (SA) and SA-plus-CF-treated plants (SA + CF). **D**, *N. tabacum* xanthi-nc plants were grown axenically for 3 weeks and treated with 1 mM SA as described in Materials and Methods; 48 h after treatment, plants were inoculated in one leaf with 4 to 5×10^7 CFU of bacterial solutions of *E. carotovora* subsp. *carotovora* in both treated (SA) and nontreated (control) plants. Bacterial growth was analyzed as described above. Six independent experiments were carried out. Standard deviation is indicated by error bars.

cal and systemic expression of a gene encoding a basic glucanase (Castresana et al. 1990) after treatment of plants with individual cell wall-degrading enzymes from *E. carotovora* subsp. *carotovora*. Our results show that pectic enzymes (endopolygalacturonase, PehA, and pectate lyases, PelA/D) induce the systemic expression of the basic glucanase gene, while cellulases do not appear to induce a systemic response. Thus, our results suggest that pectic enzymes but not cellulases are efficient elicitors of systemic defense gene expression. However, when plants were treated with a combination of cellulases and pectic enzymes, the systemic expression of the target gene appeared to be higher than that induced by individual pectic enzymes, and similar to that induced by CF, suggesting that different cell wall-degrading enzymes cooperate in the induction of a systemic response.

Previous results have shown that treatment of tobacco plants with extracellular enzymes of *E. carotovora* subsp. *carotovora* reduced plant maceration upon inoculation with *E. carotovora* subsp. *carotovora* (Palva et al. 1993). We wanted to investigate whether the induction of defense genes in response to CF and to individual extracellular enzymes of *E. carotovora* subsp. *carotovora* is correlated with the development of systemic resistance against the bacteria. Our results show that treatment of plants with CF reduces bacterial growth in planta. Moreover, treatment of plants with isolated extracellular enzymes also resulted in a reduction of bacterial growth in the plants, compared with nontreated plants. However, this increase in resistance was apparently specific to the pectic enzymes, as treatment with CelV1 had practically no effect on bacterial growth. Interestingly, treatment of plants with a combination of cellulases and pectic enzymes resulted in a systemic induced resistance comparable to the one observed after treatment with CF, suggesting that pectic enzymes and cellulases cooperate also in the induction of resistance against the pathogen. Our results show that the level of induced systemic resistance correlates well with the induction of defense gene expression by each treatment. Furthermore, we have investigated if CF from *E. carotovora* subsp. *carotovora* enhances systemic resistance to another bacterial pathogen, *X. campestris*, the causal agent of black rot disease in several plant species (Williams 1980). Our results show that CF treatment of plants inhibits the in planta growth of *X. campestris* as well, suggesting that the induced systemic resistance is not specific to *E. carotovora* subsp. *carotovora* but is effective against other bacterial plant pathogens.

Systemic resistance induced by cell wall-degrading enzymes of *E. carotovora* subsp. *carotovora* is not SA-mediated.

We have shown previously that extracellular enzymes of *E. carotovora* subsp. *carotovora* induce a rapid local and systemic expression of genes encoding PR proteins in an SA-independent fashion (Vidal et al. 1997). Our present results also show that the induced systemic resistance to subsequent infections by *E. carotovora* subsp. *carotovora* is independent of SA. This was done with transgenic NahG tobacco plants (Gaffney et al. 1993), which are unable to accumulate SA. These results correspond well with our previous observations (Vidal et al. 1997) and suggest that a novel signal transduction cascade exists that leads to the induction of defense gene expression and the enhancement of resistance against the bacte-

ria triggered by extracellular enzymes of *E. carotovora* subsp. *carotovora*. However, exogenously applied SA has been shown to induce resistance against this pathogen (Palva et al. 1994). This means that two independent mechanisms, one dependent on SA and an alternative pathway triggered by extracellular enzymes of *E. carotovora* subsp. *carotovora*, induce systemic resistance to this pathogen.

As discussed, extracellular enzymes from *E. carotovora* subsp. *carotovora* have been shown to elicit defense responses in plants, most likely by releasing cell wall fragments active as elicitors (Davis and Ausubel 1989; Davis et al. 1984; Palva et al. 1993). However, carbohydrate elicitors do not seem to act as systemic signals involved in systemic defense responses. A number of molecules have been identified that meet the criteria for involvement in the signal transduction pathway leading to plant disease resistance (Creelman et al. 1992; Lamb 1994; Raskin 1992). Among the best-studied endogenous signal molecules are SA, ethylene (Lyon et al. 1995), and jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJa) (Enyedi et al. 1992; Farmer et al. 1992). Ethylene itself induces the expression of genes encoding PR proteins (Boller et al. 1983), especially the basic isoforms of glucanases and chitinases (Boller 1988; Felix and Meins 1987; Mauch et al. 1992), the same genes primarily induced during an infection with *E. carotovora* subsp. *carotovora* (Vidal et al. 1997). The possible involvement of JA and MeJa in signaling of defense responses directed against pathogens has been reported (Mueller et al. 1993; Xu et al. 1994). The role of these molecules in the induction of plant defense genes and resistance in response to *E. carotovora* subsp. *carotovora* remains to be established.

MATERIALS AND METHODS

Plant growth conditions.

Nicotiana tabacum Petit Havana SR1, transgenic *Nicotiana tabacum* Petit Havana SR1 plants containing a basic glucanase promoter region-glucuronidase gene construct (*gn1-uidA*) (Castresana et al. 1990), transgenic *Nicotiana tabacum* nahG xanthi-nc plants expressing salicylate hydroxylase (Gaffney et al. 1993), and *Nicotiana tabacum* xanthi-nc plants were used for different experiments. Seeds were surface sterilized by incubation in saturated and filtered aqueous calcium chlorate solutions for 30 min, followed by repeated washings in sterile, distilled water. The surface-sterilized seeds were sown on sterile MS-2 medium (Murashige and Skoog 1962) plates and replanted (four plants per jar) after germination. Plants were grown for 3 weeks in a Conviron growth chamber at 26°C with a 16-h light regime ($150 \text{ to } 200 \mu\text{E} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$) before analysis. Treatment of plants was done under a constant light regime ($150 \text{ to } 200 \mu\text{E} \cdot \text{s}^{-1} \cdot \text{m}^{-2}$) at 26°C.

Bacterial growth and inoculation of plants.

E. carotovora subsp. *carotovora* strain SCC3193 (Pirhonen et al. 1988) and *X. campestris* (pathovar unspecified) strain 1068 (National Collection of Plant Pathogenic Bacteria, York, UK) were cultured overnight at 28°C in L-medium (Miller 1972). Bacteria were harvested by centrifugation (10 min at $4,000 \times g$), and resuspended into 10 mM MgSO_4 at approximately 2×10^9 CFU per ml and used to inoculate plants. Experiments in which *E. carotovora* subsp. *carotovora* growth in planta was evaluated

were done in the following manner: four plants at the time (previously treated with CF or SA) and four nontreated plants were inoculated in one leaf with 10 μ l (2 to 3 $\times 10^7$ CFU) of a bacterial suspension by gently pressing the tip of an automatic pipette against the leaf surface. After the inoculation, plants were incubated under the previously indicated plant growth conditions but in high humidity to facilitate bacterial growth. The progress of the infection was evaluated by counting the number of viable bacteria in the infected plant tissue. At the indicated times, the four infected plants were pooled, homogenized in 10 ml of 10 mM MgSO₄; the number of bacteria per plant was then determined by plating serial dilutions onto L-agar plates. Six independent experiments were done to allow statistical evaluation of the data. Experiments in which *X. campestris* growth in planta was evaluated were performed in a similar manner except that the inoculum size was 7 to 8 $\times 10^5$ CFU. Treatments with CF were done by applying 20 μ l of CF in a leaf 24 h before inoculation. Treatments with SA were done by applying SA, from a stock concentration of 100 mM, to 40 ml of plant growth media to a final concentration of 1 mM, 48 h before inoculation with bacterial suspensions.

Extracellular enzyme preparation and enzyme assays.

CFs were prepared as follows: supernatant of an overnight culture corresponding to 10⁹ CFU per ml of the strain SCC3193 was obtained by centrifugation of the bacterial culture (10 min at 4,000 $\times g$) and filter sterilized. For preparations of individual enzymes, the plasmid-borne *E. carotovora* subsp. *carotovora* genes were introduced in *E. coli* strain HB101 (Boyer and Roulland-Dussoix 1969). The strains were grown to midlogarithmic phase at 37°C in L-medium (Miller 1972) supplemented with ampicillin, and these enzymes were released from the *E. coli* periplasm by osmotic shock as previously described (Palva 1978). The enzyme solutions were filter sterilized and the enzyme activities were determined essentially as described by Pirhonen et al. (1991). The genes encoding the enzymes that were used in the experiments were the following: a gene encoding endopolygalacturonase A (*pehA*; Saarilahti et al. 1990a); cellulase S (*celS*; Saarilahti et al. 1990b); pectate lyase A and D (*pelA/D* in plasmid pHSK20; Heikinheimo et al. 1995) and cellulase V1 (*celV1*; Mäe et al. 1995). Enzyme activities of the different isolated enzymes were adjusted to the units of enzyme activity present in the total culture filtrate before the plants were treated. Experiments were done by treating plants with the following concentrations of enzyme activities: 0.6 to 0.7 units of cellulase per ml of enzyme solution; 6 to 7 units of endopolygalacturonase per ml of enzyme solution; and 0.2 to 0.3 units of pectate lyase. One unit of cellulase activity corresponds to the amount of enzyme that produced 1 μ mol of glucose equivalent per hour. One unit of endopolygalacturonase activity corresponds to the amount of enzyme that produced 0.1 μ mol of glucose equivalent per hour. Pectate lyase activity was expressed as A₅₄₈ per ml of enzyme solution per minute.

Quantitative GUS assays.

Three-week-old *N. tabacum* Petit Havana SR1 plants containing the basic glucanase promoter region-glucuronidase gene construct (*gn1-uidA*) (Castresana et al. 1990) grown as described above were used for quantitative GUS assays. Extracellular enzyme treatments were done by applying to a de-

fined leaf 20 μ l of enzyme solution (with similar activity present in the CF, as described above) pressing a pipette gently against the leaf at several locations, until approximately 50% of the leaf surface was covered by enzyme solution, causing the tissue to break in order to ensure penetration of the enzymes between the cell walls. For the analysis of systemic induction patterns, only one leaf per plant was treated with CF and the opposite, systemic leaf was analyzed for production of GUS. After the indicated times, samples from treated plant leaf tissue, corresponding to 12 plants per treatment, were collected and analyzed by the spectrophotometric assay of GUS activity (Jefferson 1987). Protein concentrations were measured with Bio-Rad (Hercules, CA) dye reagent, and the specific activity was determined by the rate of increase of GUS product over protein concentration. GUS-specific activity was determined in units, with one unit = the amount of enzyme necessary to make 1 nmol of product per min per mg of protein at 37°C.

RNA analysis.

N. tabacum Petit Havana SR1 plants grown as described above were used for RNA analysis. Extracellular enzyme treatments were done by applying to a defined leaf a total volume of 20 μ l of enzyme solution (with similar activity present in the CF, as described above) pressing a pipette gently against the leaf at several locations, until approximately 50% of the leaf surface was covered by enzyme solution, causing the tissue to break in order to ensure penetration of the enzymes between the cell walls. For the analysis of systemic induction patterns, only one leaf per plant was treated with enzyme solutions and the opposite leaf was analyzed for systemic defense gene expression. After the indicated times, total RNA from treated plant leaf tissue, corresponding to 12 plants per treatment, was prepared by phenol/chloroform extraction and LiCl precipitation as described by Verwoerd et al. (1989). Ten micrograms of total RNA was denatured in formamide, separated by electrophoresis through formaldehyde agarose gels, and blotted to nylon filters (Sambrook et al. 1989). Filters were hybridized with an *Aval*-*Clal* fragment of the coding region of the basic β -1,3-glucanase gene *gn1* (Castresana et al. 1990). Probes were labeled with [α -³²P] dATP by multipriming (Amersham, Little Chalfont, UK). Hybridizations were performed overnight at 65°C in 5 $\times$ Denhardt's solutions, 6 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1% sodium dodecyl sulfate (SDS), 100 μ g of denatured herring sperm DNA per ml, and filters were washed twice with 5 $\times$ SSC, 0.5% SDS for 15 min at 65°C and twice with 1 $\times$ SSC, 0.5% SDS for 30 min at 65°C. RNA gels were analyzed with ethidium bromide and blots were probed with a ribosomal probe to ensure equal amounts of RNA in the samples.

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LITERATURE CITED

- Alexander, D., Goodman, R. M., Gut-Rella, M., Glascock, C., Weymann, K., Friedrich, L., Maddox, D., Ahl-Goy, P., Luntz, T., Ward, E., and Ryals, J. 1993. Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. *Proc. Natl. Acad. Sci. USA* 90:7327-7331.
- Atkinson, M. M. 1993. Molecular mechanisms of pathogen recognition by plants. Pages 35-64 in: *Advances in Plant Pathology*. Vol. 10. J. H. Andrews and I. C. Tommerup, eds. Academic Press, London.
- Bol, J. F., Linthorst, H. J. M., and Cornelissen, B. J. C. 1990. Plant pathogenesis-related proteins induced by virus infection. *Annu. Rev. Phytopathol.* 28:113-138.
- Boller, T. 1988. Ethylene and the regulation of antifungal hydrolases in plants. Pages 145-174 in: *Oxford Surveys of Plant Molecular and Cell Biology*. Vol. 5. B. J. Mifflin and H. F. Mifflin, eds. Oxford University Press, Oxford.
- Boller, T., Gehri, A., Mauch, F., and Vögeli, U. 1983. Chitinase in bean leaves: induction by ethylene, purification, properties, and possible function. *Planta* 157:22-31.
- Boyer, H. W., and Roulland-Dussoix, D. 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *J. Mol. Biol.* 41:459-472.
- Brogie, K., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S., Mauvais, C. J., and Brogie, R. 1991. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. *Science* 254:1194-1197.
- Castresana, C., de Carvalho, F., Gheysen, G., Habets, M., Inzé, D., and van Montagu, M. 1990. Tissue-specific and pathogen-induced regulation of a *Nicotiana plumbaginifolia* β -1,3-glucanase gene. *Plant Cell* 2:1131-1143.
- Chang, P.-F. L., Cheah, K. T., Narasimhan, M. L., Hasegawa, P. M., and Bressan, R. A. 1995. Osmotin gene expression is controlled by elicitor synergism. *Physiol. Plant.* 95:620-626.
- Collmer, A., and Keen, N. T. 1986. The role of pectic enzymes in plant pathogenesis. *Annu. Rev. Phytopathol.* 24:383-409.
- Cosio, E. G., Frey, T., and Ebel, J. 1992. Identification of a high-affinity binding protein for a hepta- β -glucoside phytoalexin elicitor in soybean. *Eur. J. Biochem.* 204:1115-1123.
- Creelman, R. A., Tierney, M. L., and Mullet, J. E. 1992. Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. *Proc. Natl. Acad. Sci. USA* 89:4938-4941.
- Darvill, A. G., and Albersheim, P. 1984. Phytoalexins and their elicitors - A defense against microbial infection in plants. *Annu. Rev. Plant Physiol.* 35:243-275.
- Darvill, A., Augur, C., Bergmann, C., Carlson, R. W., Cheong, J.-J., Eberhard, S., Hahn, M. G., Ló, V.-M., Marfà, V., Meyer, B., Mohnen, D., O'Neill, M. A., Spiro, M. D., van Halbeek, H., York, W. S., and Albersheim, P. 1992. Oligosaccharins-oligosaccharides that regulate growth, development and defence responses in plants. *Glycobiology* 2:181-198.
- Davis, K. R., and Ausubel, F. M. 1989. Characterization of elicitor-induced defense responses in suspension-cultured cells of *Arabidopsis*. *Mol. Plant-Microbe Interact.* 2:363-368.
- Davis, K. R., Darvill, A. G., and Albersheim, P. 1986. Host-pathogen interactions XXXI. Several biotic and abiotic elicitors act synergistically in the induction of phytoalexin accumulation in soybean. *Plant Mol. Biol.* 6:23-32.
- Davis, K. R., and Hahlbrock, K. 1987. Induction of defense responses in cultured parsley cells by plant cell wall fragments. *Plant Physiol.* 84:1286-1290.
- Davis, K. R., Lyon, G. D., Darvill, A. G., and Albersheim, P. 1984. Host-pathogen interactions. XXV. Endopolygalacturonic acid lyase from *Erwinia carotovora* elicits phytoalexin accumulation by releasing plant cell wall fragments. *Plant Physiol.* 74:52-60.
- Delaney, T. P., Uknes, S., Vernooij, B., Friedrich, L., Weyman, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., Ward, E., and Ryals, J. 1994. A central role of salicylic acid in plant disease resistance. *Science* 266:1247-1250.
- Ebel, J., Bhagwat, A. A., Cosio, E. G., Feger, M., Kissel, U., Mithöfer, A., and Waldmüller, T. 1995. Elicitor-binding proteins and signal transduction in the activation of a phytoalexin defense response. *Can. J. Bot.* 73:S506-S510.
- Ebel, J., Cosio, E. G., Feger, M., Frey, T., Kissel, U., Reinold, S., and Waldmüller, T. 1993. Glucan elicitor-binding proteins and signal transduction in the activation of plant defence. Pages 477-484 in: *Advances in Molecular Genetics of Plant-Microbe Interactions*. Vol. 2. E. W. Nester and D. P. S. Verma, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Enyedi, A. J., Yalpani, N., Silverman, P., and Raskin, I. 1992. Localization, conjugation, and function of salicylic acid in tobacco during the hypersensitive reaction to tobacco mosaic virus. *Proc. Natl. Acad. Sci. USA* 89:2480-2484.
- Farmer, E. E., Johnson, R. R., and Ryan, C. A. 1992. Regulation of expression of proteinase inhibitor genes by methyl jasmonate and jasmonic acid. *Plant Physiol.* 98:995-1002.
- Farmer, E. E., Moloshok, T. D., Saxton, M. J., and Ryan, C. A. 1991. Oligosaccharide signaling in plants. Specificity of oligouronide-enhanced plasma membrane protein phosphorylation. *J. Biol. Chem.* 266:3140-3145.
- Felix, G., and Meins, F., Jr. 1987. Ethylene regulation of β -1,3-glucanase in tobacco. *Planta* 172:386-392.
- Flor, H. H. 1971. Current status of the gene-for-gene concept. *Annu. Rev. Phytopathol.* 9:275-296.
- Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H., and Ryals, J. 1993. Requirement of salicylic acid for the induction of systemic acquired resistance. *Science* 261:754-756.
- Hahn, M. G., Darvill, A. G., and Albersheim, P. 1981. Host-pathogen interactions. XIX. The endogenous elicitor, a fragment of a plant cell wall polysaccharide that elicits phytoalexin accumulation in soybeans. *Plant Physiol.* 68:1161-1169.
- Heikinheimo, R., Flego, D., Pirhonen, M., Karlsson, M.-B., Eriksson, A., Mäe, A., Kõiv, V., and Palva, E. T. 1995. Characterization of a novel pectate lyase from *Erwinia carotovora* subsp. *carotovora*. *Mol. Plant-Microbe Interact.* 8:207-217.
- Jefferson, R. A. 1987. Assaying chimeric genes in plants: The GUS gene fusion system. *Plant Mol. Biol. Rep.* 5:387-405.
- Keen, N. T. 1990. Gene-for-gene complementarity in plant-pathogen interactions. *Annu. Rev. Genet.* 24:447-463.
- Klement, Z. 1982. Hypersensitivity. Pages 149-177 in: *Phytopathogenic Prokaryotes*. Vol. 2. M. S. Mount and G. H. Lacy, eds. Academic Press, New York.
- Kotoujansky, A. 1987. Molecular genetics of pathogenesis by soft-rot *Erwinias*. *Annu. Rev. Phytopathol.* 25:405-430.
- Lamb, C. J. 1994. Plant disease resistance genes in signal perception and transduction. *Cell* 76:419-422.
- Linthorst, H. J. M. 1991. Pathogenesis-related proteins of plants. *Crit. Rev. Plant Sci.* 10:123-150.
- Lyon, G. D., Reglinski, T., and Newton, A. C. 1995. Novel disease control compounds: The potential to 'immunize' plants against infection. *Plant Pathol.* 44:407-427.
- Mäe, A., Heikinheimo, R., and Palva, E. T. 1995. Structure and regulation of the *Erwinia carotovora* subspecies *carotovora* SCC3193 cellulase gene *celVI* and the role of cellulase in phytopathogenicity. *Mol. Gen. Genet.* 247:17-26.
- Malamy, J., Carr, J. P., Klessig, D. F., and Raskin, I. 1990. Salicylic acid: A likely endogenous signal in the resistance response of tobacco to viral infection. *Science* 250:1002-1004.
- Malamy, J., and Klessig, D. F. 1992. Salicylic acid and plant disease resistance. *Plant J.* 2:643-654.
- Martin, G. B., Brommonschenkel, S. H., Chunwongse, J., Frary, A., Ganal, M. W., Spivey, R., Wu, T., Earle, E. D., and Tanksley, S. D. 1993. Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science* 262:1432-1436.
- Mauch, F., Mauch-Mani, B., and Boller, T. 1988. Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and β -1,3-glucanase. *Plant Physiol.* 88:936-942.
- Mauch, F., Meehl, J. B., and Staehelin, L. A. 1992. Ethylene-induced chitinase and β -1,3-glucanase accumulate specifically in the lower epidermis and along vascular strands of bean leaves. *Planta* 186:367-375.
- Mehdy, M. C. 1994. Active oxygen species in plant defense against pathogens. *Plant Physiol.* 105:467-472.
- Métraux, J. P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W., and Inverardi, B. 1990. Increase in salicylic acid at the onset of systemic acquired resistance in

- cucumber. *Science* 250:1004-1006.
- Miller, J. H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Mueller, M. J., Brodschelm, W., Spannagl, E., and Zenk, M. H. 1993. Signaling in the elicitation process is mediated through the octadecanoic pathway leading to jasmonic acid. *Proc. Natl. Acad. Sci. USA* 90:7490-7494.
- Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.* 15:473-497.
- Neuhaus, J.-M., Flores, S., Keefe, D., Ahl-Goy, P., and Meins, F., Jr. 1992. The function of vacuolar β -1,3-glucanase investigated by antisense transformation. Susceptibility of transgenic *Nicotiana sylvestris* plants to *Cercospora nicotianae* infection. *Plant Mol. Biol.* 19:803-813.
- Nothnagel, E. A., McNeil, M., Albersheim, P., and Dell, A. 1983. Host-pathogen interactions. XXII. A galacturonic acid oligosaccharide from plant cell walls elicits phytoalexins. *Plant Physiol.* 71:916-926.
- Palva, E. T. 1978. Major outer membrane protein in *Salmonella typhimurium* induced by maltose. *J. Bacteriol.* 136:286-294.
- Palva, T. K., Holmström, K.-O., Heino, P., and Palva, E. T. 1993. Induction of plant defense response by exoenzymes of *Erwinia carotovora* subsp. *carotovora*. *Mol. Plant-Microbe Interact.* 6:190-196.
- Palva, T. K., Hurtig, M., Saindrenan, P., and Palva, E. T. 1994. Salicylic acid induced resistance to *Erwinia carotovora* subsp. *carotovora* in tobacco. *Mol. Plant-Microbe Interact.* 7:356-363.
- Pérombelon, M. C. M., and Kelman, A. 1980. Ecology of the soft rot *Erwinias*. *Annu. Rev. Phytopathol.* 18:361-387.
- Pirhonen, M., Heino, P., Helander, I., Harju, P., and Palva, E. T. 1988. Bacteriophage T4 resistant mutants of the plant pathogen *Erwinia carotovora*. *Microb. Pathog.* 4:359-367.
- Pirhonen, M., Saari-lahti, H., Karlsson, M.-B., and Palva, E. T. 1991. Identification of pathogenicity determinants of *Erwinia carotovora* subsp. *carotovora* by transposon mutagenesis. *Mol. Plant-Microbe Interact.* 4:276-283.
- Raskin, I. 1992. Role of salicylic acid in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 43:439-463.
- Rohwer, F., Fritzemeier, K.-H., Scheel, D., and Hahlbrock, K. 1987. Biochemical reactions of different tissues of potato (*Solanum tuberosum*) to zoospores or elicitors from *Phytophthora infestans*. *Planta* 170:556-561.
- Ryals, J., Uknes, S., and Ward, E. 1994. Systemic acquired resistance. *Plant Physiol.* 104:1109-1112.
- Saari-lahti, H. T., Heino, P., Pakkanen, R., Kalkkinen, N., Palva, I., and Palva, E. T. 1990a. Structural analysis of the *pehA* gene and characterization of its protein product, endopolygalacturonase, of *Erwinia carotovora* subspecies *carotovora*. *Mol. Microbiol.* 4:1037-1044.
- Saari-lahti, H. T., Henri-sat, B., and Palva, E. T. 1990b. *CelS*: A novel endoglucanase identified from *Erwinia carotovora* subsp. *carotovora*. *Gene* 90:9-14.
- Salmond, G. P. C. 1994. Secretion of extracellular virulence factors by plant pathogenic bacteria. *Annu. Rev. Phytopathol.* 32:181-200.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. A. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Sela-Buurlage, M. B., Ponstein, A. S., Bres-Vloemans, S. A., Melchers, L. S., van den Elzen, P. J. M., and Cornelissen, B. J. C. 1993. Only specific tobacco (*Nicotiana tabacum*) chitinases and β -1,3-glucanases exhibit antifungal activity. *Plant Physiol.* 101:857-863.
- Staskawicz, B. J., Ausubel, F. M., Baker, B. J., Ellis, J. G., and Jones, J. D. G. 1995. Molecular genetics of plant disease resistance. *Science* 268:661-667.
- Stintzi, A., Heitz, T., Prasad, V., Wiedemann-Merdinoglu, S., Kauffmann, S., Geoffroy, P., Legrand, M., and Fritig, B. 1993. Plant 'pathogenesis-related' proteins and their role in defense against pathogens. *Biochimie* 75:687-706.
- Templeton, M. D., and Lamb, C. J. 1988. Elicitors and defence gene activation. *Plant Cell Environ.* 11:395-401.
- Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E., and Ryals, J. 1992. Acquired resistance in Arabidopsis. *Plant Cell* 4:645-656.
- van Loon, L. C. 1985. Pathogenesis-related proteins. *Plant Mol. Biol.* 4:111-116.
- Verwoerd, T. C., Dekker, B. M. M., and Hoekema, A. 1989. A small-scale procedure for the rapid isolation of plant RNAs. *Nucleic Acids Res.* 17:2362.
- Vidal, S., Ponce de León, I., Denecke, J., and Palva, E. T. 1997. Salicylic acid and the plant pathogen *Erwinia carotovora* induce defense genes via antagonistic pathways. *Plant J.* 11:115-123.
- Walton, J. D. 1994. Deconstructing the cell wall. *Plant Physiol.* 104:1113-1118.
- Ward, E. R., Uknes, S. J., Williams, S. C., Dincher, S. S., Wiederhold, D. L., Alexander, D. C., Ahl-Goy, P., Métraux, J.-P., and Ryals, J. A. 1991. Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell* 3:1085-1094.
- White, R. F., and Antoni-w, J. F. 1991. Virus-induced resistance responses in plants. *Crit. Rev. Plant Sci.* 9:443-455.
- Williams, P. H. 1980. Black rot: A continuing threat to world crucifers. *Plant Dis.* 64:736-742.
- Xu, Y., Chang, P.-F. L., Liu, D., Narasimhan, M. L., Raghothama, K. G., Hasegawa, P. M., and Bressan, R. A. 1994. Plant defense genes are synergistically induced by ethylene and methyl jasmonate. *Plant Cell* 6:1077-1085.
- Yang, Z., Cramer, C. L., and Lacy, G. H. 1992. *Erwinia carotovora* subsp. *carotovora* pectic enzymes: In planta gene activation and roles in soft-rot pathogenesis. *Mol. Plant-Microbe Interact.* 5:104-112.