Erwinia carotovora* subsp. *carotovora* and *Erwinia*-Derived Elicitors HrpN and PehA Trigger Distinct but Interacting Defense Responses and Cell Death in *Arabidopsis

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We have used an *hrp*-positive strain of the soft rot pathogen *Erwinia carotovora* subsp. *carotovora* to elucidate plant responses to this bacterial necrotroph. Purified virulence determinants, harpin (HrpN) and polygalacturonase (PehA), were used as tools to facilitate this analysis. We show that HrpN elicits lesion formation in *Arabidopsis* and tobacco and triggers systemic resistance in *Arabidopsis*. Establishment of resistance is accompanied by the expression of salicylic acid (SA)-dependent, but also jasmonate/ethylene (JA/ET)-dependent, marker genes *PR1* and *PDF1.2*, respectively, suggesting that both SA-dependent and JA/ET-dependent defense pathways are activated. Use of pathway-specific mutants and transgenic NahG plants show that both pathways are required for the induction of resistance. *Arabidopsis* plants treated simultaneously with both elicitors PehA, known to trigger only JA/ET-dependent defense signaling, and HrpN react with accelerated and enhanced induction of the marker genes *PR1* and *PDF1.2* both locally and systemically. This mutual amplification of defense gene expression involves both SA-dependent and JA/ET-dependent defense signaling. The two elicitors produced by *E. carotovora* subsp. *carotovora* also cooperate in triggering increased production of superoxide and lesion formation.

Additional keywords: induced resistance, programmed cell death, signal pathway.

Plants are normally resistant to a variety of potential pathogens due to both preformed and induced resistance mechanisms. These induced defense responses are activated upon recognition of a pathogen, pathogen-derived elicitor molecules, or elicitors released from the plant by the action of pathogens. Consequently, plant resistance can be triggered either by a specific recognition event between an avirulence (*avr*) gene product of a pathogen and a corresponding resistance (*R*) gene product of a host plant, as in a gene-for-gene type of resistance (Flor 1971; Keen 1990) or between unspecific elicitor molecules and plant receptors (Nürnberger 1999). Recognition of a pathogen or an elicitor is often associated with localized necrosis known as the hypersensitive response (HR). HR is charac-

terized by rapid cell death and tissue necrosis at the site of pathogen attack, resulting in restriction of pathogen growth and spreading and, thus, resistance. Such recognition events will then activate defense signaling in the surrounding tissues, leading to enhanced disease resistance also in the uninfected parts of the plant (Dangl et al. 1996; Goodman and Novacky 1994).

Recent work has demonstrated the presence of distinct signal pathways leading to activation of different sets of defense genes in plants (Glazebrook 1999; Reymond and Farmer 1998; Thomma et al. 2001a). The best-characterized of these induced defense responses is the systemic acquired resistance (SAR), triggered by e.g. necrotizing pathogens. SAR results in a long-lasting resistance in the uninfected parts of the plant against a further challenge by a broad range of pathogens (Ryals et al. 1996; Sticher et al. 1997) and is characterized by the expression of certain pathogenesis related (*PR*) genes, such as *PR1* (Uknes et al. 1992; Ward et al. 1991), also in distant parts of the plant. SAR induction is dependent on the accumulation of SA and requires the activity of the positive regulator NPR1 (Cao et al. 1994; Delaney et al. 1995). Several jasmonate (JA) and ethylene (ET) dependent resistance mechanisms that are independent of SA have also been documented (Thomma et al. 1998; 2001a). JA has been implicated as a signal molecule in wound response against feeding insects (Doares et al. 1995) as well as in resistance against the bacterial pathogen *Erwinia carotovora* (Brader et al. 2001; Norman-Setterblad et al. 2000). Both JA and ET signaling are required for resistance against fungal pathogens like *Alternaria brassicicola* and *Botrytis cinerea*, but also against *E. carotovora*, which has been shown to activate this defense pathway, leading to enhanced systemic resistance (Thomma et al. 1998; 2001a; Norman-Setterblad et al. 2000). Root-colonizing, plant growth-promoting rhizobacteria trigger induced systemic resistance (ISR) (van Loon et al. 1998). ISR is independent of SA and dependent on JA and ET and also on NPR1, the key regulator of SAR signaling (Pieterse et al. 1998; Pieterse and van Loon 1999).

Accumulating evidence argues for a crosstalk between the signal pathways (Thomma et al. 2001a). JA and ET act synergistically in inducing genes for several PR proteins, including osmotin (Xu et al. 1994), defensin (Penninckx et al. 1998), and HEL (Norman-Setterblad et al. 2000). ET has also been shown to enhance SA-mediated signaling (Lawton et al. 1994), and SA has been shown to enhance JA-dependent responses (Xu et al. 1994). On the other hand, SA and its functional analogs have also been reported to prevent the expression of JA-dependent defense genes (Gupta et al. 2000; Peña-Cortés et al.

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1993; Penninckx et al. 1996; Vidal et al. 1997), and similarly, JA has been shown to interfere with SA-dependent signaling (Niki et al. 1998). Another line of evidence indicating crosstalk between the different signal pathways comes from the studies

Fig. 1. Purified HrpN induces a hypersensitive response-like response in *Arabidopsis* and tobacco. **A**, Overexpression, purification, and Western blot detection of *Erwinia carotovora* subsp. *carotovora* HrpN protein from *Escherichia coli* strain M15. Lane 1, crude extract of M15 carrying pTPE101; lane 2, purified HrpN; lane 3, Western blot of purified HrpN using antihistidine antibody. The arrow indicates the overproduced 36-kDa HrpN. **B**, Tobacco (left) and *Arabidopsis* (right) leaves four days after infiltration with HrpN (300 µg/ml), 5 mM MES buffer (pH 6.5), or acetylated bovine serum albumin (BSA; 1,500 µg/ml). **C**, *Arabidopsis* leaves were infiltrated with 5 mM MES or HrpN (300 µg/ml), and cell death was measured as relative ion leakage (percentage of total ions ± standard error) at the indicated time points after treatment.

of MAP kinases. Recently, it was shown that MAP kinase 4 (MPK 4) has a role in crosstalk between SA- and JA-dependent signal pathways (Petersen et al. 2000); while repressing SAR response, MPK4 is required for expression of JA-responsive defense genes.

The plant defenses can be induced by the action of different elicitors, for example, proteins secreted by pathogens or the compounds of the plant cell wall released by the action of microbial enzymes (Nürnberger 1999; Boller 1995). The elicitors involved may also act simultaneously, resulting in amplified, synergistic effect on the induction of plant defense, and the effect of this simultaneous action can even be greater than the sum of the individual effects of the elicitors (Boller 1995).

We use the gram-negative phytopathogen *E. carotovora* subsp. *carotovora* as a model to probe for plant defense responses and to understand the signal processes involved in the plant-pathogen interaction. *E. carotovora* subsp. *carotovora* is a broad host range, soft rot necrotroph that uses an array of plant cell wall-degrading enzymes (PCWDE), including pectinases, cellulases, and proteases, as primary virulence factors (Kotoujansky 1987; Pérombelon and Kelman 1980). Besides macerating plant tissue during disease progression, these extracellular enzymes also trigger a signal transduction cascade leading to plant defense response and resistance (Norman-Setterblad et al. 2000; Palva et al. 1993; Vidal et al. 1997, 1998). The defense response elicited by PCWDE is independent of SA and appears to be primarily dependent on JA/ET signaling in both tobacco (Vidal et al. 1997, 1998) and *Arabidopsis* (Norman-Setterblad et al. 2000). Some strains of *E. carotovora* have also been shown to possess *hrp* (hypersensitive response and pathogenicity) genes (Mukherjee et al. 1997; Rantakari et al. 2001) that contribute to virulence. Similar to other Hrp-positive bacterial pathogens the *hrp* cluster of *E. carotovora* subsp. *carotovora* encodes proteins required for type III secretion and delivery of bacterial effector proteins to the plant cells (Rantakari et al. 2001). Harpins are acidic, glycine-rich, heat-stable proteinaceous elicitors of HR in various plant-pathogen interactions (He et al. 1993; Wei et al. 1992) and are also secreted through this system. Some harpins have also been shown to function as elicitors of induced resistance (Bauer et al. 1997; Huang et al. 1988; Wei et al. 1992; Wei and Beer 1996). The harpin of *E. amylovora* was recently shown to induce the SAR response in *Arabidopsis* (Dong et al. 1999). However, the role of harpin in sensitive hosts is less clear.

We have characterized the responses of *Arabidopsis* triggered by two types of defense elicitors, harpin HrpN and a PCWDE, polygalacturonase PehA. We used transgenic NahG plants and various signal transduction mutants to dissect the signal pathways triggered, and we demonstrated that *E. carotovora* subsp. *carotovora* and HrpN induce both SA-dependent and JA/ET-dependent signal pathways in *Arabidopsis*. Moreover, HrpN was shown to induce resistance against *Erwinia*, and this induced resistance response required both SA-dependent and JA/ET-dependent signaling pathways. Furthermore, we show that both the induction of plant defense genes and the lesion formation were strongly enhanced by the cooperative action of the two types of elicitors.

RESULTS

Purified HrpN of *E. carotovora* subsp. *carotovora* SCC1 elicits lesion formation and induces disease resistance.

To characterize the *E. carotovora* subsp. *carotovora* harpin and to elucidate its biological activity, the *hrpN* gene was first isolated from a genomic lambda library of the strain SCC1 (Rantakari et al. 2001), subcloned to an expression vector and sequenced. Comparison of the deduced amino acid sequence of

HrpN from SCC1 demonstrated clear structural similarity with harpins previously described from *E. carotovora* Ecc71 (Mukherjee et al. 1997), *E. chrysanthemi* (Bauer et al. 1995), *E. amylovora* (Wei et al. 1992) and, most recently, from *Pantoea stewartii* (Frederick et al. 2001). HrpN is 99% identical to the harpin of Ecc71 and shares approximately 48% identity with the harpins of *E. amylovora* and *E. chrysanthemi*. This structural similarity, including the presence of glycine-rich regions and the lack of cysteine, indicates that HrpN from SCC1 is a typical harpin.

To study the biological activity of *E. carotovora* subsp. *carotovora* harpin, we purified the N-terminally His₆-tagged HrpN protein produced in *Escherichia coli*. The purity of the eluted and dialyzed HrpN was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and its identity was confirmed immunologically using anti-His-Tag antibody (Fig. 1A). The biological activity of the protein was tested both in *Arabidopsis* and tobacco, in which the infiltration of 300 µg ml⁻¹ of purified HrpN caused visible symptoms within 48 to 72 h (Fig. 1B), but no lesion formation was seen when plants were treated with the controls, MES buffer and acetylated bovine serum albumin. The symptoms caused by HrpN treatment were accompanied by increased ion leakage three to four days after infiltration (Fig. 1C).

To determine the capability of HrpN to induce resistance in *Arabidopsis* against *E. carotovora* subsp. *carotovora*, wild-type Col-0 plants were locally treated with the purified HrpN protein. After this pretreatment (24 h), we challenged the plants with *E. carotovora* subsp. *carotovora* SCC1 inoculated into systemic leaves. As shown in Figure 2A, the HrpN-pretreated plants were significantly less macerated after 24 h than the MES-pretreated control plants. To quantify the effect of HrpN, we measured bacterial growth in both HrpN-treated and control plants (Fig. 2B). The results show a clear reduction of bacterial growth in HrpN-pretreated plants. After an initial multiplication, the bacterial growth was stopped in these plants, while it continued in MES-treated controls. In conclusion, HrpN treatment appears to induce plant defense and establishes resistance against *E. carotovora* subsp. *carotovora*.

HrpN and *E. carotovora* subsp. *carotovora* SCC1 induce both SA-dependent and JA/ET-dependent defense pathways.

To elucidate the identity of one or more HrpN-triggered defense pathways in *Arabidopsis*, we employed the pathway-specific marker genes *PR1*, responsive to SA, and *PDF1.2*, responsive to JA and ET. HrpN-induced expression of these genes was compared to that induced by the pathogen itself and to another *Erwinia*-derived defense elicitor polygalacturonase (PehA) that is known to induce the JA/ET-dependent defense pathways (Norman-Setterblad et al. 2000; Vidal et al. 1998). *Arabidopsis* Col-0 plants were locally inoculated with *E. carotovora* subsp. *carotovora* SCC1 bacteria (10⁶ CFU/plant) or infiltrated with preparations of HrpN or PehA, and the accumulation of *PR1* and *PDF1.2* transcripts was followed up to five days by Northern analysis (Fig. 3). The purified HrpN triggered a strong local induction of *PR1* but a relatively weak systemic response. Surprisingly, the expression of *PDF1.2* was also induced within 24 to 48 h. In accordance with previous results, PehA triggered only a weak and delayed induction of *PR1* but a strong and rapid expression of *PDF1.2*. Plants inoculated with SCC1 reacted locally with strong expression of both *PR1* as well as *PDF1.2*. All treatments induced *PDF1.2* also in systemic leaves whereas the systemic expression of *PR1* was fairly weak. These results show that both HrpN and SCC1 are capable of triggering not only SA-dependent but also JA/ET-dependent defense genes.

Both SA and JA signaling are required for establishing HrpN triggered resistance to *E. carotovora* subsp. *carotovora*.

To elucidate the importance of different signaling pathways in HrpN-induced resistance, we challenged *Arabidopsis* signal pathway mutants and transgenic plants with SCC1 bacteria. Transgenic NahG plants unable to accumulate SA were employed to characterize the need of the SA-dependent SAR pathway and JA-insensitive *coil* and *jar1* mutants were employed to elucidate the role of pathways involving JA signaling. HrpN treatment did not induce resistance to challenge inoculation by SCC1 in NahG plants but, instead, enhanced bacterial growth when compared with control plants (Fig. 4). In the JA-insensitive *coil* plants, HrpN was not capable of inducing resistance either, but HrpN-treated and control plants supported bacterial growth to a similar extent. Similar results were obtained in another type of JA-signaling mutant, *jar1*. Finally, we measured the bacterial growth in NahG *coil* plants in which both pathways were blocked. These plants showed the most severe symptoms and supported vigorous bacterial growth in HrpN-treated as well as in control plants, and the treatment with HrpN did not lead to any enhanced resistance. In *coil* as well as in NahG *coil* plants, infection was so severe

Fig. 2. HrpN induces resistance in *Arabidopsis*. Two leaves of *Arabidopsis* Col-0 plant were inoculated by infiltration with *Erwinia carotovora* subsp. *carotovora* SCC1 (indicated by white arrows) 24 h after pretreatment of two opposite leaves with HrpN (300 µg/ml) or 5 mM MES. **A**, Visual differences in HrpN and MES pretreated Col-0 plants 24 h after infection with *E. carotovora* subsp. *carotovora* SCC1 (5 × 10⁶ CFU/ml). **B**, Growth of *E. carotovora* subsp. *carotovora* SCC1 in Col-0 after pretreatment with HrpN (white bars) or MES (black bars). The plant samples were collected into 0.9% NaCl at 0, 24, and 48 h after inoculation (9.2 × 10⁵ CFU/plant). Bacterial growth was measured from five to seven parallel samples by plating serial dilutions on L-Amp (50 µg ml⁻¹) plates and is indicated as CFU/plant ± standard error.

that most of the plants were totally macerated within 48 to 72 h. Taken together, these results suggest that HrpN-induced resistance to *E. carotovora* subsp. *carotovora* SCC1 requires both SA- and JA-dependent defense signaling.

HrpN and PehA elicitors cooperate in the induction of plant defense gene expression.

Production of both elicitors HrpN and PehA by *E. carotovora* subsp. *carotovora* SCC1 and the enhanced expression of defense genes by bacterial inoculation compared with either HrpN or PehA alone (Fig. 3) led us to assess the effect of simultaneous treatment with both elicitors on wild-type *Arabidopsis*. Northern hybridization analysis (Fig. 5A and B) showed that both *PDF1.2* and *PR1* were induced both faster and more strongly by the combination of HrpN and PehA than by either of the elicitors alone. Even more remarkable was the enhanced systemic induction of both SA- and JA/ET-dependent marker genes, especially the strong systemic induction of *PR1*, hardly detectable with any of the single treatments (Fig. 5B). Even the systemic induction of *PDF1.2* was enhanced.

To study the role of the SA-dependent and SA-independent defense pathways in this enhanced plant response, we used transgenic NahG plants as well as several signal transduction mutants of *Arabidopsis*. When the JA/ET pathway was blocked, the expression of *PDF1.2* was reduced or even abolished, as seen in HrpN/PehA-treated, ET-insensitive *ein2-1*, JA-insensitive *coi1* and *jar1*, as well as in JA-deficient *triple-fad* mutants (Fig. 5C). The induction of SA-dependent *PR1*, however, was still somewhat stronger in JA/ET-pathway mutants after the double treatment when compared with that after treatment with HrpN alone (data not shown). As expected, *PR1* induction was abolished in transgenic NahG plants as well as in *npr1* mutant plants. The induction of *PDF1.2* resembled that seen in wild-type plants after treatment with PehA alone. In NahG *coi1* plants, in which both pathways are blocked, the induction of both *PDF1.2* and *PR1* was abolished almost completely. Glutathione S-transferase *GST1* accumulates in response to oxidative stress (Conklin and Last 1995). Local *GST1* induction after HrpN/PehA treatment was similar to wild-type plants in all the mutants and in transgenic NahG and

seemed to be independent of the pathways affected. However, a slight reduction in *GST1* induction was seen in *coi1* (Fig. 5C). In conclusion, HrpN and PehA synergistically induce a more rapid and strong defense response in *Arabidopsis*.

Synergistic action of HrpN and PehA trigger enhanced lesion formation and superoxide production.

The reactive oxygen species (ROS) are known to play a role in the formation of HR, a form of programmed cell death (PCD) associated with the induction of plant defense responses (Greenberg 1997). The enhanced plant response to simultaneous HrpN and PehA treatment compared with treatment with either elicitor alone prompted us to characterize lesion formation and superoxide production in these plants. As shown in Figure 6A HrpN/PehA-treated leaves showed enhanced and accelerated lesion formation as compared with treatments with single elicitors. Already, 6-h treatment resulted in the formation of clear and massive HR-like lesions. Nitroblue tetrazolium (NBT) staining was used to visualize superoxide formation in the treated leaves (Fig. 6B). The staining showed that superoxide was formed within 2 h after HrpN/PehA treatment and could not be detected at that time after treatment with either elicitor alone. The specificity of the staining in detecting superoxides was confirmed by adding superoxide dismutase (SOD) along with NBT (Fig. 6B). The extent of tissue collapse and cell death of these double-treated leaves was quantified by measuring ion leakage (Fig. 6C). Compared with plants treated with either HrpN or PehA alone, a clearly synergistic effect was seen when these elicitors were used simultaneously. There was a massive increase in ion leakage, and the reaction itself was also much more rapid (Fig. 6C). In conclusion, superoxide production and lesion formation were strongly enhanced by the synergistic action of the *Erwinia* elicitors HrpN and PehA.

DISCUSSION

Some harpins have been shown to elicit disease resistance (Bauer et al. 1997; Huang et al. 1988; Wei et al. 1992; Wei and Beer 1996), but only recently has the focus been moved toward understanding the signaling mechanisms underlying this defense response. For example, the harpin of *E. amylovora* was shown to activate disease resistance in *Arabidopsis* through SA- and NPR1-dependent signal pathways (Dong et al. 1999). In this paper, we describe purification of the harpin isolated from a necrotrophic phytopathogen *E. carotovora* subsp. *carotovora*, the response reaction it elicits in *Arabidopsis*, and present evidence on the involvement of distinct interacting signal pathways in this response.

Harpins as elicitors of HR are generally established, but their role in pathogenesis is somewhat controversial. The first isolated harpin HrpN_{Ea} from *E. amylovora* was shown to be required for disease production in a susceptible host but not for HR induction in a resistant host (Wei et al. 1992). However, the role of HrpN_{Ea} appears to vary depending on the *E. amylovora* strain and the host plant, since different reactions have been observed in different situations (Barny 1995). The role of harpins in necrotrophic bacteria, such as *E. chrysanthemi* and *E. carotovora* is also somewhat obscure. An *hrpN*_{Ech} mutant was unable to elicit HR and was less virulent than the wild type (Bauer et al. 1995), whereas an *hrpN*_{Ecc} mutant was fully pathogenic but unable to cause HR (Cui et al. 1996). The role of harpin in pathogenicity of *E. carotovora* is particularly difficult to assess due to the massive production of PCWDEs and maceration of plant tissue, which can mask a potential HR response. To avoid these difficulties, we used purified HrpN protein from *E. carotovora* subsp. *carotovora* to study its biological activity on *Arabidopsis* and tobacco and showed that it

Fig. 3. *Erwinia carotovora* subsp. *carotovora* SCC1 and elicitors HrpN and PehA induce both local and systemic expression of defense genes in *Arabidopsis*. *Arabidopsis* Col-0 plants were treated with *E. carotovora* subsp. *carotovora* suspension (3.7×10^6 CFU/ml), HrpN (300 μ g/ml), PehA (6.2 units/ml), or with controls 0.9% NaCl, 5 mM MES buffer, or PehA⁻ vector control (pUC18). **A**, Local (treated) and **B**, systemic (untreated) leaf samples were collected 0, 6, 24, 48, and 120 h after the treatment. Total RNA was extracted and analyzed by Northern hybridization with gene-specific probes for *PR1* and *PDF1.2*. As a control for equal loading, ethidium bromide staining of RNA is shown at the bottom.

was a potent inducer of defense related genes and lesion formation as well as enhanced resistance.

The use of purified HrpN and pathway-specific marker genes allowed us to elucidate the signal pathways involved in plant response to HrpN. Similar to results with *E. amylovora* harpin, the HrpN of *E. carotovora* subsp. *carotovora* was shown to induce the SAR-specific *PR1* marker gene, whose induction is dependent on SA accumulation. However, the results also show that *E. carotovora* subsp. *carotovora* HrpN additionally triggers another defense response pathway, as evidenced by induction of the JA/ET-dependent *PDF1.2* gene expression by the purified, his-tagged harpin. The transient but strong induction of the *PDF1.2* gene by HrpN is not likely to be caused by any impurity in the HrpN preparation, since there are no other visible proteins in Coomassie-stained SDS-PAGE gels, apart from HrpN. Furthermore, the MES buffer control itself did not cause necrotic lesions in plants or the induction of defense related genes, apart from some weak wound response. Moreover, the systemic induction of *PDF1.2* by HrpN is quite strong, comparable to that induced by either SCC1 bacteria or PehA treatment. Interestingly, HrpN-induced accumulation of *PDF1.2* mRNA seems to be faster in systemic than in local tissues, indicating that more than one signal is formed, one acting locally and another systemically. Evidence supporting the role for a harpin in activating several distinct signal transduction cascades has been recently presented. A harpin of *Pseudomonas syringae* pv. *syringae* has been suggested to activate multiple signaling pathways, one leading to ROS production and another to the activation of two MAP kinases, AtMPK4 and AtMPK6, via distinct pathways (Desikan et al. 2001). AtMPK6 is, in turn, activated by H₂O₂ suggesting a role for the harpin in amplifying the ROS production.

The purified HrpN was shown to induce systemic resistance in *Arabidopsis* wild-type Col-0 plants against *E. carotovora* subsp. *carotovora*. This is in agreement with previous work with *E. amylovora* and *P. syringae* pv. *syringae* harpins, which have been shown to induce SAR in *Arabidopsis* (Dong et al. 1999) and in cucumber (Strobel et al. 1996), respectively. Our results show that the HrpN-induced resistance depends on both SA and JA signaling. Bacterial growth was not arrested in SA-deficient NahG plants, indicating that induction of resistance is dependent on salicylic acid. This dependence is expected, since the harpin-induced resistance is believed to be a form of SAR characterized by *PR* gene induction. Interestingly, the JA-insensitive *coi1*, as well as *jar1*, plants showed no resistance development whatsoever and consistently, when the both signal pathways were blocked, as in the NahG *coi1* plants, the bacteria grew to the highest density. This result is somewhat contradictory to the previously studied *E. amylovora* harpin-induced resistance (Dong et al. 1999). In this study, JA-insensitive *jar1* plants were used to show that HrpN-induced resistance against *P. syringae* and *P. parasitica* is independent of jasmonic acid. However, we can conclude that the JA-dependent signal pathway contributes to the HrpN-induced resistance against *E. carotovora* subsp. *carotovora*. This is further supported by the results showing the requirement for both SA- and JA-dependent signal pathways in HrpN-induced expression of defense genes of *Arabidopsis*.

Our results demonstrate that *E. carotovora* subsp. *carotovora* strain SCC1 triggers both SA-dependent and JA/ET-dependent defense pathways and that the level of SCC1-induced *PR1* and *PDF1.2* gene expression is higher than with either of the *E. carotovora* subsp. *carotovora* elicitors HrpN or PehA alone. Accordingly, these elicitors seem to cooperate when applied together, and the induced plant response is amplified due to their synergistic action. Simultaneous activation of both SA-dependent and JA- and ET-dependent signaling

pathways accelerated and enhanced *PR1* and *PDF1.2* expression, both locally and systemically. Especially conspicuous was the strong systemic induction of *PR1*, which increased above the level of local induction. Also, the production of ROS and ion leakage were enhanced, leading to a more rapid and massive cell death. Chandra and associates (2000) have observed similar synergism with cell-suspension cultures of soybean, in which the simultaneous application of different elicitors amplified the following oxidative burst. Although we have no data of the physiological levels of either HrpN or PehA during the actual interaction between the plant and the pathogen, it is tempting to speculate that the strong plant responses caused by SCC1 are, indeed, due to synergism of these two elicitors.

Unlike the situation in ISR, the JA/ET-dependent defense triggered by *E. carotovora* is not mediated by NPR1 (Norman-Setterblad et al. 2000), the key regulator in SAR. Since the two signal pathways triggered by *Erwinia*-derived elicitors are not interconnected at NPR1, the question arises as to how this synergistic effect is achieved. Most of the phytopathogens seem to predominantly activate either SA- or JA/ET-dependent defense mechanisms in plants (Thomma et al. 1998, 2001b). In contrast, *E. carotovora* subsp. *carotovora* SCC1 is a peculiar and interesting example of a pathogen that appears to trigger plant responses dependent on both SA and JA/ET signaling. One possibility is that a signal molecule or second messenger common to both pathways is produced which, in turn, may amplify the pathway-specific signals. We have studied the enhanced state of response by using transgenic NahG plants and various signal pathway mutants. Our results suggest that this amplification is somewhat more dependent on SA than on JA and ET. Treatment of plants with both HrpN and PehA did not result in stronger induction of *PDF1.2* in *npr1* and NahG plants. In contrast, *PR1* was still more strongly induced in *coi1*, *triple-fad*, and *jar1*, as well as in *ein2-1* mutants, compared with the induction by HrpN only (data not shown). As expected, there was no or very little accumulation of *PDF1.2* in JA/ET-signaling mutants *ein2-1*, *coi1*, and *triple-fad* or *PR1* in SA-deficient NahG plants and SA-signaling mutant *npr1*. In the JA-insen-

Fig. 4. Contribution of salicylic acid- or jasmonate-dependent defense pathways in HrpN-induced resistance against *Erwinia carotovora* subsp. *carotovora* SCC1. Two leaves of *Arabidopsis* plants were inoculated by infiltration with (3×10^6 to 6×10^6 CFU/plant) *E. carotovora* subsp. *carotovora* SCC1 24 h after pretreatment of two opposite leaves with HrpN (300 µg/ml) (white bars) or 5 mM MES buffer (black bars). Growth of bacteria in Col-0 as well as in transgenic NahG and in the mutants *coi1*, *jar1*, and NahG *coi1* was measured 24 h after inoculation from five to seven parallel samples by plating serial dilutions on L-Amp (50 µg ml⁻¹) plates. Bacterial growth is indicated as CFU/plant ± standard error.

sitive mutant *jar1*, the expression of *PDF1.2* is only reduced, suggesting that the *jar1* allele is somewhat leaky. The amplification of defense gene expression in systemic tissues was even more profound, which was especially evident in the induction of *PR1*. This effect could also be achieved by first activating either of the signaling pathways independently with their cognate elicitors (data not shown).

The massive, rapidly appearing, HR-like lesions triggered by simultaneous addition of elicitors HrpN and PehA are accompanied by enhanced ion leakage and increased production of superoxide in the margins of lesions, indicating accelerated cell death. Why would such a potent pathogen as *E. carotovora* subsp. *carotovora* use an arsenal of virulence determinants to trigger multiple responses including cell death in the host? One possibility is that host responses, such as oxidative burst and cell death, could be utilized by the pathogen to facilitate infection. The enhanced plant responses could lead to ROS levels deleterious to the plant, and the dying plant cells could serve as a source of nutrients to the growing bacterial population. Although pathogen-induced PCD is normally associated with HR and disease resistance, there is accumulating evidence to also support the role of PCD in compatible interactions (Govrin and Levine 2000; Stone et al. 2000). Govrin and Levine showed that the HR triggered by the necrotrophic fungal pathogen *Botrytis cinerea* actually facilitates pathogenesis. Similarly, it would also be possible that the pathogenesis of a necrotroph like *E. carotovora* could be promoted by local plant responses such as oxidative burst and cell death triggered by the synergistic action of pathogen elicitors. However, this needs to be confirmed through further experiments, and indeed, current studies are directed to elucidate the contribution of PCD to pathogenesis of *E. carotovora*.

MATERIALS AND METHODS

Plant material and growth conditions.

Arabidopsis thaliana plants used in all experiments were derived from ecotype Colombia (Col-0). Seeds of transgenic NahG plants (Delaney et al. 1995) were provided by J. Ryals (Ciba Geigy, Research Triangle Park, NC, U.S.A.), seeds of the fatty acid desaturase *triple fad* (*fad3-2/fad7-2/fad8*) mutant by J. Browse (Washington State University, Pullman, U.S.A.), and seeds of JA-insensitive *coi1-1* (Feys et al. 1994) by J. Turner (University of East Anglia, Norwich, U.K.). Seeds of ethylene response mutant *ein2-1* (Guzmán and Ecker 1990), JA response mutant *jar1-1* (Staswick and Howell 1992), and the nonexpresser of PR genes *npr1-1* (Cao et al. 1994) were obtained from the Biological Resource Center (accession numbers CS3071, CS8072 and CS3726; Columbus, OH, U.S.A.). The *Arabidopsis* NahG *coi1* plants were generated using pollen from kanamycin-resistant NahG plants to fertilize homozygous male-sterile *coi1* plants. The F₂ progeny were germinated on Murashige and Skoog (MS) plates (Murashige and Skoog 1962) containing 100 µg of kanamycin per ml for 5 days to screen for kanamycin resistance, followed by screening for insensitivity to MeJA on MS plates containing 20 µM MeJA for the next 5 days. Homozygous plants were backcrossed with pollen from NahG plants, and the F₄ progenies were screened for homozygous NahG lines and maintained as *coi1/Coi1* hemizygous plants. All plants were grown in 1:1 vermiculite and peat mixture with a 12-h light period at 22°C, and three- to four-week-old plants were used for experiments. Tobacco (*Nicotiana tabacum* cv. SR1) was grown at 24°C with a 16-h light period in vermiculite provided with fertilizer weekly. Six-week-old plants were used for experiments.

Fig. 5. Enhancement of plant response by simultaneous activation of salicylic acid and jasmonate/ethylene defense pathways with HrpN and PehA. *Arabidopsis* Col-0 plant leaves were treated simultaneously with HrpN (300 µg/ml) and PehA (6.2 units/ml) or with the corresponding controls. **A**, Local (treated) and **B**, systemic (untreated) samples were collected. **C**, Local samples were collected from *Arabidopsis* wild-type Col-0, transgenic NahG, and mutants *coi1*, *triple-fad*, *jar1*, *ein2-1*, and NahG *coi1* after the leaves were treated simultaneously with HrpN and PehA. In all experiments, the leaf samples were collected 0, 6, 24, 48, and 120 h after the treatment. Total RNA was extracted and analyzed by Northern hybridization with gene-specific probes for *PR1*, *PDF1.2*, and *GSTI*. As a control for equal loading, ethidium bromide staining of RNA is shown at the bottom.

Bacterial strains, plasmids, and media.

E. carotovora subsp. *carotovora* strain SCC1 (Rantakari et al. 2001) was propagated in L-medium (Miller 1972) at 28°C and *Escherichia coli* strains HB101 (Boyer and Roulland-Dussoix 1969), JM109 (Yanisch-Perron et al. 1985) and M15 (pREP4) (Qiagen GmbH, Hilden, Germany) at 37°C. Ampicillin was added to a final concentration of 100 or 150 µg ml⁻¹ and kanamycin to 25 µg ml⁻¹, when required.

Elicitor preparations and treatments.

The coding region of *hrpN* was amplified by PCR using primers 5'-TATTGGATCCCTTAATTCCTCTGG-3' and 5'-TAATAAGCTTTAGCTGGAGAGC-3', using pTPE100, pBluescript SK⁻ containing the *hrpN* gene from *E. carotovora* subsp. *carotovora* SCC1 as template. The resulting 1,000-bp product was purified and cloned to the BamHI site of the pQE30 expression vector (Qiagen GmbH) to produce pTPE101. Sequencing of pTPE101 was performed by an automatic laser fluorescent sequencer (Pharmacia, Uppsala, Sweden). pTPE101 was transformed to *Escherichia coli* M15 cells in order to express HrpN. Expression of the protein was analyzed in SDS-PAGE in 10% polyacrylamide gel. Immunoblotting was performed as described by Sambrook and associates (1989) using penta-his antibody (Qiagen GmbH) in a 1:7,500 dilution. In order to purify the HrpN protein, a 100-ml culture of *Escherichia coli* strain M15 bacteria containing pTPE101 was propagated on a rotary shaker at 37°C. When the optical density at 600 nm (OD₆₀₀) reached 0.8 the expression of the HrpN gene was induced by adding 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After additional growth for 2 h, the cells were collected by centrifugation (5,000 × g for 30 min at 4°C). The extraction and elution of N-terminally his₆-tagged HrpN was done according to the manufacturer's instructions (Qiagen GmbH). After the elution, urea was removed by dialyzing against 5 mM MES (2-[N-Morpholino] ethanesulfonic acid) for 24 h at 4°C. Removal of urea was confirmed with thin layer chromatography. Sterile filtered 5 mM MES was used as a control for HrpN. PehA preparation was done according to Palomäki and Saarilahti (1997). The activity of the preparation used in the experiments was 6.2 units per ml. A preparation of pUC18 plasmid was made similarly and used as a control for PehA. The HrpN preparation and the MES control were applied to plants by infiltration with a 2-ml needleless syringe. PehA and pUC18-control were pipetted as 5 × 2.5-µl droplets on leaves. Infection with SCC1 was done by infiltrating bacterial suspension containing approximately 3 × 10⁶ to 5 × 10⁶ CFU per ml with 0.9% NaCl as a control. All the treatments were carried out in a growth chamber with a 12-h light period at 22°C, 70% humidity, and photon flux density of 200 to 220 µmol m⁻² s⁻¹.

Inoculation of plants and evaluation of infection.

For evaluation of bacterial growth in planta, three- to four-week-old plants were infected with 3 × 10⁶ to 5 × 10⁶ CFU per ml of *E. carotovora* subsp. *carotovora* SCC1. An overnight culture was centrifuged for 15 min (4,000 × g), and the pellet was resuspended in 1 ml 0.9% NaCl and was diluted to the appropriate concentration. After infiltration with a needleless syringe, the plants were kept at photon flux density of 100 to 120 µmol m⁻² sec⁻¹ at more than 90% humidity to facilitate infection. The bacterial growth was determined by homogenizing the infected plants in 20 ml 0.9% NaCl and plating serial dilutions on L-plates containing ampicillin at 50 µg ml⁻¹.

RNA blot analyses.

Total RNA from plant leaves was prepared by SDS/phenol/chloroform extraction and LiCl precipitation

(Kingston 1997). RNA was separated by electrophoresis on denaturing formaldehyde agarose gel for size fractionation and then transferred onto positively charged nylon membranes (Boehringer Mannheim, Mannheim, Germany) via capillary transfer (Sambrook et al. 1989). The amount of RNA used per lane was 5 µg. Membranes were hybridized with PCR-labeled gene-specific digoxigenin (DIG) probes. DNA probes were amplified from the cDNA of *PR-1* (Uknes et al. 1992). *PDF1.2* and *GST1* probes were obtained from the *Arabidopsis* Biological Resource Center (GenBank accession numbers T04323 and N37195). All hybridizations were performed with Dig-Easy Hyb buffer (Boehringer Mannheim) at 50°C. Membranes were washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015

Fig. 6. Simultaneous treatment with HrpN and PehA triggers accelerated lesion formation and cell death in *Arabidopsis*. *Arabidopsis* leaves were treated simultaneously with HrpN (300 µg/ml) and PehA (6.2 units/ml). Control leaves were treated in the same way with 5 mM MES buffer and a pUC18 preparation. **A**, Visible symptoms on representative *Arabidopsis* leaves 24 h after treatment with HrpN only (1), HrpN + PehA (2), PehA only (3), or MES + pUC18 (4). **B**, Superoxide accumulation in treated plants. Untreated leaves (1) and leaves 2 h after application of HrpN + PehA (2, 3), HrpN only (4), PehA only (5), or MES + pUC18 (6) were detached and were infiltrated with nitroblue tetrazolium (NBT). The presence of the dark formazan precipitate indicates the location of superoxide. The specificity of the NBT staining was verified by coinfiltration of HrpN + PehA treated leaves (3) with NBT and superoxide dismutase. Arrows indicate where PehA was applied. **C**, Cell death was measured as relative ion leakage (percentage of total ions ± standard error) at the indicated time points after treatment with MES + pUC18 (white bars), HrpN + PehA (black bars), HrpN only (bars with horizontal stripes), or PehA only (bars with vertical stripes).

M sodium citrate), 0.1% (wt/vol) SDS at room temperature for 10 min and twice in 0.1× SSC, 0.1% (wt/vol) SDS at 50°C for 15 min. Immunodetection was done with an alkaline phosphatase-conjugated antidigoxigenin antibody and was visualized with the chemiluminescent substrate CSPD according to the instructions of the manufacturer (Boehringer Mannheim).

Ion leakage and superoxide staining.

Cell death was measured by relative ion leakage (percentage of total ions plus or minus standard error) from leaves of *Arabidopsis* plants in 5 ml of 18 Mohm water shaken for 1 h. Measurements were carried out with a Mettler conductivity meter (Mettler Toledo GmbH, Greifensee, Switzerland). For detection of superoxides, *Arabidopsis* leaves were vacuum infiltrated with 10 mM NaN₃ (in 10 mM K₂PO₄, pH 7.8) and were stained in the same buffer solution containing 0.1% NBT (Boehringer Mannheim). SOD (Roche Diagnostics, Mannheim, Germany) was used at a concentration of 0.5 mg per ml, when needed. After 40 min, the leaves were cleared by boiling in alcohol and lactophenol (2:1) for 5 min and were rinsed twice with 50% ethanol (Jabs et al. 1996).

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LITERATURE CITED

Barny, M. A. 1995. *Erwinia amylovora* *hrpN* mutants, blocked in harpin synthesis, express a reduced virulence on host plants and elicit variable hypersensitive reactions on tobacco. *Eur. J. Plant Pathol.* 101:333-340.

Bauer, D. W., Wei, Z.-M., Beer, S. V., and Collmer, A. 1995. *Erwinia chrysanthemi* harpin_{Ech}: An elicitor of the hypersensitive response that contributes to soft-rot pathogenesis. *Mol. Plant-Microbe Interact.* 8:484-491.

Bauer, D. W., Zumoff, C. H., Theisen, T. M., Bogdanove, A. J., and Beer, S. V. 1997. Optimized production of *Erwinia amylovora* harpin and its use to control plant disease and enhance plant growth. *Phytopathology* 87:7.

Boller, T. 1995. Chemoperception of microbial signals in plant cells. *Annu. Rev. Physiol. Plant Mol. Biol.* 46:189-214.

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.

Brader, G., Tas, E., and Palva, E. T. 2001. Jasmonate-dependent induction of indole glucosinolates in *Arabidopsis* by culture filtrates of the non-specific pathogen *Erwinia carotovora*. *Plant Physiol.* 126:849-860.

Cao, H., Bowling, S. A., Gordon, A. S., and Dong, X. 1994. Characterization of an *Arabidopsis* mutant that is non-responsive to inducers of systemic acquired resistance. *Plant Cell* 6:1583-1592.

Chandra, S., Cessna, S. G., Yahraus, T., Devine, R., and Low, P. S. 2000. Homologous and heterologous desensitization and synergy in pathways leading to the soybean oxidative burst. *Planta* 211:736-742.

Conklin, P. L., and Last, R. L. 1995. Differential accumulation of antioxidant mRNAs in *Arabidopsis thaliana* exposed to ozone. *Plant Physiol.* 109:203-212.

Cui, Y., Madi, L., Mukherjee, A., Dumenyo, C. K., and Chatterjee, A. K. 1996. The RsmA⁺ mutants of *Erwinia carotovora* subsp. *carotovora* strain Ecc71 overexpress *hrpN_{Ecc}* and elicit a hypersensitive reaction-like response in tobacco leaves. *Mol. Plant-Microbe Interact.* 9:565-573.

Dangl, J. L., Dietrich, R. A., and Richberg, M. H. 1996. Death don't have no mercy: Cell death programs in plant-microbe interactions. *Plant Cell* 8:1793-1807.

Delaney, T. P., Friedrich, L., and Ryals, J. A. 1995. *Arabidopsis* signal transduction mutant defective in chemically and biologically induced disease resistance. *Proc. Natl. Acad. Sci. U.S.A.* 92:6602-6606.

Desikan, R., Hancock, J. T., Ichimura, K., Shinozaki, K., and Neill, S. J.

2001. Harpin induces activation of the *Arabidopsis* mitogen-activated protein kinases AtMPK4 and AtMPK6. *Plant Physiol.* 126:1579-1587.

Doares, S. H., Narváez-Vásquez, J., Conconi, A., and Ryan, C. A. 1995. Salicylic acid inhibits synthesis of proteinase inhibitors in tomato leaves induced by systemin and jasmonic acid. *Plant Physiol.* 108:1741-1746.

Dong, H., Delaney, T. P., Bauer, D. W., and Beer, S. V. 1999. Harpin induces disease resistance in *Arabidopsis* through the systemic acquired resistance pathway mediated by salicylic acid and the *NIM1* gene. *Plant J.* 20:207-215.

Feys, B. J. F., Benedetti, C. E., Penfold, C. N., and Turner, J. G. 1994. *Arabidopsis* mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. *Plant Cell* 6:751-759.

Flor, H. H. 1971. Current status of the gene-for-gene concept. *Annu. Rev. Phytopathol.* 9:275-296.

Frederick, R. D., Ahmad, M., Majerczak, D. R., Arroyo-Rodríguez, A. S., Manulis, S., and Coplin, D. L. 2001. Genetic organization of the *Pantoea stewartii* subsp. *stewartii* *hrp* gene cluster and sequence analysis of the *hrpA*, *hrpC*, *hrpN*, and *wtse* operons. *Mol. Plant-Microbe Interact.* 14:1213-1222.

Glazebrook, J. 1999. Genes controlling expression of defense responses in *Arabidopsis*. *Curr. Op. Plant Biol.* 2:280-286.

Goodman, R. M., and Novacky, A. J. 1994. The Hypersensitive Reaction in Plants to Pathogens: A Resistance Phenomenon. American Phytopathological Society Press, St. Paul, MN, U.S.A.

Govrin, E. M., and Levine, A. 2000. The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. *Curr. Biol.* 10:751-757.

Greenberg, J. T. 1997. Programmed cell death in plant-pathogen interactions. *Annu. Rev. Plant Phys. Mol. Biol.* 48:525-545.

Gupta, V., Willits, M. G., and Glazebrook, J. 2000. *Arabidopsis thaliana* *EDS4* contributes to salicylic acid (SA)-dependent expression of defense responses: Evidence for inhibition of jasmonic acid signaling by SA. *Mol. Plant-Microbe Interact.* 13:503-511.

Guzmán, P., and Ecker, J. R. 1990. Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants. *Plant Cell* 2:513-523.

He, S. Y., Huang, H.-C., and Collmer, A. 1993. *Pseudomonas syringae* pv. *syringae* harpin_{psg}: A protein that is secreted via the *hrp* pathway and elicits the hypersensitive response in plants. *Cell* 73:1255-1266.

Huang, H. C., Schuurink, R., Denny, T. P., Atkinson, M. M., Baker, C. J., Yucel, I., Hutcheson, S. W., and Collmer, A. 1988. Molecular cloning of a *Pseudomonas syringae* pv. *syringae* gene cluster that enables *Pseudomonas fluorescens* to elicit the hypersensitive response in tobacco plants. *J. Bacteriol.* 170:4748-4756.

Jabs, T., Dietrich, R. A., and Dangl, J. L. 1996. Initiation of runaway cell death in an *Arabidopsis* mutant by extracellular superoxide. *Science* 273:1853-1856.

Keen, N. T. 1990. Gene-for-gene complementarity in plant-pathogen interactions. *Annu. Rev. Genet.* 24:447-463.

Kingston, R. F. 1997. Precipitation and analysis of RNA. Pages 4.3.1-4.3.4 in: *Current Protocols in Molecular Biology*. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds. John Wiley and Sons, New York.

Kotoujansky, A. 1987. Molecular genetics of pathogenesis by soft-rot *Erwinias*. *Annu. Rev. Phytopathol.* 25:405-430.

Lawton, K. A., Potter, S. L., Uknes, S., and Ryals, J. 1994. Acquired resistance signal transduction in *Arabidopsis* is ethylene independent. *Plant Cell* 6:581-588.

Miller, J. H. 1972. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.

Mukherjee, A., Cui, Y., Liu, Y., and Chatterjee, A. K. 1997. Molecular characterization and expression of the *Erwinia carotovora* *hrpN_{Ecc}* gene, which encodes an elicitor of the hypersensitive reaction. *Mol. Plant-Microbe Interact.* 10:462-471.

Murashige T., and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* 15:473-479.

Niki, T., Mitsuhashi, I., Seo, S., Ohtsubo, N., and Ohashi, Y. 1998. Antagonistic effect of salicylic acid and jasmonic acid on the expression of pathogenesis-related (PR) protein genes in wounded mature tobacco leaves. *Plant Cell Phys.* 39:500-507.

Norman-Setterblad, C., Vidal, S., and Palva, E. T. 2000. Interacting signal pathways control defense gene expression in *Arabidopsis* in response to cell wall-degrading enzymes from *Erwinia carotovora*. *Mol. Plant-Microbe Interact.* 13:430-438.

Nümberger, T. 1999. Signal perception in plant pathogen defense. *Cell. Mol. Life Sci.* 55:167-182.

Palomäki, T., and Saariähti, H. T. 1997. Isolation and characterization of new C-terminal substitution mutations affecting secretion of polygalacturonase in *Erwinia carotovora* ssp. *carotovora*. *FEBS (Fed. Eur.*

- Biochem. Soc.) Lett. 400:122-126.
- 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.
- Peña-Cortés, H., Albrecht, T., Prat, S., Weiler, E. W., and Willmitzer, L. 1993. Aspirin prevents wound-induced gene expression in tomato leaves by blocking jasmonic acid biosynthesis. Planta 191:123-128.
- Penninckx, I. A. M. A., Eggermont, K., Terras, F. R. G., Thomma, B. P. H. J., Samblanx, G. W. D., Buchala, A., Métraux, J.-P., Manners, J. M., and Broekaert, W. F. 1996. Pathogen-induced systemic activation of a plant defensin gene in *Arabidopsis* follows a salicylic acid-independent pathway. Plant Cell 8:2309-2323.
- Penninckx, I. A. M. A., Thomma, B. P. H. J., Buchala, A., Métraux, J.-P., and Broekaert, W. F. 1998. Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in *Arabidopsis*. Plant Cell 10:2103-2113.
- Pérombelon, M. C. M., and Kelman, A. 1980. Ecology of the soft rot *Erwinias*. Annu. Rev. Phytopathol. 18:361-387.
- Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., Nielsen, H. B., Lacy, M., Austin, M. J., Parker, J. E., Sharma, S. B., Klessig, D. F., Martienssen, R., Mattsson, O., Jensen, A. B., and Mundy, J. 2000. *Arabidopsis* MAP kinase 4 negatively regulates systemic acquired resistance. Cell 103:1111-1120.
- Pieterse, C. M., van Wees, S. C. M., van Pelt, J. A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P. J., and van Loon, L. C. 1998. A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. Plant Cell 10:1571-1580.
- Pieterse, C. M., and van Loon, L. C. 1999. Salicylic acid-independent plant defense pathways. Trends Plant Sci. 4:52-58.
- Rantakari, A., Virtaharju, O., Vähämäki, S., Taira, S., Palva, E. T., Saarilahti, H. T., and Romantschuk, M. 2001. Type III secretion contributes to the pathogenesis of the soft-rot pathogen *Erwinia carotovora*: Partial characterization of the *hrp* gene cluster. Mol. Plant-Microbe Interact. 14:962-968.
- Reymond, P., and Farmer, E. E. 1998. Jasmonate and salicylate as global signals for defense gene expression. Curr. Op. Plant Biol. 1:404-411.
- Ryals, J. A., Neuenschwander, U. H., Willits, M. G., Molina, A., Steiner, H.-Y., and Hunt, M. D. 1996. Systemic acquired resistance. Plant Cell 8:1809-1819.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY, U.S.A.
- Staswick, P. E., and Howell, S. H. 1992. Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. Proc. Natl. Acad. Sci. U.S.A. 89:6837-6840.
- Sticher, L., Mauch-Mani, B., and Métraux, J. P. 1997. Systemic acquired resistance. Annu. Rev. Phytopathol. 35:235-270.
- Stone, J. M., Heard, J. E., Asai, T., and Ausubel, F. M. 2000. Simulation of fungal-mediated cell death by fumisin B1 and selection of fumisin B1-resistant (*fbr*) *Arabidopsis* mutants. Plant Cell 12:1811-1822.
- Strobel, N. E., Ji, C., Gopalan, S., Kuc, J. A., and He, S. Y. 1996. Induction of systemic acquired resistance in cucumber by *Pseudomonas syringae* pv. *syringae* 61 Hrp_{Z_{ps}} protein. Plant J. 9:431-439.
- Thomma, B. P. H. J., Eggermont, K., Penninckx, I. A. M. A., Mauch-Mani, B., Vogelsang, R., Cammue, B. P. A., and Broekaert, W. F. 1998. Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. Proc. Natl. Acad. Sci. U.S.A. 95:15107-15111.
- Thomma, B. P. H. J., Penninckx, I. A. M. A., Broekaert, W. F., and Cammue, B. P. A. 2001a. The complexity of disease signaling in *Arabidopsis*. Curr. Opin. Immunol. 13:63-68.
- Thomma, B. P. H. J., Tierens, K. F. M., Penninckx, I. A. M. A., Mauch-Mani, B., Broekaert, W. F., and Cammue, B. P. A. 2001b. Different micro-organisms differentially induce *Arabidopsis* disease response pathways. Plant Phys. Biochem. 39:673-680.
- 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., Bakker, P. A. H. M., and Pieterse, C. M. J. 1998. Systemic resistance induced by rhizosphere bacteria. Annu. Rev. Phytopathol. 36:453-483.
- Vidal, S., Ponce, D.-L., 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.
- Vidal, S., Eriksson, A. R. B., Montesano, M., Denecke, J., and Palva, E. T. 1998. Cell wall-degrading enzymes from *Erwinia carotovora* cooperate in the salicylic acid-independent induction of a plant defense response. Mol. Plant-Microbe Interact. 11:23-32.
- 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.
- Wei, Z.-M., Laby, R. J., Zumoff, C. H., Bauer, D. W., He, S. Y., Collmer, A., and Beer, S. V. 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. Science 257:85-88.
- Wei, Z.-M., and Beer, S. V. 1996. Harpin from *Erwinia amylovora* induces plant resistance. Acta Hort. 411:223-225.
- 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.
- Yanisch-Perron, C., Vieira, J., and Messing, J. 1985. Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:102-119.