Erwinia amylovora Type Three–Secreted Proteins Trigger Cell Death and Defense Responses in *Arabidopsis thaliana*

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Erwinia amylovora is the bacterium responsible for fire blight, a necrotic disease affecting plants of the rosaceous family. *E. amylovora* pathogenicity requires a functional type three secretion system (T3SS). We show here that *E. amylovora* triggers a T3SS-dependent cell death on *Arabidopsis thaliana*. The plants respond by inducing T3SS-dependent defense responses, including salicylic acid (SA)-independent callose deposition, activation of the SA defense pathway, reactive oxygen species (ROS) accumulation, and part of the jasmonic acid/ethylene defense pathway. Several of these reactions are similar to what is observed in host plants. We show that the cell death triggered by *E. amylovora* on *A. thaliana* could not be simply explained by the recognition of AvrRpt2_{ea} by the resistance gene product RPS2. We then analyzed the role of type three-secreted proteins (T3SPs) DspA/E, HrpN, and HrpW in the induction of cell death and defense reactions in *A. thaliana* following infection with the corresponding *E. amylovora* mutant strains. HrpN and DspA/E were found to play an important role in the induction of cell death, activation of defense pathways, and ROS accumulation. None of the T3SPs tested played a major role in the induction of SA-independent callose deposition. The relative importance of T3SPs in *A. thaliana* is correlated with their relative importance in the disease process on host plants, indicating that *A. thaliana* can be used as a model to study their role.

Erwinia amylovora is the bacterium responsible for fire blight, a necrotic disease affecting specifically plants of the rosaceous family, including pear and apple. The main symptoms on susceptible hosts are progressive necrosis on actively growing young aerial parts, associated with ooze production in certain climatic conditions (Paulin 1996). The bacteria may then invade woody parts and eventually kill the plant.

It was shown that, conversely to other pathosystems, fire blight disease in host plants is correlated with the induction of several defense mechanisms normally elicited by an incompatible pathogen such as *Pseudomonas syringae* pv. *tabaci* (Venisse et al. 2002). Indeed, Venisse and collaborators found a production of reactive oxygen species (ROS) in infected tissues as well as the accumulation of several pathogenesis-related (PR) proteins.

Two main pathogenicity factors are required for *E. amylovora* pathogenicity, the exopolysaccharide amylovoran and the type three secretion system (T3SS). *E. amylovora* mutants affected in amylovoran synthesis (*ams*) and mutants affected in T3SS biosynthesis (*tts*) are both nonpathogenic but they are blocked at different stages of the infection process. Indeed, the T3SS is required during early steps of infection that lead to local necrosis formation (Barny et al. 1990; Steinberger and Beer 1988) whereas amylovoran is required during later stages of the infection process which result in bacterial progression in planta (Burgert and Geider 1995).

T3SS allows secretion in the apoplast and injection into the plant cell of a number of bacterial proteins. Collectively, type three-secreted proteins (T3SPs), transiting through the T3SS, contribute to pathogenesis by controlling plant defenses and triggering cell death. *E. amylovora* T3SS also enables the pathogen to elicit a hypersensitive reaction (HR) in the non-host plant *Nicotiana tabacum* (Barny et al. 1990; Steinberger and Beer 1988). Twelve T3SPs have been identified (Nissinen et al. 2007) and several characterized. AvrRpt2_{ea} is homologous to the avirulence gene AvrRpt2_{ps} from *P. syringae* pv. *tomato*. It was shown to contribute to *E. amylovora* virulence and it is genetically recognized by the RPS2 disease resistance gene in *Arabidopsis thaliana* when expressed in *P. syringae* pv. *tomato* (Zhao et al. 2006). HrpN and HrpW belong to the harpin family of proteins; they are glycine rich and heat stable, and purified HrpN and HrpW are able to induce an HR on nonhost *N. tabacum* (Gaudriault et al. 1998; Kim and Beer 1998; Wei et al. 1992). The mechanisms by which purified HrpN provokes nonhost HR have been extensively studied (Adam et al. 1997; Baker et al. 1993; Cessna et al. 2001; El Maarouf et al. 2001; Pike et al. 1998; Reboutier et al. 2005, 2007). On *A. thaliana*, purified HrpN was found to induce local and systemic (SAR) disease resistance (Dong et al. 1999) as well as to promote plant growth (Dong et al. 2004). Yet, the exact roles of HrpN and HrpW during disease remain to be determined. HrpN was detected in the intercellular spaces of infected apple tissues by immunocytochemistry, indicating that it is secreted in planta in a T3SS-dependent way (Perino et al. 1999). Mutant analysis indicates that HrpN is an important virulence factor because *hrpN* mutants are severely affected in pathogenicity and induce symptoms that rarely evolve beyond the inoculated leaf (Barny 1995). *hrpW* mutants, on the contrary, induce symptoms as strong as the wild-type strain, indicating that HrpW is not required for full virulence (Gaudriault et al. 1998; Kim et al. 1998). Opposite roles for the two harpins are also observed on nonhost *N. tabacum*. Indeed,

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HrpN is required for full HR whereas HrpW was found to modulate HR negatively (Gaudriault et al. 1998).

The large multidomain protein DspA/E is essential to *E. amylovora* disease process because *dspA/E* mutants are nonpathogenic and unable to grow on host plants (Bogdanove et al. 1998; Gaudriault et al. 1997). It belongs to the AvrE family of T3SPs which is conserved among phytopathogenic *Pseudomonadaceae* and *Enterobacteriaceae* (Alfano et al. 2000; Bogdanove et al. 1998; Gaudriault et al. 1997; Holeva et al. 2004; Mor et al. 2001; Preston et al. 2001). Mutant analysis indicated that DspA/E is involved in callose deposition suppression during disease (Debroy et al. 2004) and is also implicated, together with HrpN, in the generation of an oxidative stress (Venisse et al. 2003). Indirect evidence for DspA/E translocation into the plant cell was obtained using a DspA/E-cya fusion protein (Bocsanczy et al. 2006) and it was further shown that transient expression of DspA/E into apple, *N. tabacum*, *N. benthamiana*, and yeast induces cell death (Boureau et al. 2006; Oh et al. 2007).

In this article, we show that infection of nonhost *A. thaliana* by *E. amylovora* leads to local necrotic symptoms. We show that several of the molecular events correlated with the development of cell death in *A. thaliana* are similar to those in host plants. Finally, we show that the importance of the T3SS and of several T3SPs in *A. thaliana* is similar to that in host plants. We conclude that *A. thaliana* is a good model to study the roles of *E. amylovora* T3SPs during plant–bacteria interaction.

RESULTS

E. amylovora induces T3SS-dependent cell death in *A. thaliana* leaves.

Infiltration of *A. thaliana* leaves with *E. amylovora* led to the formation of a necrotic symptom (Fig. 1A). In apple leaves, *E. amylovora* also induces a necrotic symptom following infiltration (Fig. 1B). However, in apple leaves, the necrosis progresses along the midrib after a few days (data not shown) whereas it is restricted to the infected area in *A. thaliana*. As in apple shoots (Boureau et al. 2006; Venisse et al. 2002), the development of necrotic lesions in *A. thaliana* was correlated with electrolyte leakage as early as 10 h postinoculation (hpi) (Fig. 1C) and the production of ROS (Fig. 1D) 24 hpi. We found that *E. amylovora* was able to grow in *A. thaliana* leaves 24 or 48 hpi depending on experiments (Fig. 1E). At the microscopic level, *E. amylovora*-induced cell death could be visualized by trypan blue staining (Fig. 2B) and toluidin blue staining of transverse sections of infected *A. thaliana* leaves 24 hpi (Fig. 2E). The chloroplasts of these dying cells were swollen and the plasma membrane appeared separated from the cell wall (Fig. 2E). At later time points, a collapse of the cells was observed (data not shown). Bacteria were found in the intercellular spaces of parenchyma as well as in the substomatal spaces (Fig. 2E and G). We found no bacteria in xylem vessels (not shown). All these symptoms were dependent upon a functional T3SS. Indeed, the *E. amylovora* *tts* mutant, which lacks a functional T3SS, induced almost no symptoms both at the macroscopic (Fig. 1F) and the microscopic level (Fig. 2C and F). Consistently, the *tts* mutant induced no electrolyte leakage (Fig. 1C) and almost no H₂O₂ production (Fig. 1D) and was unable to grow in *A. thaliana* leaves (Fig. 1E), although we were able to detect bacteria in infected leaves 24 hpi (Fig. 2F and H).

Cell death is associated with T3SS-dependent triggering of plant defense pathways.

Phytohormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) have been shown to be involved in the triggering

of plant defenses. Moreover, it was shown that, in apple leaves, infection by *E. amylovora* is associated with the activation of number of defense genes (Bonasera et al. 2006; Venisse et al. 2002). In order to determine whether defense reactions mediated by the three major defense signaling pathways were activated in *A. thaliana* leaves following infiltration with *E. amylovora*, we analyzed the expression of several marker genes of each defense pathway by reverse-transcriptase polymerase chain reaction (RT-PCR). We chose *PAD4*, *EDS5*, and *PR1* as markers of the SA pathway; *PDF1-2a* and *CHI-B* as marker genes induced synergistically by ET and JA pathways (Glazebrook et al. 2003); and *AtERF1*, a gene induced by ET (Fujimoto et al. 2000). We also monitored the expression of *AOX1a* which is a marker of oxidative stress (Simons et al. 1999). The expression patterns of these genes in mock-treated leaves and leaves inoculated with *E. amylovora* wild-type or *tts* mutant strains at different time points after inoculation is shown in Figure 3A. The expression of all genes tested, except for *PDF1-2a*, was enhanced 24 hpi with the *E. amylovora* wild-type strain. *AtERF1* and *AOX1a*, however, were only weakly induced. No significant induction of any of these genes was observed in mock-treated control leaves. Up to 48 hpi, none of the genes analyzed were induced by the *tts* mutant (Fig. 3A). In some replicas, however, we found that induction of some defense marker genes such as *EDS5*, *AOX1a*, and *AtERF1* appeared as soon as 6 hpi. In these experiments, the *tts* mutant was able to induce most defense marker genes analyzed 24 hpi but always to a lesser extent than wild-type *E. amylovora*.

In *A. thaliana*, SA can be produced via two metabolic pathways (Shah 2003): one pathway is dependent on an isochorismate synthase (ICS) while the other is dependent upon phenylalanine ammonia-lyase (PAL) activity. Several reports have indicated that the ICS-dependent pathway is predominant in *A. thaliana* (Wildermuth et al. 2001). The *A. thaliana* *sid2* mutant lacks a functional *ICS1* gene and displays reduced SA levels in response to infection by *P. syringae* (Nawrath and Métraux 1999; Wildermuth et al. 2001). We investigated whether the induction of the *PR1* gene by *E. amylovora* was *SID2/ICS1* dependent. We compared the accumulation of *PR1* transcripts in the *sid2* mutant with that in wild-type plants following inoculation with *E. amylovora*. We found almost no *PR1* accumulation in response to *E. amylovora* in the *sid2* mutant (Fig. 3B). This result indicates that *PR1* induction by *E. amylovora* in *A. thaliana* leaves requires mostly the *SID2/ICS1*-dependent SA biosynthesis pathway. In order to determine whether absence of induction SA-dependent defenses had an impact on *E. amylovora*-induced cell death, we inoculated the *A. thaliana* *sid2* mutant with wild-type *E. amylovora*. Our results show that induction of necrotic symptoms by *E. amylovora* was similar in wild-type and *sid2* mutant plants (Fig. 3C).

Cell death is associated with T3SS-dependent triggering of SA independent-callose deposition.

Plants often respond to a pathogen attack by inducing callose deposition to reinforce the cell wall (Benhamou 1995). We investigated whether this was also the case in *A. thaliana* infected with *E. amylovora*. We found that wild-type *E. amylovora* induces callose deposition 8 hpi in *A. thaliana* leaves (Fig. 3D; top panels). This induction was dependent upon a functional T3SS because the *tts* mutant induces almost no callose deposition. This result surprised us because it was shown previously that proteins of the AvrE family, such as DspA/E of *E. amylovora*, are able to suppress SA-dependent callose deposition in *A. thaliana* (Debroy et al. 2004). Therefore, we tested whether the induction of the callose deposition by wild-type *E. amylovora* was *SID2/ICS1* dependent. Callose accumulated in the *sid2* mutant to the same level as in Col-0 plants following

inoculation with wild-type *E. amylovora* (Fig. 3D, bottom panels). This indicates that the callose deposition observed following inoculation with the *E. amylovora* wild-type strain does not require the SID2/ICS1-dependent SA biosynthesis pathway.

DspA/E and HrpN are important for cell death induction, whereas HrpW acts as a negative regulator of cell death on *A. thaliana*.

In order to determine the involvement of different T3SPs in the triggering of necrotic lesions in *A. thaliana*, we infiltrated *A. thaliana* leaves with three *E. amylovora* mutants—*dspA/E*, *hrpN*, and *hrpW*—affected in the biosynthesis of the T3SPs DspA/E, HrpN, and HrpW, respectively. Both *dspA/E* and *hrpN* mutants induced fewer necrotic symptoms on *A. thaliana* leaves than wild-type *E. amylovora* (Fig. 4A). Consistently, *dspA/E* and *hrpN* mutants induced less electrolyte leakage than

wild-type bacteria on *A. thaliana* leaves (Fig. 4B). However, the *hrpW* mutant induced necrotic symptoms to the same level as wild-type bacteria and induced slightly more electrolyte leakage than wild-type bacteria (Fig. 4A and B).

Therefore, the *E. amylovora* T3SS-dependent necrosis on *A. thaliana* leaves involved both DspA/E and HrpN. The higher electrolyte leakage observed following inoculation with the *hrpW* mutant suggests that, as already observed in tobacco (Gaudriault et al. 1998), HrpW acts as a negative regulator of plant cell death induced by *E. amylovora* in *A. thaliana*.

DspA/E and HrpN are involved in the induction of plant defenses.

In order to determine the relative importance of *E. amylovora* T3SPs in the induction of *A. thaliana* defenses, we analyzed plant defense responses following inoculation with vari-

Fig. 1. Symptoms induced by *Erwinia amylovora* on *Arabidopsis thaliana* leaves. **A**, Typical necrosis induced by *E. amylovora* on *Arabidopsis* Col-0 leaves 4 days postinoculation. Arrows indicate leaves infiltrated with *E. amylovora*. **B**, Symptoms induced on leaves of apple seedlings 48 h after infiltration with *E. amylovora* wild-type and type-three secretion (*tts*) mutant strains. **C**, Electrolyte leakage induced in *A. thaliana* leaf disks after infiltration with *E. amylovora* wild-type and *tts* strains. **D**, Detection of H_2O_2 in *A. thaliana* leaves inoculated with *E. amylovora*. The lower half of each leaf was infiltrated with *E. amylovora* wild-type and *tts* mutant strains or water. Leaves were stained with 2,7-dichlorofluorescein diacetate 6 and 24 hpi. **E**, Growth curve of *E. amylovora* wild-type and *tts* mutant strains in *A. thaliana* leaves. **F**, Symptoms induced on leaves of *A. thaliana* 48 h following infiltration with *E. amylovora* wild-type and *tts* mutant strains and symptom severity scale. Ea = wild-type *E. amylovora* and dpi and hpi = days and hours, respectively, postinoculation.

ous *E. amylovora* mutants. Using RT-PCR, we analyzed the modulation of *A. thaliana* defense marker genes 6 and 24 hpi with *E. amylovora* mutant strains affected in the biosynthesis of DspA/E (*dspA/E*), HrpW (*hrpW*), and HrpN (*hrpN*) T3SPs. Our results show that, for all defense marker genes tested here, induction is as strongly reduced in response to *E. amylovora dspA/E* mutant as in response to *E. amylovora tts* mutant (Fig.

5A). In response to the *hrpN* mutant, most marker genes showed a reduced induction compared with wild-type *E. amylovora* induction whereas *PR1* was induced to the same level as in response to wild-type *E. amylovora*. Induction of defense marker genes was similar in response to *hrpW* mutant and wild-type *E. amylovora*. In order to confirm our results concerning *PR1*, we used *PR1::GUS* transgenic *A. thaliana*. We measured

Fig. 2. Microscopic analysis of the *Erwinia amylovora* infection process in *Arabidopsis thaliana* leaves. Trypan blue staining of dead cells in leaves inoculated with **A**, water, **B**, wild-type *E. amylovora*, or **C**, *E. amylovora* type-three secretion (*tts*) mutant 24 h postinoculation (hpi). Dead cells are stained dark blue. The wild-type strain of *E. amylovora* was able to induce cell death in *A. thaliana* leaves as early as 24 hpi, before macroscopic symptoms were visible. Thin sections of **D** through **F**, toluidin blue-stained or **G** and **H**, anti-LPS-stained leaves inoculated with **D**, water; **E** and **G**, wild-type *E. amylovora*, or **F** and **H**, *E. amylovora tts* mutant 24 hpi; b = bacteria, cp = chloroplast, dc = dead cells, is = intercellular space, and ss = substomatal space. Bar length: 0.5 mm (**A** through **C**) and 20 μ m (**D** through **H**).

β -glucuronidase (GUS) accumulation 24 hpi using a fluorimetric assay. Our results confirm our RT-PCR data (Fig. 5B).

It was shown previously that purified HrpN protein is able to induce SAR and *PR1* when sprayed on *A. thaliana* leaves (Dong et al. 1999). We were surprised that both *hrpN* and *hrpW* mutants were able to induce *PR1* expression to the same level as wild-type *E. amylovora*. Therefore, we analyzed GUS

accumulation in *PR1::GUS* transgenic plants following infiltration with purified HrpN and HrpW proteins from *E. amylovora*. Results show that purified HrpN and HrpW do not induce *PR1* gene expression in *A. thaliana* leaves challenged by purified proteins 24 hpi (Fig. 5C). The discrepancy between our results and those of Dong and associates could come from the purification methods employed because they used a crude

Fig. 3. Expression of defense-related marker genes and cell-wall based defenses in response to *Erwinia amylovora*. **A**, Expression of *Arabidopsis thaliana* defense marker genes in response to infiltration by water (mock), *E. amylovora* wild-type (*Ea*), and *E. amylovora* type-three secretion (*tts*) mutant monitored by reverse-transcriptase polymerase chain reaction (RT-PCR) at different time points after inoculation. **B**, Expression of the *PR1* and *CHI-B* marker genes 24 h after inoculation in *A. thaliana* wild-type and *sid2* mutant plants monitored by RT-PCR. **C**, Symptoms induced on *A. thaliana* leaves of wild-type Col-0 and *sid2* mutant 48 h after infiltration with wild-type *E. amylovora*. Symptom severity scale is shown in Figure 1F. **D**, Callose deposition detected with aniline blue in Col-0 and *sid2* *A. thaliana* leaves 8 h after inoculation with *E. amylovora* wild-type (*Ea*) and *tts* mutant. The mean number of callose deposits per picture (0.6 mm²) is indicated for each treatment. The mean number of callose deposits in Col-0 and *sid2* leaves was not significantly different (Fisher's exact test, $\alpha = 0.05$). Bar length = 200 μ m.

extract of *Escherichia coli* overexpressing HrpN whereas we purified HrpN through an affinity column.

Callose deposition is not dependent upon DspA/E, HrpN, or HrpW.

Because *E. amylovora*-induced callose deposition was found to be T3SS dependent, we tested the involvement of T3SPs DspA/E, HrpN, and HrpW in this process. Following inoculation with *dspA/E*, *hrpN*, or *hrpW* mutants, leaves accumulated as many spots of callose as in response to wild-type *Erwinia amylovora* (data not shown), indicating that none of the three tested T3SPs play an essential role in callose deposition in *A. thaliana* leaves. HrpW and HrpN proteins are known to be secreted in the apoplast during the interaction (Perino et al. 1999); therefore, we also tested whether purified HrpW and HrpN proteins were able to induce callose deposition when inoculated on *A. thaliana* leaves. Results show that almost no callose accumulation could be observed following inoculation with the purified protein (data not shown).

RPS2 is not required for induction of symptoms and defense genes.

Zhao and associates (2006) showed recently that the T3E AvrRpt2_{EA} contributes to *E. amylovora* virulence on pear. This group also showed that AvrRpt2_{EA} is genetically recognized by *A. thaliana* RPS2 when expressed in *P. syringae* pv. *tomato*. This led us to ask whether the necrotic symptoms and associated responses observed in *A. thaliana* leaves following inoculation with *E. amylovora* were the result of an AvrRpt2_{EA}–RPS2 interaction. In order to answer this question, we inoculated the *A. thaliana* *rps2* mutant (Kunkel et al. 1993) with wild-type *E. amylovora*. Our results show that induction of necrotic symptoms by *E. amylovora* was similar in wild-type and *rps2* mutant plants (Fig. 6A); the slight differences observed in Figure 6A were not significant (Fisher's exact test, $\alpha = 0.05$). We also wondered whether defense gene expression was a result of an AvrRpt2_{EA}–RPS2 interaction. The SA-dependent defense marker gene *PR1* and ET/JA-dependent defense marker gene *CHI-B* were both strongly induced in wild-type and *rps2* mutant plants 24 hpi (Fig. 6B). Finally, we tested whether callose deposition was the result of an AvrRpt2_{EA}–RPS2 interaction (Fig. 6C). Results show that callose deposition was similar in wild-type and *rps2* mutant plants. Altogether, our data indicate that the responses of *A. thaliana* to *E. amylovora* analyzed here cannot be attributed to an interaction between AvrRpt2_{EA} and RPS2.

DISCUSSION

E. amylovora, the fire blight pathogen, induces a progressive necrotic cell death on its host plant which locally resembles an incompatible reaction by many features (Venisse et al. 2001, 2002, 2003). In this article, we analyzed *A. thaliana* responses following inoculation with *E. amylovora*. Our aim was to compare the death response induced on *A. thaliana* to the one already described on the host plant and to analyze to what extent the two types of cell deaths are similar.

As in the host plant, the cell death response induced by *E. amylovora* on *A. thaliana* is dependent upon a functional T3SS. This response is accompanied by a T3SS-dependent growth of *E. amylovora* inside the plant tissue during the first 2 days following inoculation, indicating that cell death elicited by the bacteria is at first beneficial. In *A. thaliana*, however, *E. amylovora* does not maintain growth after several days or progress throughout the plant, indicating either that *A. thaliana* successfully restrains *E. amylovora* growth or that *A. thaliana* is missing susceptibility factors that would allow successful invasion (Panstruga 2005).

The cell death triggered by wild-type *E. amylovora* on *A. thaliana* was observed in the 12 ecotypes of the *A. thaliana* core collection that we tested (data not shown) (McKhann et al. 2004), suggesting that it is a general mechanism. Moreover, this cell death could not be simply explained by the recognition of AvrRpt2_{EA} by the resistance gene product RPS2 because no difference could be observed in terms of necrosis timing or apparition of plant defense between Col-0 and *rps2* plants when challenged by wild-type *E. amylovora*. Redundancy of T3SPs could not be invoked either because DspA/E and HrpN were found to be essential to trigger *E. amylovora*-induced cell death on *A. thaliana*. Therefore, it appears that, when delivered by *E. amylovora* instead of *P. syringae*, AvrRpt2_{EA} is not able to trigger RPS2 resistance on *A. thaliana*. It is possible, however, that recognition of AvrRpt2_{EA} by the resistance gene product RPS2 is counteracted by another *E. amylovora* T3SP. On the other hand, it was shown previously that AvrRpt2_{EA} is not translocated or secreted as well by *P. syringae* pv. *tomato* as AvrRpt2_{ps} (Zhao et al. 2006). Therefore, one could speculate that AvrRpt2_{EA} is badly translocated by *E. amylovora* and plays a role only at later time points than those analyzed in this study.

The cell death induced by *E. amylovora* in *A. thaliana* was correlated with induction of major plant defense pathways and SA-independent callose deposition. Concerning the JA and ET pathways, we observed only a partial induction by *E. amylovora*

Fig. 4. Role of different type-three-secreted proteins in the triggering of necrosis by *Erwinia amylovora*. **A**, Symptoms induced on leaves of *Arabidopsis thaliana* 48 h after inoculation with *E. amylovora* wild-type (*Ea*) and type-three secretion (*tts*), *dspA/E*, *hrpN*, and *hrpW* mutant strains. Symptom severity scale is shown in Figure 1F. Stars indicate significant differences from the wild type (Fisher's exact test, $\alpha = 0.05$). **B**, Electrolyte leakage induced in *A. thaliana* leaf disks after infiltration with *E. amylovora* wild-type and *tts*, *dspA/E*, *hrpN*, and *hrpW* mutant strains.

because the JA- and ET-dependent gene *PDF1.2a* was not induced by *E. amylovora*. None of these plant defense reactions was detected following inoculation with the *tts* mutant. This was unexpected because callose deposition is often induced by pathogen-associated molecular patterns (PAMPs) present at the surface of *tts* mutants of different phytopathogenic bacteria such as *Xanthomonas* spp. (Brown et al. 1995) or *Pseudomonas* spp. (Bestwick et al. 1995; Debroy et al. 2004; Hauck et al. 2003). This is also the case for the ET-dependent gene *AtERF1* which has been found to be induced by PAMPs during the first 24 h following infection by *P. syringae* (Thilmony et al. 2006). This suggests that *E. amylovora* could have developed defense-avoiding strategies by masking its potential PAMPs, as was also suggested by Cesbron and associates (2006), and is only perceived

as a threat for the plant once it deploys its T3SS and T3SPs. None of the well-described T3SPs we tested (AvrRpt2, HrpN, HrpW, or DspA/E) seems to play a major role in the induction of callose deposition. Whether it is the T3SS by itself that is recognized or another, as-yet-undescribed *E. amylovora* T3SP remains to be determined.

Our analysis of the reactions elicited by *E. amylovora* *hrpN* and *dspA/E* mutants on *A. thaliana* indicates that, when delivered by the bacteria, HrpN and DspA/E play a major role in the induction of cell death and associated plant defense responses. These two T3SPs are also required to efficiently trigger disease on the host plant (Barny 1995; Bogdanove et al. 1998; Gaudriault et al. 1997; Wei et al. 1992). On the other hand, electrolyte leakage measurements indicate that the T3SP HrpW acts

Fig. 5. Role of different type-three-secreted proteins in the triggering of defense genes. **A**, Expression of *Arabidopsis thaliana* defense marker genes in response to infiltration by water, *Erwinia amylovora* wild-type (*Ea*) and type-three secretion (*tts*), *dspA/E*, *hrpN*, and *hrpW* mutant strains monitored by reverse-transcriptase polymerase chain reaction 6 and 24 h after inoculation. **B**, Quantification of the relative β -glucuronidase (GUS) activity in *A. thaliana* plants carrying the *PR1::GUS* construct 24 h following inoculation with wild-type *E. amylovora* (*Ea*) or *tts*, *dspA/E*, *hrpN*, and *hrpW* mutant strains; u = untreated plants. **C**, Quantification of the relative GUS activity in *A. thaliana* plants carrying the *PR1::GUS* construct 24 h following inoculation with purified HrpN and HrpW proteins. Stars indicate significant differences from the wild type (Fisher's exact test, $\alpha = 0.05$).

as a negative modulator of the cell death process triggered by *E. amylovora* on *A. thaliana*. This situation is equivalent to those observed on *N. tabacum* (Gaudriault et al. 1998) and on host apple plants (unpublished results). Therefore, the three effectors DspA/E, HrpN, and HrpW, when delivered by the bacteria, modulate the cell death process both on nonhost *A. thaliana* and on host plant in the same direction, suggesting that these effectors fulfill the same biological function in both situations.

In *A. thaliana* leaves infiltrated with purified HrpN and HrpW, we did not observe callose deposition, *PR1* expression, or necrotic symptoms (data not shown). This situation is clearly different from the one observed on tobacco, where both purified proteins are strong HR inducers (Kim et al. 1998; Wei

et al. 1992), but is reminiscent of the situation observed on host apple plants where purified HrpN was found to be unable to induce cell death (Reboutier et al. 2007). This clearly differentiates *E. amylovora*-induced cell death in *A. thaliana* and tobacco and indicates, again, that *E. amylovora*-induced cell death in *A. thaliana* resembles that in the host plant.

In contrast to host apple plants in which callose deposition is efficiently suppressed by the DspA/E effector protein (Debroy et al. 2004), DspA/E does not suppress callose deposition induced by *E. amylovora* in *A. thaliana*. This surprised us because AvrE, the functional homolog of DspA/E in *P. syringae*, was shown to suppress callose deposition induced by *P. syringae* in *A. thaliana* (Debroy et al. 2004). However, callose deposition induced by *P. syringae* was shown to require the SA pathway (Debroy et al. 2004) whereas we show, in this article, that callose deposition induced by *E. amylovora* is independent of SA. This could explain why DspA/E does not repress *E. amylovora*-induced callose deposition in *A. thaliana*. It is possible, however, that DspA/E is able to suppress *P. syringae*-induced callose deposition.

We observed a good correlation between the level of necrosis induced by the different *E. amylovora* mutants and the level of induction of different defense reactions analyzed in this article. These inductions are similar to the defense reactions observed on host plant after infection; indeed, *E. amylovora* induces accumulation of ROS as well as several *PR* genes (Venisse et al. 2001, 2002). In that sense, induction of these defense pathways could be seen as collateral damage resulting from the induced necrosis. Alternatively, some defense reactions, such as *E. amylovora*-induced callose deposition, could be seen as genuine defense reactions triggered by the plant to circumvent *E. amylovora* growth. Further studies using relevant *A. thaliana* mutants will help to understand the exact role of each plant-defense pathway during the interaction.

Given the similarities we observed between cell deaths induced by *E. amylovora* on *A. thaliana* and host plants, one could wonder whether the interaction on *A. thaliana* is an HR or an aborted disease. Indeed, *E. amylovora*-induced cell death in *A. thaliana* is not less similar to an aborted disease than to a typical HR. These similarities also indicate that *A. thaliana* can be used as a model to study the role of *E. amylovora* T3SPs during the early events of the interaction.

MATERIALS AND METHODS

Plant growth and inoculation conditions.

A. thaliana seed from the Col-0 ecotype were obtained from the Institut National de la Recherche Agronomique (INRA) Versailles collection. The *sid2-1* null mutant was donated by J.-P. Métraux (Switzerland). Seed of the *rps2-201* mutant (Kunkel et al. 1993) were provided by the NASC (Scholl et al. 2000). The seed were sown in nonsterile soil and then vernalized by incubation for 2 days at 4°C. The resulting seedlings were allowed to grow for 2 weeks and were then potted individually into separate pots. The plants were allowed to grow for an additional 3 weeks before inoculation. The 5-week-old plants were watered 16 h before inoculation to stimulate the stomata opening, thereby facilitating inoculation. Inoculations were performed using a blunt syringe. Plants were always subjected to a cycle of 8 and 16 h of light and darkness, respectively, at 19°C, with 70% relative humidity.

Bacterial strains and harpins purification.

The bacterial strains used in this study were the wild-type strain *E. amylovora* CFBP1430 (Paulin and Samson 1973) and the following isogenic mutants: PMV6023 (*hrcV::MdlIPR13*) (Gaudriault et al. 1997), PMV6112 (*hrpN::MudIIPR13*) (Barney

Fig. 6. Role of *Arabidopsis thaliana* RPS2 gene in necrosis and defense gene triggering by *Erwinia amylovora*. **A**, Symptoms induced on *A. thaliana* leaves of wild-type Col-0 and *rps2* mutant 48 h after infiltration with wild-type *E. amylovora*. The slight differences in symptom distribution were not significant (Fisher's exact test, $\alpha = 0.05$). Symptom severity scale is shown in Figure 1F. **B**, Expression of the *PR1* and *CHI-B* marker genes 24 h after inoculation in *A. thaliana* wild-type and *rps2* mutant plants monitored by reverse-transcriptase polymerase chain reaction. **C**, Callose deposition detected with aniline blue in leaves 8 h after inoculation by *E. amylovora* wild-type and type-three secretion (*tts*) mutant 8 h after infiltration on *A. thaliana* wild-type and *rps2* mutant. The mean number of callose deposits per picture (0.6 mm²) is indicated for each treatment. The mean number of callose deposits in Col-0 and *rps2* leaves was not significantly different (Fischer's exact test, $\alpha = 0.05$). Bar length = 200 μ m.

1995), M52 (*dspA/E::uidA-Kan*) (Gaudriault et al. 1997), and M56 (*hrpW::MudI1734*) (Gaudriault et al. 1998), representative of *tts*, *hrpN*, *dspA/E*, and *hrpW* mutants, respectively. *E. amylovora* strains were grown in Luria-Bertani (LB) medium (Miller 1972). Bacterial strains were stored at -70°C in liquid cultures containing 40% glycerol. For plant inoculations, the bacterial inoculums were prepared as follows. An aliquot of the glycerol stock culture was streaked on agar medium and grown for 24 h at 30°C . Bacteria grown on petri dishes were used to inoculate a liquid culture. After 8 h of growth, 100 μl of the culture were plated on agar medium without antibiotic and allowed to grow overnight. The bacteria were then suspended in the appropriate solution and inoculum density was adjusted to $0.1 (10^7 \text{ CFU ml}^{-1})$.

The harpins *hrpN* and *hrpW* were polymerase chain reaction (PCR)-amplified with high-fidelity TAQ (Roche Molecular Systems, Meylan, France) using pMAB 40 as a template and the following primers: N-for 5'-AAC GGA TCC ATG AGT CTG AAT ACA AGT GGG, N-rev 5'-AAA CTC GAG TTA AGC CGC GCC CAG CTT GCC, W-for 5'-AAA GGA TCC ATG TCA ATT CTT ACG CTT AAC, and W-rev 5'-AAA CTC GAG TCA GCC ACC TTC AGG TTG GCG. The PCR-amplified products were digested with *Bam*H1 and *Xho*1, cloned into the pGEX6P vector (Amersham, Tokyo), and sequenced. The plasmids containing *hrpN* and *hrpW*, named pMAB164 and pMAB165, respectively, were used to transform *Escherichia coli* BL21. HrpW and HrpN were purified to homogeneity from recombinant *E. coli* BL21 strains as described previously (Reboutier et al. 2007). Harpins were infiltrated into *A. thaliana* leaves at a concentration of 400 nM each. This concentration corresponds to 15 $\mu\text{g ml}^{-1}$ for HrpN and 18 $\mu\text{g ml}^{-1}$ for HrpW. At this concentration, both proteins elicit an HR on tobacco. We also inoculated *A. thaliana* leaves with HrpN and HrpW at concentrations of 150 and 180 $\mu\text{g ml}^{-1}$, respectively, and still found no necrosis and no induction of the *PR1::GUS* fusion (data not shown).

Electrolyte leakage determination.

A. thaliana (ecotype Columbia) leaf disks (5 mm in diameter) were infiltrated with bacterial suspension of the wild-type strain or *tts*, *hrpN*, *dspA/E*, or *hrpW* mutants in assay medium (0.5 mM 2-N-morpholino-ethanesulfonic acid and 0.5 mM CaCl_2 , pH 6). Disks were blotted dry for 30 min and incubated in vials with assay medium under light conditions, at 25°C , with continuous stirring. Three repetitions of six disks in 2.5 ml of medium per treatment were carried out. Conductivity probes (Tacussel XE 120) were permanently immersed in each vial and connected to a computer-monitored conductivimeter.

Plant inoculations.

A. thaliana leaves were infiltrated with bacterial suspensions at a concentration of 10^7 CFU ml^{-1} in sterile water or with harpins at a concentration of 400 nM in 10 mM Tris-HCl (pH 8) using a needleless syringe. Symptom severity was scaled as shown in Figure 2D. Fisher's exact test was used to compare symptom distributions (Simple Interactive Statistical Binomial website). Bacterial counting was performed by grinding infected leaves in sterile water using a pestle and sterile sand. The resulting suspension was used for serial dilutions followed by plating on LB medium. Each experiment was repeated at least twice.

H_2O_2 detection.

H_2O_2 detection method was adapted from Zhang and associates (2004). At different time points following half-leaf infiltration with different bacterial strains or water, leaves were immersed in a 300- μM 2,7-dichlorofluorescein diacetate solution

and vacuum infiltrated. Whole-leaf images were taken using an Olympus SZX12 binocular magnifier. Green fluorescence was detected with an HQ510 1p emission filter. Experiments were repeated twice.

Trypan blue staining.

Trypan blue staining of dead cells was done according to Mauch-Mani and Slusarenko (1994). Inoculated *A. thaliana* leaves were sampled at 24 hpi and immersed into appropriate tubes containing the following solution: for 120 ml, 0.02 g of trypan blue (Acros Organics), 10 ml of phenol, 10 ml of lactic acid, 10 ml of glycerol, 10 ml of water, and 80 ml of 96% ethanol. The tubes were then placed for 1 min in a boiling water bath and leaves were left overnight in the staining solution at room temperature. Leaves were then destained several times by replacing the staining solution with aqueous chloral hydrate solution (2.5 g/ml) (Sigma-Aldrich, St. Louis) and observed with a binocular magnifier.

Measurement of GUS activity using a fluorometric assay.

This measurement assay was modified from Jefferson and associates (1987). Inoculated leaf disks (8 mm) were sampled 24 hpi and total proteins were extracted in GUS buffer (50 mM Na_2HPO_4 and 10 mM EDTA, pH 7.0). Total protein concentration was determined using the Bradford method (Bradford 1976). Protein (2 μg per sample) added to 150 μl of GUS buffer containing 2 mM 4-methylumbelliferyl- β -D-glucuronide (MUG) was disposed in a 96-well plate. GUS activity was determined at 37°C by measuring V_{max} of degradation of MUG into 4-MU (measured by fluorescence at emission 465 nm when excitation wavelength is 365 nm) using the cytoCalc software (PerSeptive Biosystems, Ramsey, MN, U.S.A.). Experiments were repeated twice with similar results. Fisher's exact test ($\alpha = 0.05$) was used to compare medians.

RNA extraction and RT-PCR analysis.

For RNA extraction, infiltrated leaves were cut off at the indicated times, immediately frozen in liquid nitrogen, and then stored at -70°C . Total RNA was prepared by phenol-chloroform extraction followed by LiCl precipitation as described by Verwoerd and associates (1989). For RT-PCR analysis, first-strand cDNA was synthesized using Superscript reverse transcriptase (Invitrogen, Cergy Pontoise, France) from 1 μg of DNase-treated (Invitrogen) total RNA in a 2- μl reaction volume. cDNA was precipitated and resuspended in 50 μl of water; then, 1 μl of the cDNA was subjected to PCR in a 20- μl reaction volume. PCR runs were of 25 to 30 cycles, each cycle consisting of 94°C for 30 s, 52 to 55°C for 30 s, and 72°C for 1 min, with a final step of 72°C for 2 min to complete polymerization. For each gene, an equal number of PCR cycles was performed on the different samples. The difference of intensity between samples, observed after gel electrophoresis, reflects the relative abundance of the corresponding transcript in the total RNA of the different samples. Each experiment was repeated three times and representative gels are presented. The gene-specific primers used in this analysis were as previously described (Fagard et al. 2007): *CHIB-F* (At3g12500), 5'-TTG TCC TGC TAG AGG TTT CTA CAC T-3'; *CHIB-R*, 5'-ATC AAG ATT ACC ACC AGG ATT AAC A-3'; *PDF1-2a-F* (At5g44420), 5'-TCA TGG CTA AGT TTG CTT CCA TCA TCA CCC-3'; and *PDF1-2a-R*, 5'-GTA GAT TTA ACA TGG GAC G-3'.

Microscopy, immunolabeling, and callose staining.

For light microscopy (LM), small fragments of leaves were sampled from the infected zone 24 hpi. Fragments of leaves inoculated with sterile water were collected as control. Samples

were fixed for 3 h in a solution consisting of 4% paraformaldehyde, 1% glutaraldehyde in 0.05 M piperazine-N, and N'-bis (2-ethanesulfonic acid) buffer, pH 7.3, under intermittent vacuum. They were washed four times in the same buffer and dehydrated in a graded ethanol series before embedding in a LR White resin (The London Resin Co., Hampshire, U.K.). For LM, transverse semi-thin (0.5 µm) sections were cut with a diamond knife (Diatome histo, Bienne, Switzerland). Some sections were stained with 0.1% toluidine blue (Touzard & Matignon, Paris) for 3 min, washed, air dried, and mounted in Eukitt medium (O. Kindler, Freiburg, Germany). They were then observed with a Zeiss Axiophot light microscope (Zeiss, Oberkochen, Germany). For immunolabeling of bacterial cells, we used a rabbit antiserum directed to *Erwinia amylovora* LPS (gift of J. P. Paulin, INRA Angers) diluted 1/30,000 and then a goat anti-rabbit immunoglobulin labeled with fluorescein isothiocyanate diluted in PBSB-T as already described (Perino et al. 1999). Fluorescence was observed with a Zeiss microscope (excitation filter for 450- to 490-nm wavelength and barrier filter for 520-nm wavelength). Experiments were repeated twice with similar results. Representative pictures are shown.

For callose detection, the leaves were collected 8 hpi and cleared by immersion in an alcoholic lactophenol solution by the method of Shipton and Brown (1962) modified by Adam and Sommerville (1996). They were rinsed in 50% ethanol, then in water. Callose was detected by staining for 30 min in a 150 mM K₂HPO₄ (pH 9.5) buffer containing 0.01% aniline blue (Sigma-Aldrich). Each leaf was mounted in 50% glycerol and observed with a Zeiss fluorescence microscope (excitation filter 365 nm and barrier filter 420 nm). Experiments were repeated twice with similar results. Representative pictures are shown. The number of callose deposits per picture was determined using ImageJ (National Institutes of Health, Bethesda, MD, U.S.A.) and compared using Fisher's exact test ($\alpha = 0.05$). We analyzed 25 to 30 pictures corresponding to more than five independent leaves for each treatment.

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LITERATURE CITED

- Adam, L., and Somerville, S. C. 1996. Genetic characterization of five powdery mildew disease resistance loci in *Arabidopsis thaliana*. *Plant J.* 9:341-356.
- Adam, A. L., Pike, S., Hoyos, M. E., Stone, J. M., Walker, J. C., and Novacky, A. 1997. Rapid and transient activation of a myelin basic protein kinase in tobacco leaves treated with harpin from *Erwinia amylovora*. *Plant Physiol.* 115:853-861.
- Alfano, J. R., Charkowski, A. O., Deng, W. L., Badel, J. L., Petnicki-Ocwieja, T., van Dijk, K., and Collmer, A. 2000. The *Pseudomonas syringae* Hrp pathogenicity island has a tripartite mosaic structure composed of a cluster of type III secretion genes bounded by exchangeable effector and conserved effector loci that contribute to parasitic fitness and pathogenicity in plants. *Proc. Natl. Acad. Sci. U.S.A.* 97:4856-4861.
- Baker, C. J., Orlandi, E. W., and Mock, N. M. 1993. Harpin, an elicitor of the HR in tobacco caused by *E. amylovora*, elicits active oxygen production in suspension cells. *Plant Physiol.* 102:1341-1344.
- Barny, M. A. 1995. *Erwinia amylovora* hrpN mutants blocked in harpin synthesis express a reduced virulence on host plant and elicit variable hypersensitive reactions on tobacco. *Eur. J. Plant Pathol.* 101:333-340.
- Barny, M. A., Guinebrière, M. H., Marçais, B., Coissac, E., Paulin, J. P., and Laurent, J. 1990. Cloning of a large gene cluster involved in *Erwinia amylovora* CFBP1430 virulence. *Mol. Microbiol.* 4:777-786.
- Benhamou, N. 1995. Immunocytochemistry of plant defense mechanisms induced upon microbial attack. *Microsc. Res. Tech.* 31:63-78.
- Bestwick, C. S., Bennet, M. H., and Mansfield, J. W. 1995. Hrp mutant of *Pseudomonas syringae* pv. *phaseolicola* induces cell wall alteration but not membrane damage leading to the hypersensitive reaction in lettuce. *Plant Physiol.* 108:503-516.
- Bocsanczy, A. M., Nissinien, R. M., Oh, C. S., and Beer, S. V. 2006. DspE, an effector of *Erwinia amylovora* is translocated into the plant cells. *Acta Hort.* 704:467-471.
- Bogdanove, A. J., Kim, J. F., Wei, Z., Kolchinsky, P., Charkowski, A. O., Collmer, A., and D. W., Beer, S. V. 1998. Homology and functional similarity of an hrp-linked pathogenicity locus, *dspEF*, of *Erwinia amylovora* and the avirulence locus *avrE* of *Pseudomonas syringae* pv. *tomato*. *Proc. Natl. Acad. Sci. U.S.A.* 95:1325-1330.
- Bonaser, J. M., Kim, J. F., and Beer, S. V. 2006. PR genes of apple: Identification and expression in response to elicitors and inoculation with *Erwinia amylovora*. *BMC Plant Biol.* 6:23.
- Boureau, T., El Maarouf-Bouteau, H., Garnier, A., Brisset, M. N., Perino, C., Pucheu, I., and Barny, M. A. 2006. DspA/E, a type III effector essential for *Erwinia amylovora* pathogenicity and growth in planta, induces cell death in host apple and non host tobacco plants. *Mol. Plant-Microbe Interact.* 19:16-24.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-54.
- Brown, I., Mansfield, J., and Bonas, U. 1995. *hrp* genes in *Xanthomonas campestris* pv. *vesicatoria* determine the ability to suppress papillae deposition in pepper mesophyll cells. *Mol. Plant-Microbe Interact.* 8:825-836.
- Burgert, P., and Geider, K. 1995. Molecular analysis of the *ams* operon required for exopolysaccharide synthesis of *Erwinia amylovora*. *Mol. Microbiol.* 15:917-933.
- Cesbron, S., Paulin, J. P., Tharaud, M., Barny, M. A., and Brisset, M. N. 2006. The alternative sigma factor HrpL negatively modulates the flagellar system in the phytopathogenic bacterium *Erwinia amylovora* under hrp-inducing conditions. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett.* 257:221-227.
- Cessna, S. G., Messerli, M. A., Robinson, K. R., and Low, P. S. 2001. Measurement of stress induced Ca²⁺ pulses in single aequorin-transformed tobacco cells. *Cell Calcium* 30:151-156.
- Debroy, S., Thilmony, R., Kwack, Y. B., Nomura, K., and He, S. Y. 2004. A family of conserved bacterial effectors inhibits salicylic acid-mediated basal immunity and promotes disease necrosis in plants. *Proc. Natl. Acad. Sci. U.S.A.* 101:9927-9932.
- 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 NIM gene. *Plant J.* 20:207-215.
- Dong, H. P., Peng, J., Bao, Z., Meng, X., Bonaser, J. M., Chen, G., Beer, S. V., and Dong, H. 2004. Downstream divergence of the ethylene signaling pathway for harpin-stimulated *Arabidopsis* growth and insect defense. *Plant Physiol.* 136:3628-3638.
- El-Maarouf, H., Barny, M. A., Rona, J. P., and Bouteau, F. 2001. Harpin, a hypersensitive response elicitor from *Erwinia amylovora*, regulates ion channel activities in *Arabidopsis thaliana* suspension cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 497:82-84.
- Fagard, M., Dellagi, A., Roux, C., Perino, C., Rigault, M., Boucher, V., Shevchik, V., and Expert, D. 2007. *Arabidopsis thaliana* expresses multiple lines of defense to counter-attack *Erwinia chrysanthemi*. *Mol. Plant-Microbe Interact.* 7:795-805.
- Fujimoto, S. Y., Ohta, M., Usui, A., Shinshi, H., and Ohme-Takagi, M. 2000. A *thaliana* ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. *Plant Cell* 12:393-404.
- Gaudriault, S., Malandrin, L., Paulin, J. P., and Barny, M. A. 1997. DspA, an essential pathogenicity factor of *Erwinia amylovora* showing homology with AvrE of *Pseudomonas syringae*, is secreted via the Hrp secretion pathway in a DspB-dependent way. *Mol. Microbiol.* 26:1057-1069.
- Gaudriault, S., Brisset, M. N., and Barny, M. A. 1998. HrpW of *Erwinia amylovora*, a new Hrp-secreted protein. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 428:224-228.
- Glazebrook, J., Chen W., Estes, B., Chang, H.-R., Nawrath, C., Métraux, J.-P., Zhu, T., and Katagiri, F. 2003. Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. *Plant J.* 34:217-228.
- Hauck, P., Thilmony, R., and He, S. Y. 2003. A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defense in susceptible *Arabidopsis* plants. *Proc. Natl. Acad. Sci. U.S.A.* 100:8577-8582.
- Holeva, M. C., Bell, K. S., Hyman, L. J., Avrova, A. O., Whisson, S. C., Birch, P. R., and Toth, I. K. 2004. Use of a pooled transposon mutation grid to demonstrate roles in disease development for *Erwinia caroto-*

- vora* subsp. *atroseptica* putative type III secreted effector (DspE/A) and helper (HrpN) proteins. *Mol. Plant-Microbe Interact.* 17:943-950.
- Jefferson, R. A., Kavanagh, T. A., and Bevan, M. W. 1987. GUS fusions: Beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO (Eur. Mol. Biol. Organ.) J.* 20:3901-3907.
- Kim, J. F., and Beer, S. V. 1998. HrpW of *Erwinia amylovora*, a new harpin that contains a domain homologous to pectate lyases of a distinct class. *J. Bacteriol.* 180:5203-5210.
- Kunkel, B. N., Bent, A. F., Dahlbeck, D., Innes, R. W., and Staskawicz, B. J. 1993. RPS2, an *Arabidopsis* disease resistance locus specifying recognition of *Pseudomonas syringae* strains expressing the avirulence gene *avrRpt2*. *Plant Cell* 5:865-875.
- Mauch-Mani, B., and Slusarenko, A. J. 1994. Systemic acquired resistance in *Arabidopsis thaliana* induced by a predisposing infection with a pathogenic isolate of *Fusarium oxysporum*. *Mol. Plant-Microbe Interact.* 7:378-383.
- McKhann, H. I., Camilleri, C., Bérard, A., Bataillon, T., David, J. L., Reboud, X., Le Corre, V., Caloustian, C., Gut, I. G., and Brunel, D. 2004. Nested core collections maximizing genetic diversity in *Arabidopsis thaliana*. *Plant J.* 38:193-202.
- Miller, J. H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, U.S.A.
- Mor, H., Manulis, S., Zuck, M., Nizan, R., Coplin, D. L., and Barash, I. 2001. Genetic organization of the *hrp* gene cluster and *dspAE/BF* operon in *Erwinia herbicola* pv. *gypsophylae*. *Mol. Plant-Microbe Interact.* 14:431-436.
- Nawrath, C., and Métraux, J. P. 1999. Salicylic acid induction-deficient mutants of *A. thaliana* express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. *Plant Cell* 11:1393-1404.
- Nissinen, R. M., Ytteberg, A. J., Bogdanove, A. J., Van Wijk, K. J., and Beer, S. V. 2007. Analyses of the secretomes of *Erwinia amylovora* and selected *hrp* mutants reveal novel type III secreted proteins and an effect of HrpJ on extracellular harpin levels. *Mol. Plant. Pathol.* 8:55-57.
- Oh, C. S., Martin, G. B., and Beer, S. V. 2007. DspA/E, a type III effector of *Erwinia amylovora* is required for early rapid growth in *Nicotiana benthamiana* and causes NbSGT1-dependent cell death. *Mol. Plant Pathol.* 8:255-265.
- Panstruga, R. 2005. Serpentine plant MLO proteins as entry portals for powdery mildew fungi. *Biochem. Soc Trans.* 33:389-392.
- Paulin, J. P. 1996. Control of fire blight in European pome fruits. *Outlook Agric.* 25:49-55.
- Paulin, J. P., and Samson, R. 1973. Le feu bactérien en France II. Caractères des souches d'*Erwinia amylovora* (Burril) Winslow et al. 1920 isolées du foyer franco-belge. *Annu. Rev. Phytopathol.* 5:389-397.
- Perino, C., Gaudriault, S., Vian, B., and Barny, M. A. 1999. Visualization of harpin secretion in planta during infection of apple seedlings by *Erwinia amylovora*. *Cell. Microbiol.* 1:131-141.
- Pike, S. M., Ádám, A. L., Pu, X. A., Hoyos, M. E., Laby, R., Beer, S. V., and Novacky, A. 1998. Effects of *Erwinia amylovora* harpin on tobacco leaf cell membranes are related to leaf necrosis and electrolyte leakage and distinct from perturbations caused by inoculated *E. amylovora*. *Physiol. Mol. Plant Pathol.* 53:39-60.
- Preston, G. M., Jackson, R. and Rainey, P. B. 2001. Characterization of the type III secreted protein RopE from *Pseudomonas fluorescens* SBW25. *Mol. Microbiol.* 41:999-1014.
- Reboutier, D., Frankart, C., Vedel, R., Brault, M., Duggleby, R. G., Rona, J. P., Barny, M. A., and Bouteau, F. 2005. A CFTR chloride channel activator prevents HrpN(ea)-induced cell death in *Arabidopsis thaliana* suspension cells. *Plant Physiol. Biochem.* 43:567-572.
- Reboutier, D., Frankart, C., Briand, J., Biligui, B., Laroche, S., Rona, J. P., Barny, M. A., and Bouteau, F. 2007. The HrpNea Harpin from *Erwinia amylovora* triggers differential responses on the nonhost *Arabidopsis thaliana* cells and on the host apple cells. *Mol. Plant-Microbe Interact.* 20:94-100.
- Scholl, R. L., May S. T., and Ware, D. H. 2000. Seed and molecular resources for *Arabidopsis*. *Plant Physiol.* 124:1477-1480.
- Shah, J. 2003. The salicylic acid loop in plant defense. *Curr. Opin. Plant Biol.* 6:365-371.
- Shipton, W. A., and Brown, J. F. 1962. A whole-leaf clearing and staining technique to demonstrate host-pathogen relationships of wheat stem rust. *Phytopathology* 52:1313.
- Simons, B. H., Millenaar, F. F., Mulder, L., Van Loon, L. C., and Lambers, H. 1999. Enhanced expression and activation of the alternative oxidase during infection of *A. thaliana* with *Pseudomonas syringae* pv. *tomato*. *Plant Physiol.* 120:529-538.
- Steinberger, E. S., and Beer, S. V. 1988. Creation and complementation of pathogenicity mutants of *Erwinia amylovora*. *Mol. Plant-Microbe Interact.* 1:135-144.
- Thilmony, R., Underwood, W., and He, S. Y. 2006. Genome-wide transcriptional analysis of the *Arabidopsis thaliana* interaction with the plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000 and the human pathogen *Escherichia coli* O157:H7. *Plant J.* 46:34-53.
- Venisse, J. S., Gullner G., and Brisset, M. N. 2001 Evidence for the involvement of an oxidative stress in the initiation of infection of pear by *Erwinia amylovora*. *Plant Physiol.* 125:2164-2172.
- Venisse, J. S., Malnoy, M., Faize, M., Paulin, J. P., and Brisset, M. N. 2002. Modulation of defense responses of *Malus spp.* during compatible and incompatible interactions with *Erwinia amylovora*. *Mol. Plant-Microbe Interact.* 15:1204-1212.
- Venisse, J. S., Barny, M. A., Paulin, J. P., and Brisset, M. N. 2003. Involvement of three pathogenicity factors of *Erwinia amylovora* in the oxidative stress associated with compatible interaction in pear. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 537:198-202.
- Verwoerd, T. C., Dekker, B. M., and Hoekema, A. 1989. A small-scale procedure for the rapid isolation of plant RNAs. *Nucleic Acids Res.* 17:2362.
- 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.
- Wildermuth, M. C., Dewdney, J., Wu, G., and Ausubel, F. 2001. Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* 414:562-565.
- Zhang, C., Gutsche, A. T., and Shapiro, A. D. 2004. Feedback control of the *Arabidopsis* hypersensitive response. *Mol. Plant-Microbe Interact.* 17:357-365.
- Zhao, Y., He, S. Y., and Sundin, G. W. 2006. The *Erwinia amylovora* *AvrRpt2_{ea}* gene contributes to virulence on pear and *AvrRpt2_{ea}* is recognized by *Arabidopsis* RPS2 when expressed in *Pseudomonas syringae*. *Mol. Plant-Microbe Interact.* 19:644-654.

AUTHOR-RECOMMENDED INTERNET RESOURCE

Simple Interactive Statistical (SISA) Binomial website:
www.quantitativeskills.com/sisa/distributions/binomial.htm