The Role of Pectate Lyase and the Jasmonic Acid Defense Response in *Pseudomonas viridiflava* Virulence

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Pseudomonas viridiflava is a common pathogen of *Arabidopsis thaliana* in wild populations, yet very little is known about mechanisms of resistance and virulence in this interaction. We examined the induced defense response of *A. thaliana* to several strains of *P. viridiflava* collected from this host by quantifying the expression of *PR-1* and *LOX2/PDF1.2*, which serve as markers for induction of the salicylic and jasmonic acid (JA) pathways, respectively. Growth of these strains then was assessed on Col-0, the *fad3/7/8* and *coi1-1* mutants deficient in JA- and ethylene (ET)-induced defense responses, and the *sid2-1* mutant deficient in salicylic acid-induced defense responses. All strains of *P. viridiflava* induced high expression of *LOX2* and *PDF1.2* on Col-0. In contrast, *PR-1* expression was delayed and reduced relative to *PDF1.2* expression. Additionally, three of four *P. viridiflava* strains were more virulent on *fad3/7/8* relative to Col-0, whereas all strains were more virulent on *coi1-1* relative to Col-0, indicating that *P. viridiflava* generally may be suppressed by JA/ET-mediated defense responses. In contrast, no increase in the growth of *P. viridiflava* strains was observed in the *sid2-1* mutant relative to Col-0. Parallel experiments were performed with the closely related *P. syringae* pv. *tomato* for comparative purposes. In addition, we assessed the role of pectate lyase and the alternative sigma factor HrpL in *P. viridiflava* virulence on *A. thaliana* and found that pectate lyase activity is correlated with virulence, whereas the removal of pectate lyase or HrpL significantly reduced virulence.

Additional keyword: salicylic acid.

Plants use a variety of mechanisms to minimize pathogen damage through resistance and to reduce the deleterious effects of damage through tolerance. Plant resistance to microbial pathogens involves recognition of the invading pathogen followed by the elicitation of a complex defense response. It is likely that many defense genes have been shaped by coevolution between plants and pathogens (Stahl and Bishop 2000), and our understanding of the functional diversity and molecular evolution of these genes (Bergelson et al. 2001; Mauricio et

al. 2003; Stahl et al. 1999; Tian et al. 2002) benefits from an understanding of the evolutionary forces that drive plant–pathogen coevolution. However, empirical investigations of selection on interacting resistance and virulence genes in *Arabidopsis thaliana* and its microbial pathogens have been limited by an incomplete understanding of the genetic basis for virulence and resistance in naturally occurring interactions. One noteworthy exception is the interaction between *A. thaliana* and its oomycete pathogen, *Hyaloperonospora parasitica*, where both resistance (*R*) and avirulence (*avr*) genes are known and patterns of polymorphism are consistent with coevolutionary models involving balancing selection (Allen et al. 2004; Rose et al. 2004). *A. thaliana* also commonly interacts with many bacterial pathogens in natural populations, providing the opportunity to develop a more complete picture of plant–bacteria coevolution by studying the evolution of resistance and virulence genes involved in these interactions.

Pseudomonas viridiflava is one of the most prevalent species of bacteria found in Midwestern populations of *A. thaliana* (Goss et al. 2005; Jakob et al. 2002). *P. viridiflava* is closely related to *P. syringae* (Goss et al. 2005; Yamamoto et al. 2000) and shows extensive genetic variation for virulence on *A. thaliana*. Similarly, genotypes of *A. thaliana* collected from these populations exhibit substantial genetic variation for resistance to *P. viridiflava* (Jakob et al. 2002).

A. thaliana is known to use at least three primary signaling molecules in the defense response to microbial pathogens: salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). SA is required for plant *R*-gene-mediated resistance (Gaffney et al. 1993; Malamy et al. 1990; Métraux et al. 1990; Rairdan and Delaney 2002), and also is required for systemic acquired resistance (SAR) against many different microbial pathogens, including *P. syringae* (Durrant and Dong 2004; Ryals et al. 1996; Uknes et al. 1992). In contrast, studies of JA and ET have revealed a much broader role for these molecules in response to varied damage, including damage from a wide variety of microbial pathogens and insects, and abiotic damage (Kariola et al. 2003; Reymond et al. 2004; Thaler et al. 2004; Vijayan et al. 1998). Recent work indicates that JA and ET induce an overlapping set of defense genes, whereas SA induces a distinct set of defense genes, suggesting that these signaling molecules may be used selectively to induce specific defense responses (Glazebrook 2005; Kunkel and Brooks 2002).

Although very little is known about virulence mechanisms in *P. viridiflava*, other macerating or soft-rotting pathogens such as *P. viridiflava* use pectate lyases to break down plant cell walls and release nutrients (Barras et al. 1994; Collmer and Keen 1986). Bacterial pathogens also are known to have pathogenicity islands (PAIs) that contain genes involved in the

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type III secretion system (TTSS) that delivers effector and Avr proteins to host cells (Casper-Lindley et al. 2002; Deng et al. 2003; Hueck 1998; Wu et al. 2003). In *P. syringae*, a close relative of *P. viridiflava*, there is a PAI composed of three components, a conserved *hrp/hrc* gene cluster in the center, flanked by an exchangeable and a conserved effector locus (Alfano et al. 2000). Sequence homology with *P. syringae* facilitated the identification of two distinct PAIs in *P. viridiflava* containing a TTSS and several potential effector and *avr* genes (Araki et al. 2006). Some strains of *P. viridiflava* possess the tripartite structure of the PAI found in *P. syringae* (T-PAI), whereas other strains have a single-component (S-PAI) containing only the conserved *hrp/hrc* gene cluster. The T- and S-PAI occupy different chromosomal locations; however, only one or the other PAI is present in an individual strain (Araki et al. 2006). The presence of two distinct PAIs in *P. viridiflava* was unexpected, but provides the opportunity to evaluate the role of polymorphism at a large and complex genomic region in *P. viridiflava* virulence, and to determine whether these two very different PAIs influence the defense response of *A. thaliana* to *P. viridiflava* infection.

In this article, we report on experiments conducted to characterize the interaction between *P. viridiflava* and one of its natural hosts, *A. thaliana*. To determine how *A. thaliana* responds to this pathogen, we measured expression of *PR-1*, lipoxigenase2 (*LOX2*), and *PDF1.2* in both S-PAI and T-PAI strains. *PR-1* is used conventionally as a marker for induction of the SA-mediated defense response (Ryals et al. 1996; Uknes et al. 1992) whereas *LOX2*, which is required for JA synthesis (Bell et al. 1995), and *PDF1.2*, which requires JA and ET for transcription, were used as markers for induction of the JA/ET-mediated defense response (Penninckx et al. 1998). We followed these expression experiments with growth assays on *A. thaliana* mutants deficient in the SA-mediated (*sid2-1* [Nawrath and Métraux 1999; Wildermuth et al. 2001]) or JA/ET-mediated (*fad3/7/8* [McConn and Browse 1996] and *coi1* [Feys et al. 1994, Xie et al. 1998]) defense response. These experiments were conducted in parallel on *P. syringae* pv. *tomato* (DC3000) for comparative purposes. Finally, we investigated the role of pectate lyase and type III secretion in *P. viridiflava* virulence.

RESULTS

T-PAI and S-PAI strains of *P. viridiflava* induce the JA/ET-mediated defense response more strongly than the SA-mediated defense response in *A. thaliana*.

The T- and S- PAI strains differ in the composition of genes that are homologous to known *avr* or effector genes in other pathogens (Araki et al. 2006). To determine whether PAI type changes the induced defense response of *A. thaliana*, levels of *PR-1* and *PDF1.2* expression were measured as markers for induction of SA- and JA/ET-mediated defense responses, respectively, at 1, 2, and 4 days following inoculation for S-PAI (RMX23.1 and RMX3.1b) and T-PAI (LP23.1 and PNA3.3a) strains. Expression of *PR-1* and *PDF1.2* also was measured following infection with two strains of *P. syringae* pv. *tomato* (DC3000) for comparison. One strain, DC3000/*avrRpt2*, possesses a vector-borne copy of *avrRpt2* that causes a hypersensitive response on the Col-0 ecotype (Whalen et al. 1991), whereas a second strain, DC3000, lacks *avrRpt2*. These tests were conducted on the Col-0 wild type as well as on two mutants: the *sid2-1* mutant deficient in the SA-induced defense response and the *fad3/7/8* mutant deficient in the JA/ET-induced defense response.

T-PAI and S-PAI strains both induced *PDF1.2* expression in Col-0 (Fig. 1 through C). *PDF1.2* expression was induced

within 24 h after infection, and expression ranged from 2.1- to 6.4-fold greater than buffer-infected controls (Fig. 1A). *PDF1.2* expression became more variable by day 2, ranging from 8.1- to 21.3-fold greater than controls (Fig. 1B). Strains that induced relatively low *PDF1.2* expression on day 2 induced high expression 4 days postinfection, whereas strains that induced high expression on day 2 showed a decline in expression by day 4 (Fig. 1C). However, there was no effect of PAI type on *PDF1.2* expression ($F_{1,48} = 0.002$, $P = 0.9608$), suggesting that differences in the complement of *avr* or effector genes between PAI types did not alter the induction of JA/ET-mediated defense. In contrast, the *P. syringae* DC3000/*avrRpt2* and DC3000 strains showed very little induction of *PDF1.2* expression in Col-0 (Fig. 1D through F).

PDF1.2 expression was reduced in *sid2-1* relative to Col-0 following infection with *P. viridiflava* ($F_{1,48} = 4.92$, $P = 0.0313$), suggesting that the SA signaling pathway may contribute to *P. viridiflava*-induced *PDF1.2* expression. The opposite pattern was found following infection with *P. syringae* DC3000/*avrRpt2*, where *PDF1.2* expression was greater in *sid2-1* than Col-0 ($F_{1,18} = 6.89$, $P = 0.0172$), indicating that the SA signaling pathway may suppress *P. syringae* DC3000/*avrRpt2* *PDF1.2* expression. This latter observation is consistent with the presence of negative crosstalk between the SA and JA pathways (Kunkel and Brooks 2002). However, we failed to detect a difference in *PDF1.2* induction by DC3000 in *sid2-1* and Col-0 ($F_{1,18} = 0.12$, $P = 0.9157$). Finally, both *P. viridiflava* and *P. syringae* show consistently low *PDF1.2* expression on the *fad3/7/8* mutant deficient in the JA/ET-mediated defense response, supporting a causal relationship between JA pathway induction and *PDF1.2* expression, as has been noted previously (Penninckx et al. 1996).

Following the *PDF1.2* expression results, expression of *LOX2* was measured to further verify differential induction of the JA pathway by *P. viridiflava* and *P. syringae*. All four strains of *P. viridiflava* showed elevated *LOX2* expression, with expression ranging from 1.98- to 9.66-fold greater than controls (mean 4.21) (Fig. 2A). Similar to *PDF1.2* expression, there was no effect of PAI type on *LOX2* expression ($F_{1,36} = 0.51$, $P = 0.4807$). *P. viridiflava* induction of *LOX2* expression was significantly lower in *sid2-1* relative to Col-0 ($F_{1,36} = 9.41$, $P = 0.0041$), and, as expected, *LOX2* expression was very low in *fad3/7/8* following infection by either *P. viridiflava* or *P. syringae* (Fig. 2A). However, unlike *PDF1.2* expression, which was very low following *P. syringae* infection in Col-0 (Fig. 1 D through F), *P. syringae* induced up to 5.96 greater *LOX2* expression in Col-0 relative to controls (mean 2.08) (Fig. 2A). Interestingly, qualitatively similar results were observed by Spoel and associates (2003), who found that *LOX2* expression in Col-0 was induced following infection by *P. syringae*. Thus, although it is clear that *P. syringae* induces *LOX2* expression, these results further support the differential induction of JA-mediated defense response by *P. viridiflava* and *P. syringae*.

Infection with *P. viridiflava* triggered a 2.2- to 6.9-fold increase in *PR-1* expression in Col-0 on day 2 relative to buffer-infected controls, indicating that *P. viridiflava* also induces the SA-mediated defense response (Fig. 1A and B). Again, there was no effect of PAI type on *PR-1* expression ($F_{1,45} = 0.14$, $P = 0.7113$). As expected, *P. syringae* DC3000/*avrRpt2* induced significantly greater *PR-1* expression than DC3000 in Col-0 ($F_{1,18} = 8.38$, $P = 0.0096$), with a 44.4-fold increase at 24 h postinfection (Fig. 1D). By days 2 and 4 postinfection, *PR-1* expression did not differ between DC3000/*avrRpt2* and DC3000 ($F_{1,18} = 0.04$, $P = 0.8472$ and $F_{1,18} = 0.04$, $P = 0.8485$, respectively), ranging from 12.5 to 21.9 (Fig. 1E and F).

Expression of *PR-1* in the *fad3/7/8* mutant after inoculation with *P. viridiflava* ranged from 4.9- to 9.7-fold greater than

buffer-inoculated controls on day 1 (Fig. 1A) and increased to a maximum of 23.2-fold greater expression by day 2 after infection (Fig. 1B and C). Expression levels were significantly greater than in Col-0 ($F_{1,45} = 66.16$, $P < 0.0001$). Although a comparison of buffer-infected plants revealed strong evidence of elevated constitutive expression of *PR-1* in *fad3/7/8* relative to Col-0 ($F_{1,22} = 5.15$, $P = 0.0339$), it also is evident that *PR-1* expression increased beyond this constitutive level following infection by *P. viridiflava* relative to buffer-infected controls (Fig. 1A through C). Likewise, data from day 4 postinfection show that *PR-1* expression was greater in *fad3/7/8* relative to Col-0 for *P. syringae* ($F_{1,18} = 7.63$, $P = 0.0128$). This experiment shows that the JA and ET signaling pathway can suppress the induction of *PR-1* by both *P. viridiflava* and *P. syringae* and, thus, provides evidence consistent with an antagonistic effect of JA and ET on the SA-mediated defense response. Furthermore, *PR-1* expression data from the Col-0 and *fad3/7/8* demonstrate that both T-PAI and S-PAI strains of *P. viridiflava* have the ability to induce the SA-mediated defense response. However, insofar as quantitative expression of *PR-1* reflects quantitative differences in the SA-mediated defense response, it also is clear that *P. viridiflava* induces SA-mediated defense

in Col-0 at levels far below those observed with the DC3000/*avrRpt2* and DC3000 strains of *P. syringae*.

Growth of *P. viridiflava* is enhanced in mutants deficient in the JA-mediated defense response but not the SA-mediated defense response for both T-PAI and S-PAI strains.

Bacterial growth was measured to investigate the effects of SA- or JA/ET-mediated defense responses on the virulence of *P. viridiflava* using Col-0, *sid2-1*, and *fad3/7/8*. In addition, the growth of DC3000 and DC3000/*avrRpt2* was measured to compare how these two related isolates responded to SA- and JA/ET-mediated defense in *A. thaliana*.

In an analysis of *P. viridiflava* growth, there was a strong interaction between strain (nested within PAI type) and plant genetic background ($F_{4,108} = 4.41$, $P = 0.0024$) (Fig. 3A). In three of the four *P. viridiflava* strains, growth was significantly greater in the *fad3/7/8* background relative to Col-0 (RMX23.1a: $F_{1,108} = 24.7$, $P < 0.0001$; LP23.1a: $F_{1,108} = 24.24$, $P < 0.0001$; PNA3.3a: $F_{1,108} = 7.9$, $P = 0.0059$, and RMX3.1b: $F_{1,108} = 1.13$, $P = 0.29$), while growth in *sid2-1* remained indistinguishable from growth in Col-0 (Fig. 3A). Furthermore, PAI

Fig. 1. Difference in fold expression of *PR-1* and *PDF1.2* in infected Col-0, *sid2-1*, and *fad3/7/8* plants relative to mock-infected controls. Expression was measured on A, day 1, B, day 2, and C, day 4 postinfection for *Pseudomonas viridiflava* S-PAI strains RMX23.1a (R) and RMX3.1b (X) and T-PAI strains LP23.1a (L) and PNA3.3a (P) and on D, day 1, E, day 2, and F, day 4 postinfection for *P. syringae* pv. tomato DC3000 (V) and DC3000/*avrRpt2* (A). Note different Y axes scales. Bars represent means and standard error of two or three independent replications.

type had a strong effect, with S-PAI strains outperforming T-PAI strains ($F_{1,108} = 14.12$, $P = 0.0003$), but there was no interaction between PAI type and plant genetic background ($F_{1,108} = 0.41$, $P = 0.6628$), suggesting that the putative growth benefits of the S-PAI were not mediated by differences in SA or JA/ET defense responses.

To further verify that the JA pathway plays a major role in the defense of *A. thaliana* to *P. viridiflava*, a second experiment was conducted with the *coi1-1* mutant that is insensitive to coronatine and methyl jasmonate and, thus, not able to confer a normal JA-mediated defense response (Feys et al. 1994, Xie et al. 1998). *P. viridiflava* growth was significantly higher in *coi1-1* than Col-0 for all strains (RMX23.1a: $F_{1,168} = 21.9$, $P = 0.001$; LP23.1a: $F_{1,168} = 29.1$, $P = 0.0001$; PNA3.3a: $F_{1,168} = 13.5$, $P = 0.0003$; and RMX3.1b: $F_{1,168} = 11.2$, $P = 0.001$) (Fig. 4). Once again, S-PAI strains experienced greater growth than T-PAI strains in *coi1-1* ($F_{1,168} = 15.4$, $P = 0.0001$). Thus, *P. viridiflava* shows a strong increase in growth on two independent mutants that are deficient in JA pathway signaling.

In *P. syringae*, the presence of *avrRpt2* had a large effect on bacterial growth in Col-0 (Fig. 3B), which is known to possess the cognate *R* gene, *Rps2*, and there was an expected statistical interaction between the presence or absence of *avrRpt2* and the plant's genetic background ($F_{2,54} = 5.81$, $P = 0.0052$). DC3000 showed elevated growth relative to DC3000/*avrRpt2* in all backgrounds (Col-0: $F_{1,54} = 89.04$, $P < 0.0001$; *sid2-1*: $F_{1,54} = 27.52$, $P < 0.0001$; and *fad3/7/8*: $F_{1,54} = 88.41$, $P < 0.0001$). However, as expected, DC3000/*avrRpt2* experienced dramatically elevated growth in *sid2-1* relative to Col-0 ($F_{1,54} = 31.28$, $P < 0.0001$), due to the known requirement of SA accu-

mulation in the interaction between *AvrRpt2* and *RPS2* in Col-0 (Delaney et al. 1994; Nawrath and Métraux 1999). Consequently, although the SA-mediated defense response suppresses *P. syringae* growth, it has no effect on *P. viridiflava*, which instead is deterred by the JA-mediated defense response.

Pectate lyase of *P. viridiflava* contributes to virulence on Col-0.

Pectate lyases are important virulence mechanisms in many soft-rotting and macerating pathogens. Macerating symptoms caused by *P. viridiflava* prompted an investigation of pectate lyase in our wild-collected *P. viridiflava* strains. DNA sequencing of the pectate lyase gene in two S-PAI and two T-PAI strains revealed a 1,140-bp open reading frame (GenBank nos. DQ004275 to DQ004278). Amino acid identity of the predicted pectate lyase gene was 99.7% between the two T-PAI strains and 96.0% between the two S-PAI strains. Between the groups, amino acid identities ranged from 96.5 to 98.9%. A comparison of the phylogenetic relationship of pectate lyase sequences (data not shown) with a consensus tree of five randomly chosen genomic fragments from *P. viridiflava* (Goss et al. 2005) shows that pectate lyase sequence diversity reflects the larger pattern of genome divergence between strains. Specifically, the S-PAI strain, RMX3.1b, which derives from a generally distinct clade of *P. viridiflava* (clade B) is the most divergent in pectate lyase sequence relative to the remaining three strains that derive from clade A (Goss et al. 2005).

Pectate lyase activity was detected by observing pectate degradation in polygalacturonic acid containing plates with varying pH (Schaad et al. 2001). Seven strains of *P. viridiflava*

Fig. 2. Difference in fold expression of *LOX2* in infected Col-0, *sid2-1*, and *fad3/7/8* plants relative to mock-infected controls. Expression was measured on day 1, 2, and 4 postinfection with **A**, *Pseudomonas viridiflava* strains and **B**, *P. syringae* pv. *tomato* DC3000 and DC3000/*avrRpt2*. Bars represent means and standard error of two independent replications.

were tested, including the American Type Culture Collection 13223 *P. viridiflava* type strain. Plates were scored for pitting at 4 days after inoculation with 10 μ l of a *P. viridiflava* solution (optical density at 600 nm [OD₆₀₀] = 0.1). No pitting appeared in the acidic pH plates. However, all *P. viridiflava* strains produced distinctive pitting rings on the plates at neutral (6.9 to 7.1) and basic (8.3 to 8.5) pH values, indicating pectate lyase activity. In contrast, *P. syringae* pv. *tomato* did not develop pitting at any of these pH values. Furthermore, a BLAST search using the *P. viridiflava* pectate lyase open reading frame revealed no putative pectate lyase genes in *P. syringae* pv. *tomato*. These results confirm pectate lyase activity in *P. viridiflava* and suggest that pectate lyase may represent a major difference in the biology of *P. viridiflava* and *P. syringae*.

In a second experiment, variation in pectate lyase activity was quantified among 10 T-PAI and 10 S-PAI strains by measuring the diameter of the pitting ring using basic pH value (8.3

to 8.5) plates. In addition, pectate lyase enzyme activity was quantified after growing bacteria in minimal media containing polygalacturonic acid as a carbon source (Matsumoto et al. 2003). There were striking differences between PAI types, with S-PAI strains producing significantly larger pitting rings ($F_{1,19} = 12.09$, $P < 0.01$) and exhibiting greater than twofold higher enzyme activity ($F_{1,19} = 5.24$, $P < 0.05$) than T-PAI strains. To examine whether pectate lyase activity influences virulence, bacterial growth for seven S-PAI and six T-PAI *P. viridiflava* strains was assessed on Col-0 after infection at 10⁵ CFU/ml. Bacterial growth on day 4 was positively correlated with pectate lyase activity for the S-PAI strains ($r_{1,6} = 0.80$, $P = 0.0297$) but not for the T-PAI strains ($r_{1,5} = 0.04$, $P = 0.9378$) (Fig. 5), suggesting that pectate lyase may be an important virulence factor on *A. thaliana*, at least for the S-PAI strains.

Next, a pectate lyase mutant (*pel*⁻) was generated to confirm a causal role for pectate lyase in *P. viridiflava* virulence. The *pel*⁻ was created in the S-PAI strain RMX23.1a by marker-exchanged mutagenesis (Ried and Collmer 1987). RMX23.1a (wild type) and the *pel*⁻ mutant exhibited indistinguishable growth in liquid media over 16 h and there was no difference in colony appearance on agar plates. We complemented *pel*⁻ with the plasmid pME6010 containing the pectate lyase gene from RMX23.1a (*pel*⁻pME*pel*⁺). pME6010 is a commonly used vector that is stable in plant-associated *Pseudomonads* (Heeb et al. 2000). As an additional control, we also generated a strain of *pel*⁻ complemented with an empty pME6010 plasmid (*pel*⁻pME).

Polymerase chain reaction (PCR) was used to confirm the presence of the pectate lyase gene in RMX23.1a and the complemented *pel*⁻pME*pel*⁺ as well as the absence of the pectate lyase gene from *pel*⁻ and the *pel*⁻pME vector control. All strains also were examined for macerating activity on potato tuber slices to confirm the presence or absence of pectate lyase activity. The *pel*⁻ mutant and the *pel*⁻pME plasmid control showed no rotting activity on potato, whereas the complemented *pel*⁻pME*pel*⁺ macerated the potato as heavily as the wild-type strain (results not shown). This suggests that a single pectate lyase gene is responsible for the soft-rot activity in RMX23.1a.

Next, we tested the effect of pectate lyase on virulence in planta by infecting Col-0 with all strains and measuring bacterial growth on day 4 postinfection at 100 and 85% humidity. There was a strong effect of both strain ($F_{3,70} = 10.55$, $P < 0.0001$) and level of humidity ($F_{1,70} = 83.97$, $P < 0.0001$) on bacterial growth, but no significant interaction between these effects ($F_{3,70} = 1.45$, $P = 0.2349$). Overall, *pel*⁻ exhibited reduced growth compared with the wild-type strain ($F_{1,70} = 8.81$, $P = 0.0041$) and *pel*⁻pME showed reduced growth relative to the complemented *pel*⁻pME*pel*⁺ ($F_{1,70} = 21.89$, $P < 0.0001$) (Fig. 6). No significant difference was found between the growth of wild-type and *pel*⁻pME*pel*⁺ strains ($F_{1,70} = 0.01$, $P = 0.9414$) or between the *pel*⁻ and *pel*⁻pME strains ($F_{1,70} = 0.25$, $P = 0.6173$). Furthermore, macerated leaves were observed following infection with the wild-type strain but not with *pel*⁻ (Fig. 7). These results demonstrate that pectate lyase elevates bacterial virulence and contributes to disease symptoms on *A. thaliana*.

Evidence for a functional TTSS in *P. viridiflava*.

Virulence in many bacterial pathogens is dependent upon a TTSS that delivers effectors to host cells using a pilus (Hueck 1998). A recent genomic survey revealed two distinct PAIs in *P. viridiflava* containing genes similar to those encoding the TTSS in *P. syringae* (Araki et al. 2006). Consistent with this genomic survey, both S-PAI and T-PAI strains are capable of pilus production, the type III secretion apparatus (Fig. 8). Pili

Fig. 3. Growth of **A**, *Pseudomonas viridiflava* and **B**, *P. syringae* pv. *tomato* (DC3000) in *Arabidopsis thaliana* Col-0 wild type, the salicylic acid-deficient *sid2-1*, and the jasmonic acid-deficient *fad3/7/8* mutant at day 4 postinfection. Infection concentration was 10⁵ CFU/ml for *P. viridiflava* and 10⁴ CFU/ml for DC3000; bars represent mean and standard error of four independent replications. Asterisks indicate a significant difference between a mutant relative to Col-0 within bacterial strains ($P < 0.05$).

initially were visualized on *P. syringae* pv. *tomato* (DC3000) grown on minimal media that is known to induce pilus production using an electron microscope (Mudgett and Staskawicz 1999; Roine et al. 1997); no pili were observed when bacteria were grown on King's B (KB) media. When grown on minimal media, pili also were visible on both T-PAI (LP23.1a) (Fig. 8A) and S-PAI (RMX23.1a) (Fig 8B) strains, whereas no pili were found on these strains when grown on KB media. To further explore the potential role of type III secretion in virulence, the alternative sigma factor HrpL was removed by marker-exchanged mutagenesis in strain LP23.1a (*hrpL*⁻). PCR was used to confirm the absence of *hrpL* in this strain, and there was a complete loss of ability to induce the hypersensitive response (HR) on tobacco in *hrpL*⁻ (Fig. 9). The *hrpL*⁻ strain exhibited reduced growth on Col-0 plants relative to LP23.1a following inoculation at 10⁵ CFU/ml ($t = 2.51$, $df = 15$, $P = 0.0126$), and there was a similar loss of virulence in *hrpL*⁻pME relative to *hrpL*⁻pME*hrpL*⁺ ($t = 4.62$, $df = 18$, $P < 0.0001$). Complementation with a plasmid-borne copy of *hrpL* completely restored virulence (Fig. 10) as well as the capacity to induce wild-type levels of the HR on tobacco (Fig. 9). The presence of a physical gene cluster encoding a TTSS (Araki et al. 2006), pilus production, and loss of virulence in *hrpL*⁻ are all consistent with a role for type III secretion in virulence.

DISCUSSION

P. viridiflava is a common pathogen of *A. thaliana* in the Midwestern United States, although little is known about mechanisms of resistance and virulence in this interaction. One important exception is a recent genomic survey in *P. viridiflava* that revealed two distinct PAIs of differing gene composition; strains of *P. viridiflava* possess either the S-PAI or the T-PAI, but none of 286 strains surveyed possess both PAIs (Araki et al. 2006). Both S-PAI and T-PAI contain a conserved *hrp/hrc* gene cluster encoding genes with high homology to those involved in the TTSS in *P. syringae*. However, these two PAIs also differ in several *avr* and effector homologs that could influence the induced defense response of *A. thaliana* and *P. viridiflava* virulence.

Despite these differences in gene composition between the S-PAI and T-PAI, expression levels of *PR-1*, *PDF1.2*, and *LOX2* provide no evidence that PAI type modifies either the SA- or the JA-mediated defense response to *P. viridiflava* infection. It is clear, on the other hand, that *A. thaliana* shows a

very different induced defense response to *P. syringae* pv. *tomato* and *P. viridiflava*: *P. syringae* pv. *tomato* primarily induced the SA-mediated defense response whereas *P. viridiflava* strongly induced the JA/ET-mediated defense response and exhibited a relatively weak induction of the SA-mediated defense response. Expression patterns differed not only on the Col-0 wild type but also in *sid2-1*. In the presence of a compromised SA signaling pathway, *PDF1.2* expression was suppressed for *P. viridiflava*. A qualitatively similar result was found following infection by *Alternaria brassicicola* on *sid2* (*eds16*) (Nawrath and Métraux 1999), indicating that the SA signaling pathway can have a positive effect on the JA/ET-mediated defense response. A very different pattern was observed following *P. syringae* infection. *PDF1.2* expression on the *sid2-1* mutant was elevated for DC3000/*avrRpt2* but unchanged for DC3000 relative to Col-0. Together, these results suggest that the influence of the SA signaling pathway on JA/ET-mediated defense can vary depending upon pathogen species and the presence of *avr* genes.

Fig. 5. Correlation between pectate lyase activity and bacterial growth on day 4 postinfection in *A. thaliana* Col-0 ecotype for seven single-component pathogenicity island (S-PAIs) (◆) and six tripartite (T)-PAI (*) strains of *Pseudomonas viridiflava*. Bacterial growth values are means from 4 to 12 independent leaves infected with 5×10^5 CFU/ml. Values for pectate lyase enzyme activity derive from the means of three independent measurements of the pitting ring (cm). The correlation between growth and pectate lyase activity was significant for S-PAI strains ($r = 0.80$, $P = 0.029$) but not for T-PAI strains.

Fig. 4. Growth of *Pseudomonas viridiflava* strains in *Arabidopsis thaliana* Col-0 wild type and *coi1-1* at day 2 postinfection, which reflected peak bacterial performance. Infection concentration was 10⁵ CFU/ml; bars represent mean and standard error of eight independent replications. Asterisks indicate a significant differences between *coi1-1* and Col-0 within bacterial strains ($P < 0.05$).

Although *P. viridiflava* primarily induced the JA/ET defense response, it also showed some *PR-1* expression indicative of SA defense induction. Many soft-rot and macerating pathogens harbor functional TTSSs involved in the induction of the HR and SA-mediated defense (Bauer et al. 1994, 1995; Lehtimäki et al. 2003; Rantakari et al. 2001; Yang et al. 2002) and are known to induce both SA- and JA/ET-mediated defense responses (Kariola et al. 2003; Thomma et al. 1998, 2001). For example, the HrpN effector from the soft-rot pathogen *Erwinia carotovora* induces both SA- and JA/ET-mediated defense responses (Kariola et al. 2003). Although a combination of SA and JA/ET defense induction may be a simple by-product of virulence effectors, such as HrpN, it also has been hypothesized that pathogens with a necrotrophic life style may adaptively induce SA to facilitate local cell death and the release of nutrients from plant cells (Govrin and Levine 2000). Thus, as a by-product of virulence or through adaptive evolution, the induction of both SA and JA/ET defense responses may be general to many soft-rot and macerating pathogens.

Assays of bacterial growth largely complement the *PR-1*, *LOX2*, and *PDF1.2* expression studies: *P. viridiflava* generally performed best on the *fad3/7/8* and *coi1* mutant deficient in the JA-mediated defense response. One *P. viridiflava* strain, RMX3.1b, partly deviated from this pattern by exhibiting equivalent growth in Col-0, *sid2-1*, and *fad3/7/8*. In contrast, *P. syringae*, particularly the DC3000/*avrRpt2* strain, performed best on the *sid2-1* mutant, and previous work has demonstrated that the growth of DC3000 and other *P. syringae* strains is restricted in *coi1* (Feys et al. 1994; Klock et al. 2001). Increased growth of *P. viridiflava* on *coi1* is consistent with other fungal and soft-rot pathogens that induce the JA/ET-mediated defense response (Norman-Setterblad et al. 2000; Thomma et al. 1998; Vijayan et al. 1998). The contrasting growth of *P. viridiflava* and *P. syringae* on *coi1* may derive from both the suppressive effects of the JA and ET defense response on *P. viridiflava* and the phytotoxin coronatine. This molecular mimic of JA is known to cause chlorosis and increase the virulence of *P. syringae* on *Arabidopsis thaliana* (Mittal and Davis 1995) but is unrecognized in *coi1* plants. Beresewill and associates (1994) failed to amplify part of the *CFL* gene (coronafacate ligase) within the coronatine biosynthetic gene cluster in *P. viridiflava*, and we also failed to amplify this region in *P. viridiflava* as well as the coronafac acid genes *CFA6* and *CFA7* using primers based on *P. syringae* pv. *tomato* DC3000 (unpublished

data). Together, the elevated growth of *P. viridiflava* on *fad3/7/8* and *coi1-1* plants and the elevated expression of *LOX2* and *PDF1.2* provide strong evidence that *P. viridiflava* both induces and is suppressed by the JA/ET-mediated defense response.

Although there are likely to be many differences between *P. viridiflava* and its close relative *P. syringae* that contribute to their divergent induced response on *A. thaliana*, the macerating disease symptoms present on *P. viridiflava*-infected plants, but not on *P. syringae* plants, prompted further investigation into its potential causes. There was a significantly positive correlation between pectate lyase activity and virulence among seven *P. viridiflava* strains carrying the S-PAI, which is estimated to reside in 90% of wild *P. viridiflava* strains (Araki et al. 2006). Following these results, a pectate lyase mutant, *pel*⁻, was generated within an S-PAI strain that exhibited reduced virulence relative to the wild-type strain. The confirmation of pectate lyase as a significant virulence determinant necessary for optimal growth of this bacterium in its host is consistent with work in other systems (Collmer and Keen 1986; Liao et al. 1988; Lojkowska et al. 1995; López-Solanilla et al. 2001; Toth et al. 2003). However, unlike other soft-rotting plant pathogens that have multiple pectate lyase genes (Kelemu and Collmer 1993), the loss of macerating symptoms with the *pel*⁻ strain suggests the presence of a single active pectate lyase gene.

Despite the importance of pectate lyase to S-PAI strain virulence, pectate lyase activity in T-PAI strains was not correlated with virulence. T-PAI strains also showed reduced pectate lyase activity relative to S-PAI strains even though the pectate lyase gene does not reside within either PAI. We sequenced a pectate lyase gene showing 82 to 84% amino acid identity to other *P. viridiflava* strains (Liao et al. 1996) but found only a single nonsynonymous change that distinguishes S- from T-PAI strains. Consequently, the basis for the difference in pectate lyase activity between S- and T-PAI strains is currently unknown. One possibility is that S- and T-PAI strains differ in cis- or trans-acting elements affecting pectate lyase expression. It is worth noting that, although S-PAI strains exhibit greater pectate lyase activity, T-PAI strains induce a more rapid and extensive HR on tobacco (Araki et al. 2006). Again, there is no obvious explanation currently available for this difference: both the S-PAI and T-PAI contain the conserved *hrp/hrc* gene cluster involved in type III secretion, and both produce a visible type III secretion apparatus. Furthermore, initial work in the T-PAI strain LP23.1a shows that removal of HrpL, a key

Fig. 6. Growth of *Pseudomonas viridiflava* single-component pathogenicity island (S-PAI) strain RMX23.1a, the pectate lyase mutant (*pel*⁻), plasmid control (*pel*⁻pME), and the pectate lyase rescued strain (*pel*⁻pME*pel*⁺) in *Arabidopsis thaliana* Col-0 wild type at day 4 postinfection. All strains were generated in the RMX23.1a background. Infection concentration was 10⁵ CFU/ml; bars represent one standard error of the mean. Different letters above bars represent significant differences between strains (*P* < 0.05).

regulatory element of the *hrp/hrc* cluster (Fouts et al. 2002; Xiao et al. 1994), reduces the growth of LP23.1a. These results suggest a potential divergence in virulence strategy between S-PAI and T-PAI strains. However, further work is needed to determine the role of pectate lyase activity and HrpL in T-PAI strain and S-PAI strain virulence, respectively, and, more generally, to identify additional virulence factors, including effectors or sensitivity to antimicrobial peptides (*sap*) factors that contribute to virulence in other soft-rotting pathogens (Bauer et al. 1994, 1995; López-Solanilla et al. 2001; Yang et al. 2002).

Fig. 7. Disease symptoms caused by *Pseudomonas viridiflava* at day 2 postinfection with 10^5 CFU/ml; all strains were generated in the RMX23.1a background. **A**, Col-0 plants infected with strain RMX23.1a or the *pel*⁻ mutant lacking pectate lyase, **B**, Wu-0 plants infected with strain RMX23.1a, and **C**, Wu-0 plants infected with *pel*⁻ complemented with pectate lyase on pME6010 (*pel*⁻pME_{pel}⁺) or the uncomplemented *pel*⁻ strain.

In conclusion, *P. viridiflava* exhibits strong induction of the JA/ET-mediated defense response in its host *A. thaliana* and exhibits enhanced growth on plants that are impaired in JA production or perception, indicating that the JA/ET signaling pathway is likely to contribute to the genetic basis for variation in resistance and virulence in this interaction. Furthermore, we show that pectate lyase is an important virulence factor. Additional work is needed to understand how PAI type influences *P. viridiflava* virulence through pectate lyase activity or other potential virulence mechanisms. Finally, the contrasting pattern of defense induction and growth on mutants deficient in these defense pathways for the closely related *P. viridiflava* and *P. syringae* indicates that the genes involved in plant–bacteria coevolution are likely to be as diverse as the pathogens themselves.

MATERIALS AND METHODS

Plant material.

We used the *A. thaliana* mutant *sid2-1* that is defective in the isochorismate synthase enzyme required for SA synthesis and, thus, not able to exhibit SAR (Nawrath and Métraux 1999; Wildermuth et al. 2001). Mutant *sid2-1* was provided by J. Greenberg (University of Chicago). The *fad3-2*, *fad7-2*, *fad8* triple mutant (*fad3/7/8*) is incapable of producing linolenic acid and is deficient in JA accumulation (McConn and Browse 1996). It was provided by D. Cipollini (Wright State University, Dayton, OH, U.S.A.). Seed for *coi1-1*, insensitive to coronatine and methyl jasmonate (Feys et al. 1994, Xie et al. 1998), were provided by A. Bahrami (Harvard University) and plants were selected on MS basal media supplemented with methyl jasmonate (Sigma-Aldrich, St. Louis) as documented (Feys et al. 1994). Plants were infected as described earlier (Jakob et al. 2002) after growing for 4 weeks under short day conditions at 20°C.

Bacterial strains and infection experiments.

P. viridiflava and *P. syringae* strains used were described elsewhere (Goss et al. 2005; Jakob et al. 2002; Korves and Bergelson 2003). Preparation of bacterial infection solution, infection, and measurement of bacterial titer in the plant leaf has been described in Jakob and associates (2002). Briefly, two leaves per plant were infected with a blunt-end syringe and one leaf per plant was harvested at days 1, 2, and 4 for quantification of bacterial titer. Mock-infected plants were harvested at the same time as controls and no bacteria were found in mock-inoculated plants. *P. viridiflava* strains KNOX3.4a, ME3.1a, RMX23.1a, RMX3.1b, BOG1.1a, KY5.1a, SP1.1a (all S-PAI) and LP23.1a, PNA3.3a, LU5.1a, SP3.1a, ME210.1b, and PT220.1a, (all T-PAI) were used for S- and T-PAI virulence assays on Col-0. The experiment with Col-0, *sid2-1*, and *fad3/7/8* was carried out two times with four replications per treatment, whereas the *coi1-1* experiment was performed once with eight replications. A three-way analysis of variance (ANOVA) was used to determine the effect of PAI type and plant ecotype on bacterial growth, where ecotype, day, and strain (nested within PAI-type) were considered fixed effects. Planned contrast tests were used to compare treatments and strains.

Virulence assays of the *pel*⁻ and associated strains were performed twice. In the first of these experiments, plants were grown at high humidity (100%) whereas, in the second experiment, reported here, plants were grown at both 100 humidity and 85% humidity. The latter experiment was initiated with 12 replicate plants per strain per environment, although heavy disease symptoms on leaves in the 100% humidity environment reduced sample size. Leaves were harvested at the day of infection and at day 4 postinfection (experiment 1) or only at

day 4 postinfection (experiment 2). These data were analyzed with a two-way ANOVA, where strain and level of humidity were considered fixed effects and planned contrast tests were used to compare individual strains. Growth of *hrpL*⁻ and the associated strains was assayed twice with qualitatively similar results. In both experiments, plants were grown at 85% humidity. In the second experiment, reported here, there were 15 to 19 replicates per strain, and growth again was quantified at day 4 postinfection.

Electron microscopy.

To prepare for electron microscopy, bacterial strains were incubated at 28°C on KB plates for 48 h. A single colony then was incubated overnight in liquid KB. Next, 1.5 ml of bacterial solution was spun for 5 min at 3,000 rpm, the supernatant was discarded, and the pellet was resuspended in 1 ml of minimal media (MM). This solution (500 µl) was added to 5 ml of MM (Mudgett and Staskawicz 1999) and incubated overnight at room temperature. The next morning, 1.5 ml of this solution was centrifuged as described above and the pellet was resuspended in 100 µl of MM. After spreading 50 µl onto an MM plate, the plate was incubated for 18 h at room temperature. To apply bacteria to a carbon grid for viewing, a single drop of water was placed upon a colony to suspend bacteria, and carbon

grids were placed on the surface of the water. Carbon grids were washed three times with sterile water and stained with 1% uranyl acetate. Samples were viewed with a Philips CM120 Transmission Electron Microscope (TECNAI F30ST).

Pectate lyase sequencing.

Bacterial DNA was extracted following standard protocols (Ausubel et al. 1996). For amplification of the pectate lyase gene, we used the following primers based on the GenBank *P. viridiflava* pectate lyase gene (L38901) and partly positioned in the conserved region of the gene (Liao et al. 1996): Pv212f: 5'-GCAACCACCGCTGGGCCACCCAGAAC-3' and Pv1240r: 5'-CTTGCCGGCACCGGCCTTGGC-3'. To obtain the 5' and 3' ends of the putative pectate lyase gene, we used a fosmid library of strain RMX23.1a created with CopyControl Fosmid Library Production Kit (Epicentre, Madison, WI, U.S.A.) and screened for positive clones harboring the pectate lyase gene by PCR with the following primers: Pvcont-182r: 5'-CGCCTTGCTTCGCTGACATCG-3' and Pvcont-749f: 5'-CAGCACCAGCCACAAAATCTA-3'. PCR products and fosmid clones were sequenced using a CEQ8000 capillary sequencer (Beckman Coulter Inc., Fullerton, CA, U.S.A.). Polymorphism analysis was performed with DnaSP v. 4.00 (Rozas et al. 2003).

Fig. 8. Pilus induction on minimal media plates visualized by electron microscopy. Arrow indicates pili (larger structures are flagella). **A**, *Pseudomonas viridiflava* LP23.1a harboring the tripartite pathogenicity island. **B**, *P. viridiflava* RMX23.1a harboring the single-component pathogenicity island.

Pitting test.

Plates for the pitting test were prepared following the protocol of Hildebrand's media for pectate degradation using polygalacturonic acid (Sigma-Aldrich) (Schaad et al. 2001). pH values were adjusted after autoclaving to acidic (pH 4.9 to 5.1), neutral (pH 6.9 to 7.1), and basic (pH 8.3 to 8.5). The following bacterial strains were incubated on KB plates at 28°C and a single colony was further incubated in liquid KB overnight: S-PAI: KNOX3.4a, ME3.1a, RMX23.1a, BOG1.1a, BOG4.2a, BOR1.3d, KY5.1a, KY7.1d, SP1.1a, and SP12.1a; and T-PAI: LP23.1a, PNA3.3a, LU5.1a, LU9.1e, LU13.1a, LU18.1a, LU19.1a, SP3.1a, ME210.1b, and PT220.1a. The next morning, 1.5 ml of bacterial solution was centrifuged for 5 min at 3,000 rpm, the supernatant was discarded, and the pellet was resuspended in 1 ml of 10 mM MgSO₄ buffer. The bacterial solution was adjusted to OD₆₀₀ = 0.1 and 10 µl of this

Fig. 9. Dependence of hypersensitive response in tobacco on HrpL. Infection concentration of all strains was 10⁸ CFU/ml. Symbols: + indicates section of leaf infiltrated with strain possessing HrpL and – indicates section of leaf infiltrated with strain lacking HrpL. All strains were generated in the LP23.1a background. The leaf on the left is infected with *hrpL*⁻ complemented with *hrpL* on pME6010 (+) and *hrpL*⁻ carrying empty pME6010 (-). The leaf on the right is infected with wild-type strain LP23.1a (+) and the *hrpL*⁻ knockout (-).

solution was applied to the pectate degradation plates. Plates were incubated at 28°C for 4 days and the diameter of the pitting ring was measured. This experiment was repeated three times.

Pectate lyase enzyme assay.

To assay pectate lyase activity, bacterial strains were incubated in liquid minimal yeast media (MYM) overnight (Liao et al. 1988) and, the following morning, the bacterial solution was diluted 1:10 and bacteria were grown to an approximate density of 0.9 CFU/ml. The exact OD₆₀₀ was measured for each sample. Bacterial solution (1 ml) was centrifuged at 11,000 rpm for 2 min, and the pellet was washed once in 50 mM Tris-HCl (pH 8.0), centrifuged again, and then resuspended in 1 ml of the same buffer. Subsequently, cells were disrupted by sonication and cell debris removed by centrifugation for 30 min at 11,000 rpm. The clear supernatant was used to determine intracellular pectate lyase activity by adding 20 to 980 µl of reaction buffer (0.2% polygalacturonic acid [Sigma-Aldrich], 100 mM Tris-HCl [pH 8.5], and 1 mM CaCl₂) and kept at 25°C. Immediately after mixing and 20 min later, absorbance at 232 nm was measured with a Hitachi U-1100 spectrophotometer. Enzyme activity was defined as the amount of enzyme that produced a change in absorbance of 0.001 at 232 nm/min (Matsumoto et al. 2003), as determined by the formation of unsaturated uronide (Liao et al. 1988). This experiment was repeated twice. Statistical analysis of pectate lyase activity was conducted with a one-way ANOVA in which *P. viridiflava* strain nested within PAI type was treated as a random effect. Consequently, the *F* value for PAI type was calculated using the MS value of strain nested within PAI type as the denominator.

Pectate lyase and *hrpL* mutant construction.

The pectate lyase gene from strain RMX23.1a, including the up and downstream region, had been sequenced from a fosmid library (see above) and was knocked out by marker-exchange mutagenesis (Ried and Collmer 1987). A 672-bp PCR product corresponding to the region immediately upstream of the pectate lyase gene was amplified using high-fidelity polymerase *Pfu* (Stratagene, La Jolla, CA, U.S.A.) with the forward primer, containing an *Apa*I restriction site, TTTTGGGCCCTG AAAGTGTATCTGCGAATCC; and the reverse primer, containing an *Eco*RI restriction site, TTTTGAATTCACCATTTA ATCTGATCCTTGA. Similarly, a 597-bp PCR product immediately downstream of pectate lyase gene was amplified using

Fig. 10. Growth of *Pseudomonas viridiflava* LP23.1a, the HrpL mutant (*hrpL*⁻), the plasmid control (*hrpL*⁻pME), and the *hrpL* rescued strain (*hrpL*⁻pME*hrpL*⁺) in Col-0 on day 4 postinfection. All strains were generated in the LP23.1a background. Infection concentration was 10⁵ CFU/ml; bars represent the mean and standard error of 15 to 19 independent replications. Different letters above bars represent significant differences between strains (*P* < 0.05).

the forward primer with an *EcoRI* restriction site, TTTTGAA TTCCTGACGTTTGACCTGTTGA; and the reverse primer, containing an *SpeI* restriction site, TTTTACTAGTCATTCGT GGCTGGGGTTGG. Both PCR products were ligated into vector pBAV209 digested with *Apal* and *SpeI* (pBAV209 was provided by B. Vinatzer, Virginia Polytechnic Institute and State University, Blacksburg, VA, U.S.A.), electroporated into *Escherichia coli* DH5 α and screened for kanamycin resistance (50 μ g/ml). Positive clones were used for chromosomal gene exchange by triparental mating with the *P. viridiflava* RMX23.1a wild-type strain and the RK600 helper plasmid and selected on plates containing kanamycin and nitrofurantoin (100 μ g/ml). Clones were tested for the insert by PCR and evaluated for sucrose sensitivity on KB plates containing 10% sucrose. Strains were grown without selection for a second crossover event and single colonies then were selected for kanamycin susceptibility. Potential pectate lyase deletion strains grew on KB but not on KB with kanamycin and were further tested by PCR and a rotting test on potato tubers to confirm pectate lyase identity. One confirmed pectate lyase deletion strain, *pel*⁻, was complemented with plasmid pME6010 (Heeb et al. 2000) expressing the pectate lyase gene driven by its own promoter.

Marker-exchange mutagenesis also was used to knock out *hrpL* in *P. viridiflava* strain LP23.1a (referred to as *hrpL*⁻) using the following primer pairs: forward primer with the *Apal* site, TTTTGGGCCCCATCGCTTTTCGCCGCATCA, and reverse primer with the *EcoRI* site, TTTTGAATTCCAGCAC ATCGACCCGTTG; and forward primer with the *EcoRI* site, TTTTGAATTC CTTGGGAACATGGGGGCTA, and reverse primer with the *SpeI* site, TTTTACTAGTATGCGTGCCAAA CTTGCGT. The *hrpL*⁻ mutant exhibited a complete loss of the HR on tobacco, and the absence of the *hrpL* gene was verified using a pair of flanking primers (HrpLf and HrpLr) (Jakob et al. 2002) and an internal set of primers (HrpL202F: GCAAGC AAGCCGCAAACCTG and HrpL439R: CCTGATAGTTGCC GTCCAT). The *hrpL*⁻ mutant was complemented with *hrpL* on the plasmid pME6010 (Heeb et al. 2000), which restored its ability to induce the HR on tobacco at wild-type levels.

Quantitative real-time PCR.

Plants used in this experiment were completely randomized within flats. Following bacterial inoculation, three infected plants per treatment were harvested immediately (day 0) and at days 1, 2, and 4 postinfection. These leaves were transferred into liquid nitrogen, and RNA was extracted using RNeasy mini kit (Qiagen, Valencia, CA). On-column DNase digestions was performed to remove DNA. For reverse transcription, oligo (dT)₁₂₋₁₈ primers, 10 mM dNTP mix, and water were added to 5 μ g of RNA following the manufacturer's protocol (Invitrogen, Carlsbad, CA, U.S.A.). The 20- μ l reaction mixture was heated to 65°C for 2 min, and then a mixture of 4 μ l of 5 $\times$ first-strand buffer and 2 μ l of 0.1 M dithiothreitol was mixed gently into the reaction and incubated at 37°C for 2 min. Next, 1 μ l of M-MLV reverse transcriptase was added and the mixture was incubated at 25°C for 10 min, followed by incubation at 37°C for 50 min. The reaction ultimately was inactivated by incubation at 70°C for 15 min. cDNA was diluted in 100 μ l of water and used as template for the following quantitative real-time PCR. Primers for *PR-1* and *EF1 α* were created with the ABI Primer Express Software v2.0 as follows: *EF1 α* -forward, 5'-CATCATCATGAA CCACCCTGG-3' and *EF1 α* -reverse, 5'-GTAAGAGGTGTGGCAATCCA-3'; and *PR-1*-forward, 5'-TCGGAGCTACGCAGAACAACT-3' and *PR-1*-reverse, 5'-TCTCGCTAACCCACATGTTTCAC-3'. Primers for *PDF1.2* and *LOX2* are described by Schenk and associates (2003) and Kishimoto and associates (2005), respectively. Hot-

start polymerase *Takara Taq* HS (Takara Bio Inc., Shiga, Japan) was used in a mix with SYBR green (Stratagene). cDNA template (1.5 μ l) was used in a 20 μ l of reaction mix containing 0.2 μ M primer. The housekeeping gene *EF1 α* was used as internal standard to normalize differences in template quantity. Quantitative PCR was carried out using an ABI 7900 sequence detector with PCR cycling conditions as follows: 3 min at 95°C, followed by 40 cycles at 95°C for 30 s, 59°C for 40 s, and 72°C for 1 min 15 s. All reactions were checked for their dissociation curves. Fold expression differences were calculated as 2^{-(Δ t gene of interest) - (Δ t housekeeping gene)}. A dilution series of the housekeeping gene was run to make sure that each amplification cycle difference corresponded to a twofold change. Two PCR replicates from two to three independent biological replications were run for each ecotype by bacterial strain combination. A three-way ANOVA was used for statistical analysis of gene expression where ecotype, day, and strain (nested within PAI type) were considered fixed effects.

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