The *Pseudomonas syringae* *avrRpt2* Gene Contributes to Virulence on Tomato

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In order to cause disease on plants, gram-negative phytopathogenic bacteria introduce numerous virulence factors into the host cell in order to render host tissue more hospitable for pathogen proliferation. The mode of action of such bacterial virulence factors and their interaction with host defense pathways remain poorly understood. *avrRpt2*, a gene from *Pseudomonas syringae* pv. *tomato* JL1065, has been shown to promote the virulence of heterologous *P. syringae* strains on *Arabidopsis thaliana*. However, the contribution of *avrRpt2* to the virulence of JL1065 has not been examined previously. We show that a mutant derivative of JL1065 that carries a disruption in *avrRpt2* is impaired in its ability to cause disease on tomato (*Lycopersicon esculentum*), indicating that *avrRpt2* also acts as a virulence gene in its native strain on a natural host. The virulence activity of *avrRpt2* was detectable on tomato lines that are defective in either ethylene perception or the accumulation of salicylic acid, but could not be detected on a tomato mutant insensitive to jasmonic acid. The enhanced virulence conferred by the expression of *avrRpt2* in JL1065 was not associated with the suppression of several defense-related genes induced during the infection of tomato.

Additional keywords: effector protein, pathogenesis, type III secretion.

Although the leaf apoplast constitutes a potentially abundant and easily accessible source of nutrients for bacteria, it is colonized by only a select number of pathogenic bacterial species (Alfano and Collmer 1996). This implies that the apoplastic space is inhospitable to the majority of bacterial species and is exploited exclusively by those capable of changing it into a more favorable growth environment. Apoplastic bacterial pathogens must have evolved not only the ability to obtain nutrients and water from the plant but also to overcome host defense mechanisms. To accomplish this, phytopathogenic bacteria rely on specialized secretion pathways to deliver virulence factors into the apoplast and host cells (Alfano and Collmer 1996).

A well-studied apoplastic colonizer is *Pseudomonas syringae*, the causal agent of foliar spots and blights on a wide range of hosts (Schroth et al. 1981). As for many other gram-negative phytopathogens, pathogenicity of *P. syringae* is dependent on a specialized secretion apparatus called the type III

secretion system (TTSS) that functions to deliver bacterial proteins, known as effectors, into the host cell (Buttner and Bonas 2002; Jin et al. 2003). Mutations that disable the secretory function of the TTSS abrogate the ability of *P. syringae* to cause disease, indicating that the activities of TTSS effectors are essential for the pathogenicity of the bacteria (Lindgren et al. 1986). Although numerous TTSS effectors recently have been identified from several different pathogenic *P. syringae* strains (Collmer et al. 2002; Greenberg and Vinatzer 2003), only a few of these effectors have been shown to contribute to pathogen virulence on susceptible hosts and their modes of action remain poorly understood.

In response to microbial attack, plants activate an extensive array of defenses. Studies using the genetically tractable model host *Arabidopsis thaliana* have greatly advanced our understanding of these responses by uncovering numerous factors involved in their regulation (Glazebrook 1999; Nimchuk et al. 2003). Several plant hormone signaling pathways contribute to the outcome of host–pathogen interactions. The hormone salicylic acid (SA) plays a central role in host defense activation in response to *P. syringae*, such that *Arabidopsis* lines that have reduced levels of SA are more susceptible to infection by the pathogen (Delaney et al. 1994; Glazebrook et al. 1996; Nawrath and Metraux 1999). Jasmonic acid (JA) and ethylene are two other major plant hormones implicated in regulating responses to *P. syringae*; however, their roles are less well understood than that of SA (Bent et al. 1992; Kunkel and Brooks 2002; Pieterse and van Loon 1999).

Although not as amenable to genetic analysis as *Arabidopsis*, tomato (*Lycopersicon esculentum*) is another host of *P. syringae* that commonly is used to study plant–pathogen interactions. Analyses of lines impaired in phytohormone signaling have revealed the involvement of SA, ethylene, and JA in regulating susceptibility to *P. syringae* in tomato. Loss of responsiveness to JA in the *JA insensitive 1* (*jai1-1*) tomato mutant prevents *P. syringae* pv. *tomato* DC3000 from growing to high levels in planta and causing disease (Li et al. 2001; Zhao et al. 2003). On the other hand, reduced ethylene sensitivity due to mutation of the ethylene receptor gene *Never ripe* (*Nr*) results in a decrease in the severity of disease symptoms elicited by DC3000 but does not detectably affect bacterial growth (Lund et al. 1998). In tomato, expression of the *P. putida* gene encoding salicylate hydrolase (*NahG*), which catalyzes the breakdown of SA, impairs the resistance to DC3000 that results from the overexpression of the disease resistance gene *Prf* (Oldroyd and Staskawicz 1998). However, the contribution of SA to host defense activation during a susceptible interaction with *P. syringae* pv. *tomato* remains unclear (Li et al. 2002).

Numerous defense-related host genes are strongly induced early during resistant interactions with *P. syringae* and, therefore, their expression often is used to assess the activation of

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defenses (Tao et al. 2003; Zhou et al. 1997). Some of these genes—for example, the pathogenesis-related (*PR*) genes—encode proteins with demonstrated antimicrobial activity and, thus, may play a direct role in restricting pathogen virulence (Van Loon and Van Strien 1999). Given that plants are able to deploy extensive antimicrobial defense pathways, it would be expected that bacterial pathogens have evolved mechanisms to overcome these defenses. Accordingly, several *P. syringae* TTSS effectors recently have been shown to suppress the induction of some *PR* genes induced during the infection of *Arabidopsis* and tobacco (Bretz et al. 2003; Chen et al. 2004; Jamir et al. 2004). Although this activity may suggest that these TTSS effectors contribute to virulence by suppressing host defense responses, direct evidence for such a mechanism remains limited.

avrRpt2, a gene from *P. syringae* pv. *tomato*, encodes a TTSS effector with demonstrated virulence activity. Thus, *P. syringae* strains carrying *avrRpt2* cause more severe disease on susceptible lines of *Arabidopsis* (Chen et al. 2000, 2004; Guttman and Greenberg 2001). *AvrRpt2* expressed in transgenic plants also promotes the virulence of *P. syringae*, indicating that the protein exerts its virulence activity from inside the plant cell (Chen et al. 2000). The mechanisms underlying *avrRpt2* virulence activity on susceptible *Arabidopsis* plants are not well understood. *avrRpt2* virulence activity appears to be largely independent of SA accumulation because it is detectable in *Arabidopsis* plants that fail to accumulate SA (Chen et al. 2004). This activity also is retained in the JA-insensitive *Arabidopsis* mutant *coronatine-insensitive1* (*coi1*), indicating that functional JA signaling is not essential for *avrRpt2* virulence activity in *Arabidopsis* (Chen et al. 2000). The ability of bacterially delivered *AvrRpt2* to suppress the induction of several *PR* genes over the course of infection raises the possibility that *AvrRpt2* exerts its virulence activity by suppressing host defense responses in *Arabidopsis* (Chen et al. 2004). The recent discovery that *AvrRpt2* appears to encode a cysteine protease that triggers the disappearance of the *Arabidopsis* RIN4 protein, a positive regulator of plant defenses, has provided additional insight into the mode of action of *AvrRpt2* within the plant cell (Axtell et al. 2003; Axtell and Staskawicz 2003; Mackey et al. 2003). However, *AvrRpt2* virulence activity is largely independent of RIN4 disappearance (Belkhadir et al. 2004; Lim and Kunkel 2004b), and it is presently unclear whether the predicted cysteine protease activity of *AvrRpt2* plays a role in its virulence function in *Arabidopsis* (Lim and Kunkel 2004b).

To date, most analyses of the virulence activity of *AvrRpt2* have been carried out using *P. syringae* pv. *tomato* DC3000 as the infectious agent and *Arabidopsis* as the host. However, because DC3000 does not carry an endogenous copy of *avrRpt2*, the gene must be supplied in trans by heterologous expression from a freely replicating plasmid. *avrRpt2* originally was isolated from *P. syringae* pv. *tomato* JL1065 (Whalen et al. 1991). Unlike DC3000, which is highly virulent on *Arabidopsis*, JL1065 is pathogenic on tomato but not on *Arabidopsis*. Thus, despite being highly convenient and powerful, the *Arabidopsis*-DC3000 pathosystem does not allow us to study *avrRpt2* virulence in an evolutionary relevant context and may be prone to potential artifacts arising from the expression of a foreign gene in a heterologous system. Therefore, to extend our understanding of the function of *avrRpt2*, we examined its contribution to the virulence of its source strain JL1065. Here, we show that a mutant derivative of JL1065 deficient in *avrRpt2* function is severely impaired in its ability to cause disease on tomato. This indicates that *avrRpt2* also acts as a virulence gene in its native strain by enhancing the ability of JL1065 to cause disease on its natural host. In an attempt to understand the mechanism underlying the virulence activity of *avrRpt2* in

this context, we analyzed the interaction between *avrRpt2* virulence activity and host pathways implicated in the regulation of disease susceptibility in tomato. We present evidence suggesting that *avrRpt2* virulence activity in tomato is largely independent of the ethylene and SA response pathways. We also show that the enhanced virulence conferred by the native expression of *avrRpt2* in JL1065 is not associated with the suppression of several defense-related genes induced during the infection of tomato.

RESULTS

Disruption of *avrRpt2* reduces the virulence of *P. syringae* pv. *tomato* JL1065 on tomato.

We have shown previously that *avrRpt2* enhances the virulence of *P. syringae* pv. *tomato* DC3000 on *Arabidopsis* (Chen et al. 2000). Given that *avrRpt2* originally was isolated from *P. syringae* pv. *tomato* JL1065, a natural pathogen of tomato (Dong et al. 1991; Whalen et al. 1991), we sought to determine whether *avrRpt2* also acts as a virulence factor for its native strain. Therefore, we generated a mutant derivative of JL1065 in which the endogenous chromosomally encoded copy of *avrRpt2* was replaced with a version disrupted by an omega cassette (discussed below). We then tested whether the *avrRpt2* mutant strain, named ML100, was altered in its ability to cause disease on tomato when compared with JL1065. Tomato plants infected by vacuum infiltration with JL1065 exhibited severe wilting of leaflets, followed by the striking abscission of entire leaves by 5 to 6 days postinoculation (dpi) (Fig. 1A; Table 1). In contrast, tomato plants infected with ML100 exhibited only mild leaflet wilting, followed by occasional and delayed abscission of their lowermost leaves (Fig. 1A; Table 1). Leaflets infected with JL1065 developed numerous individual, water-soaked lesions that coalesced to form extensive confluent necrotic areas, whereas leaflets infected with ML100 developed fewer lesions and necrotic areas of smaller size (Figs. 1B and 2; Table 1).

To quantify the difference in disease severity produced by the two strains, we used electrolyte leakage as a measure of disease-associated cell injury or death (Lund et al. 1998; Rate and Greenberg 2001). Consistent with the reduction in the severity of symptoms elicited by ML100, significantly lower levels of electrolyte leakage were observed from ML100-infected plants than from JL1065-infected tomato plants (Fig. 3A; Table 1). To ensure that the reduction in virulence of ML100 was due specifically to the disruption of *avrRpt2*, we assayed the virulence of ML100 carrying a plasmid-borne copy of the wild-type *avrRpt2* gene (ML100(*avrRpt2*)). When vacuum infiltrated into tomato, this complemented strain produced disease symptoms that were as severe as those elicited by JL1065 (Fig. 1). Moreover, similarly high levels of electrolyte leakage were measured from plants infected with ML100(*avrRpt2*) and JL1065 (data not shown). These results demonstrate that *avrRpt2* acts as a virulence factor in JL1065 by enhancing the ability of this strain to cause disease on tomato.

We then asked whether the reduction in disease symptom severity resulting from loss of *avrRpt2* is associated with a reduction in the ability of ML100 to grow in planta. JL1065 multiplied by six to seven orders of magnitude on susceptible tomato plants by 6 dpi (Fig. 3B; Table 1). By this stage, ML100 usually grew to levels approximately 10-fold lower than JL1065 (Fig. 3B; Table 1). The difference between the growth of the wild-type and mutant strains was more pronounced when we infected older tomato plants or when plants were grown in a greenhouse rather than in growth chambers prior to infection (data not shown). Thus, although it is not essential for JL1065 to multiply to high levels on tomato,

avrRpt2 enhances the levels of in planta growth attained by its native strain. The reduction in the virulence of JL1065 resulting from the loss of *avrRpt2* was observed consistently on four different tomato cultivars, including Rio Grande, Pearson, Moneymaker, and Castlemart (Figs. 1 and 2; Table 1).

Interaction between *avrRpt2* virulence activity and the ethylene, SA, and JA response pathways.

To initiate an analysis of the mode of action of *avrRpt2* virulence in tomato, we examined the interaction between *avrRpt2* and the ethylene, SA, and JA response pathways. Because these pathways had been examined previously for their role in regulating the response of tomato only to strain DC3000, we first wanted to determine whether they also are involved in regulating host susceptibility to strain JL1065. Therefore, we compared the virulence of JL1065 between tomato mutant lines defective in either the perception or accumulation of ethylene, SA, or JA and the corresponding wild-type progenitor lines. In parallel with these experiments, we infected the tomato mutants and their wild-type progenitors with the *avrRpt2* mutant ML100 to test the impact of the defects in hormone signaling on the virulence activity of *avrRpt2*.

Ethylene-insensitive *Nr* tomato mutants were less susceptible than the wild-type progenitor Pearson to JL1065 infection. Leaflets of *Nr* plants underwent less severe necrosis than those of Pearson plants (Fig. 2A; Table 1) and leaves of *Nr* plants did not undergo abscission following infiltration with JL1065 (Table 1). Although JL1065 caused milder disease on *Nr* than on Pearson plants, it multiplied to similar levels on both tomato lines (Table 1), indicating that ethylene perception is more important for disease symptom development induced by JL1065 than for the growth of the pathogen, as has been observed with strain DC3000 (Lund et al. 1998). The *avrRpt2* mutant strain ML100 induced milder disease symptoms than JL1065 on wild-type

Pearson plants, as shown by the reduction in the extent of necrosis (Figs. 1B and 2A) and electrolyte leakage from ML100-infected leaflets at 6 dpi (Table 1). ML100 also induced milder disease than JL1065 on *Nr* plants. This was evident from the reduction in the extent of necrosis on leaflets of *Nr* plants infected with ML100 when scored late during the course of infection (Fig. 2A). We could not consistently detect a significant difference in electrolyte leakage measurements between *Nr* plants infected with JL1065 and with ML100 at 6 dpi because *Nr* plants developed only mild necrosis in response to JL1065. Consistent with *avrRpt2* virulence being maintained in *Nr* plants, ML100 achieved 5- to 10-fold lower levels of growth than JL1065 on *Nr* plants at 6 dpi (Table 1). Thus, the virulence ac-

Fig. 1. Loss of *avrRpt2* results in a reduction in the severity of disease symptoms produced by *Pseudomonas syringae* pv. *tomato* JL1065 on tomato. **A**, Whole-plant symptoms at 6 days postinoculation. Tomato plants (cv. Rio Grande 76S) were vacuum infiltrated with JL1065 (left), the *avrRpt2* mutant ML100 (center), and the mutant complemented with a plasmid-borne copy of *avrRpt2*, ML100(*avrRpt2*) (right) at 1×10^4 CFU/ml. **B**, Underside of individual tomato leaflets (cv. Pearson) 5 days after vacuum infiltration with JL1065 (left), ML100 (center), and ML100(*avrRpt2*) (right) at 1×10^4 CFU/ml. Experiments were repeated at least three times with similar results.

Fig. 2. Disease symptom production by *Pseudomonas syringae* pv. *tomato* JL1065 and the *avrRpt2* mutant ML100 on wild-type tomato and mutant lines defective in the accumulation or perception of ethylene, salicylic acid (SA), or jasmonic acid (JA). **A**, Representative leaflets from wild-type and *Nr* Pearson plants at 11 days postinoculation (dpi). **B**, Representative leaflets from transgenic plants expressing NahG and wild-type Moneymaker plants at 6 dpi. The apparent increase in disease symptom severity shown in the JL1065-infected NahG plants was not reproducibly observed in several other independent experiments. **C**, Representative leaflets from wild-type and *jai1-1* Castlemart plants at 6 dpi. Plants were vacuum infiltrated with either JL1065 or ML100 at 1×10^4 CFU/ml. Experiments were repeated three times with similar results.

tivity of *avrRpt2* was clearly detectable on a tomato line that exhibits a severe reduction in ethylene sensitivity. Collectively, these results indicate that, although wild-type ethylene sensitivity is required for the full virulence of JL1065 on tomato, it is largely dispensable for the virulence activity of *avrRpt2*.

To test the involvement of SA in both defense against JL1065 infection and the virulence activity of *avrRpt2*, we infected a transgenic tomato line expressing NahG and its wild-type progenitor Moneymaker with either JL1065 or ML100. The ability of JL1065 to grow and to cause disease was not reproducibly different on Moneymaker and NahG plants (Fig. 2B; Table 1). As observed on cvs. Pearson and Rio Grande, ML100 grew to lower levels and produced milder disease than JL1065 on Moneymaker plants. A similar difference in the virulence of the two strains was observed on NahG plants (Fig. 2B; Table 1). These results suggest that SA does not play a significant role in defense against JL1065 in tomato plants and that *avrRpt2* works either independently of SA signaling or downstream of SA accumulation to exert its virulence activity.

To examine the interaction between *avrRpt2* virulence activity and JA-dependent signaling, we infected the JA-insensitive tomato mutant *jail-1* and its wild-type progenitor Castlemart with JL1065 or ML100. JL1065 multiplied to high levels in Castlemart plants by 6 dpi but its growth was reduced by two to three orders of magnitude in the *jail-1* mutant (Table 1). Furthermore, this severe restriction in growth was associated with an absence of disease development on infected *jail-1* plants (Fig. 2C; Table 1). Thus, as has been observed with DC3000 (Zhao et al. 2003), host responsiveness to JA is essential for the ability of JL1065 to multiply and cause disease on tomato. By 6 dpi, the *avrRpt2* mutant ML100 grew to lower levels and caused milder disease symptoms than JL1065 on Castlemart plants, indicating that the virulence activity of *avrRpt2* can be detected in this tomato cultivar (Fig. 2C; Table 1). Although ML100 often grew to lower levels than JL1065 on *jail-1* plants by 6 dpi, the difference in the growth levels achieved by these two strains was not statistically significant. We were unable to assess the effect of JA insensitivity on the ability of *avrRpt2* to promote disease

symptoms because *jail-1* plants did not develop symptoms in response to JL1065 infection.

The virulence activity of *avrRpt2* is not associated with the suppression of defense-related genes in tomato.

In both *Arabidopsis* and tomato, infection by *P. syringae* induces the expression of numerous defense-related genes (Van Loon and Van Strien 1999; Zhou et al. 1997). In *Arabidopsis*, the induction of several *PR* genes by DC3000 infection is attenuated if the pathogen expresses *avrRpt2*, which may indicate that the latter exerts its virulence activity by suppressing host defense responses (Chen et al. 2004). To test whether *avrRpt2* has a similar effect on the expression of defense-related genes induced by JL1065 infection in tomato, we compared the expression pattern of a select set of defense-related genes between tomato plants infected with JL1065 and ML100. We chose to analyze the expression of a set of genes for which induction is correlated with resistance to *P. syringae*, including *PR-1b1*, *PR-1a1*, genes encoding chitinase and β -1-glucanase, and the ethylene biosynthesis gene *ACO1* (Jia and Martin 1999; Tornero et al. 1994; van Kan et al. 1992). The expression of all five genes was induced in tomato within 3 to 5 dpi with both JL1065 and ML100, and the timing and level of induction of these genes were not reproducibly different between plants infected with either strain (Fig. 4). Thus, the reduction in virulence of the *avrRpt2* mutant ML100 on tomato is not associated with a detectable increase in transcript levels of the defense-related genes tested.

DISCUSSION

Genetic screens aimed at identifying *P. syringae* virulence factors have been largely unsuccessful at uncovering a role in disease for individual putative TTSS effectors. This difficulty has been attributed to a high degree of functional redundancy among TTSS effectors that could result in extremely subtle phenotypes arising from their individual loss (Collmer et al. 2002; Greenberg and Vinatzer 2003). An alternative approach that has proven useful for uncovering the virulence activity of

Table 1. Virulence activity of *avrRpt2* on wild-type tomato and mutant lines defective in the accumulation or perception of ethylene, salicylic acid (SA), or jasmonic acid (JA)^a

Host ^b	Strain ^c	Growth (log [CFU/cm ²]) ^d	Conductivity (μ MHO/ml) ^e	Abscission ^f	Necrosis ^g
Pearson	JL1065	8.30 $\pm$ 0.15	15.4 $\pm$ 1.0	Severe	Severe
	ML100	7.21 $\pm$ 0.35**	7.6 $\pm$ 0.7**	Attenuated	Attenuated
<i>Nr</i>	JL1065	8.27 $\pm$ 0.17	9.2 $\pm$ 1.4	None	Attenuated
	ML100	7.73 $\pm$ 0.09**	8.5 $\pm$ 2.0	None	Attenuated
Moneymaker	JL1065	8.52 $\pm$ 0.22	13.7 $\pm$ 3.1	Severe	Severe
	ML100	7.72 $\pm$ 0.29**	4.4 $\pm$ 1.8**	Attenuated	Attenuated
NahG	JL1065	8.37 $\pm$ 0.19	9.0 $\pm$ 1.8	Severe	Severe
	ML100	7.63 $\pm$ 0.10*	3.0 $\pm$ 0.7*	Attenuated	Attenuated
Castlemart	JL1065	7.78 $\pm$ 0.07	Nd	Severe	Severe
	ML100	7.08 $\pm$ 0.10**	Nd	Attenuated	Attenuated
<i>jail-1</i>	JL1065	3.54 $\pm$ 0.67	Nd	None	None
	ML100	2.57 $\pm$ 0.17	Nd	None	None

^a Tomato lines were vacuum infiltrated with JL1065 or ML100 at 1×10^4 CFU/ml JL1065 or ML100. Values represent the average of four replicates $\pm$ standard error. Asterisks indicate a significant difference between the averages of JL1065 and ML100 treatments (Student's *t* test, ** and * = *P* < 0.05 and 0.1, respectively). All experiments were repeated at least twice with similar results.

^b Wild-type isogenic tomato lines used as controls for the ethylene-insensitive *Nr*, SA-deficient NahG, and JA-insensitive *jail-1* lines were Pearson, Moneymaker, and Castlemart, respectively.

^c *Pseudomonas syringae* pv. *tomato* JL1065 carries wild-type *avrRpt2* and *P. syringae* pv. *tomato* ML100 is a mutant derivative of JL1065 defective for *avrRpt2*.

^d Bacterial numbers were measured in infected tissue at 0 and 6 days postinoculation (dpi). Bacterial levels measured on day 0 were similar for all treatments (data not shown).

^e Electrolyte leakage was measured from infected plants at 6 dpi. Nd = not done due to absence of necrosis on infected *jail-1* plants.

^f Refers to the detachment of leaf petioles from the main stem of infected plants scored at 6 dpi. JL1065 treatment typically resulted in the abscission of 50% or more of leaves (severe), whereas ML100 resulted in the abscission of fewer than 35% of leaves (attenuated).

^g Refers to the appearance of patches of necrotic tissue formed by the coalescence of individual water-soaked lesions on infected leaflets at 6 dpi. Severe = necrosis on 75 to 90% of leaflets and attenuated = necrosis on fewer than 50% of leaflets.

some TTSS effectors is the use of heterologous pathosystems. For instance, loss of the *avrPto* gene does not produce a detectable reduction in the virulence of its native strain DC3000; however, heterologous expression of *avrPto* in *P. syringae* pv. *tomato* T1 enhances the virulence of the latter on tomato (Shan et al. 2000). Similarly, the virulence activity of *avrRpt2* initially was characterized by heterologous expression in DC3000 (Chen et al. 2000, 2004). Although useful, heterologous systems raise concerns about artifacts that might result from overexpression of virulence genes in non-native strains (Charkowski et al. 1998; Shan et al. 2000). However, we show here that loss of *avrRpt2* from its native strain, JL1065, also results in a significant reduction in the virulence of the bacteria on its natural host, tomato. Thus, we now have both an evolutionarily relevant context for the study of *avrRpt2* virulence function and the possibility of comparing the mode of action of *avrRpt2* between two different host systems, *Arabidopsis* and tomato.

Much less is known about host processes and signaling pathways that govern the outcome of *P. syringae* infection in tomato than in *Arabidopsis*. This constitutes one reason why the degree of functional conservation of pathogen-response pathways between these two plant species has remained poorly studied until recently (Ekengren et al. 2003). Of the tomato

signaling pathways examined to date, the one responding to ethylene clearly is required for full susceptibility to *P. syringae* (Lund et al. 1998), making it a likely candidate for targets of bacterial virulence factors like AvrRpt2. The observation that *avrRpt2* virulence activity is maintained on *Nr* plants suggests that AvrRpt2 does not rely on the *Nr*-dependent ethylene response pathway to promote the virulence of JL1065. It remains possible that modulation of ethylene signaling could contribute to the virulence activity of *avrRpt2*, given that the *Nr* mutant does not appear to be completely impaired in ethylene sensitivity (Lanahan et al. 1994). In *Arabidopsis*, impairment of ethylene responsiveness due to a mutation in the *ethylene insensitive 2 (EIN2)* gene reduces the severity of disease symptoms elicited by DC3000 (Bent et al. 1992). This suggests that, in *Arabidopsis* as in tomato, ethylene responsiveness plays a role in regulating symptom development induced by *P. syringae*. However, the involvement of ethylene signaling in the virulence activity of *avrRpt2* in *Arabidopsis* has not yet been examined.

Our ability to clearly detect the virulence activity of *avrRpt2* in NahG tomato lines suggests that *avrRpt2* works either independently of the SA response pathway or downstream of SA accumulation to exert its virulence activity in tomato. This finding is in agreement with our previous observation that *avrRpt2* virulence activity functions independently or downstream of SA accumulation in *Arabidopsis* (Chen et al. 2004). Intriguingly, we also found that NahG transgenic tomato plants were not reproducibly more susceptible to infection by JL1065 than the wild-type isogenic lines. This result contrasts dramatically with the enhanced disease susceptibility exhibited by *Arabidopsis* lines impaired in SA accumulation (Delaney et al. 1994; Glazebrook et al. 1996; Nawrath and Metraux 1999) and may point to a different role for SA in regulating host response against *P. syringae* infection in tomato and *Arabidopsis*. SA accumulation also has been reported to be differentially required for defense against fungal pathogens in tobacco and tomato (Achuo et al. 2002).

Fig. 3. Quantitative assessment of *avrRpt2* virulence activity on tomato. **A**, Loss of *avrRpt2* results in a reduction in disease-induced tissue damage as assessed by ion leakage from infected leaflets. Tomato plants (cv. Rio Grande 76S) were vacuum infiltrated with 10 mM magnesium chloride (mock) or *Pseudomonas syringae* pv. *tomato* JL1065 or the *avrRpt2* mutant ML100 at 1×10^4 CFU/ml. Values represent the average of three replicates $\pm$ standard error. **B**, *avrRpt2* promotes the growth of JL1065 on tomato. Treatments were as described for **A** with the inclusion of the *avrRpt2* mutant complemented with a plasmid-borne copy of the gene, ML100(*avrRpt2*). Values represent the average of three replicates $\pm$ standard error. Experiments were repeated at least three times with similar results.

Fig. 4. AvrRpt2 does not suppress the induction of transcripts of defense-related genes in tomato. Tomato plants (cv. Pearson) were vacuum infiltrated with *Pseudomonas syringae* pv. *tomato* JL1065 or the *avrRpt2* mutant ML100 at 1×10^4 CFU/ml. For each treatment, tissue was harvested from three infected plants at the indicated days postinoculation (dpi) and pooled prior to RNA extraction. Approximately 15 μ g of total RNA was loaded in each lane. The resulting RNA blot was sequentially hybridized with DNA probes for *PR-1a1*, *PR-1b1*, genes encoding chitinase or β -1-3-glucanase (*B-1-GLU*), and *ACO1*. The elevated levels of *PR-1b* and *ACO1* transcripts in plants infected with JL1065 at 3 dpi were not reproducibly detected in independent experiments. Ethidium bromide-stained rRNA bands (bottom panel) show loading of similar levels of total RNA in all lanes. This experiment was repeated at least twice with similar results.

JA is another plant signaling molecule that has been shown to play an important role in regulating disease production by *P. syringae* in tomato (Zhao et al. 2003), leading us to test the involvement of JA-dependent signaling in *avrRpt2* virulence activity. For the purpose of this experiment, we took advantage of the *jail-1* tomato line that is deleted for a tomato homolog of the *Arabidopsis COI1* gene (Feys et al. 1994; Li et al. 2004). Under the conditions of our assays, we were unable to consistently detect a statistically significant difference between the growth of JL1065 and the *avrRpt2* mutant strain ML100 on the *jail-1* tomato line. This could be an experimental limitation imposed by the severe reduction in susceptibility to JL1065 infection observed on *jail-1* plants. However, these results may suggest that, in tomato, *avrRpt2* virulence activity is dependent on JA signaling. Thus, AvrRpt2 could work by interacting directly with JA signaling pathways. Alternatively, the reliance of *avrRpt2* virulence activity on *COI1*-dependent signaling might be indirect. We observed that the contribution of *avrRpt2* to both bacterial growth and symptom development is manifested relatively late during the course of infection (Figs. 1 and 3B). This suggests that *avrRpt2* virulence activity is additive or synergistic to that of other virulence factors that function earlier during the course of infection. If these virulence factors function in a JA-dependent fashion, then abrogation of JA signaling in the *jail-1* mutant would preclude detection of their activity and, consequently, that of AvrRpt2. The *COI1*-dependent pathway appears to be an important target for multiple virulence factors that contribute to the pathogenicity of DC3000 on tomato (Zhao et al. 2003). This virulence strategy is likely to be conserved in JL1065, given the severe reduction in its virulence on *jail-1* plants. The lack of detectable *avrRpt2* virulence activity in the *jail-1* tomato mutant stands in contrast to our observation that *avrRpt2* is able to enhance the growth of DC3000 on the JA-insensitive *Arabidopsis* mutant *coi1* (Chen et al. 2000). However, this result is not surprising given that JA signaling has been shown to be differentially required in *Arabidopsis* and in tomato for the regulation of important host processes such as reproductive development (Feys et al. 1994; Li et al. 2004).

In *Arabidopsis*, the virulence activity of *avrRpt2* is associated with the suppression of *PR* gene induction in infected plants (Chen et al. 2000, 2004). *PR* gene suppression is also an attribute of the DC3000 effector HopPtoD2 which, when mutated, impairs bacterial virulence on *Arabidopsis* (Bretz et al. 2003; Espinosa et al. 2003). Despite being correlated with the virulence activity of these two effector proteins, the role of *PR* gene suppression in promoting the ability of *P. syringae* to cause disease is unclear. Strikingly, AvrRpt2 suppresses *PR* gene expression only when delivered to *Arabidopsis* by DC3000 but not when expressed at low levels in transgenic plants (Chen et al. 2004). That the latter nevertheless exhibit enhanced susceptibility to infection contradicts the hypothesis that the reduction in *PR* gene expression associated with the bacterial delivery of AvrRpt2 contributes directly to enhanced host susceptibility. Here, we show that the dramatic reduction in disease symptom severity resulting from the loss of *avrRpt2* from JL1065 is not associated with a detectable alteration of the expression pattern of a subset of defense-related genes. This suggests that, in tomato, the suppression of defense pathways regulating the expression of these defense-related genes is unlikely to be an important mechanism underlying the virulence activity of *avrRpt2*. However, it is possible that *avrRpt2* could have a suppressive effect on the expression of other as yet unknown defense-related genes. This question can be more adequately addressed by genome-wide expression profiling experiments.

There are likely to be several general barriers to infection present in most plant species (e.g., metabolic limitation) that must be overcome by all *P. syringae* pathovars for successful

plant parasitism. This early step in pathogenesis would be predicted to depend on the activity of conserved proteins that would contribute to the core repertoire of *P. syringae* virulence factors (Alfano et al. 2000; Greenberg and Vinatzer 2003). In addition to these conserved factors, individual *P. syringae* pathovars and strains are likely to possess strain-specific virulence factors that have evolved to facilitate parasitism of specific hosts. *avrRpt2* can promote the virulence of three different *P. syringae* strains, JL1065, DC3000, and *P. syringae* pv. *maculicola* 4326, on their respective plant hosts (Chen et al. 2000; Guttman and Greenberg 2001; this work). These findings suggest that the modifications made to the host by AvrRpt2 to exert its virulence activity are beneficial for *P. syringae* pathovars in general. However, it is currently unclear whether the virulence target or targets and, hence, the mode of action of AvrRpt2 are conserved in different hosts. As studies in *Arabidopsis* continue to illuminate the mechanisms of AvrRpt2 virulence (Axtell et al. 2003; Belkhadir et al. 2004; Chen et al. 2004; Lim and Kunkel 2004a and b; Mackey et al. 2003) we can start to address this question through the application of novel molecular genetic tools in tomato (Ekengren et al. 2003).

MATERIALS AND METHODS

Bacterial strains and plasmids.

P. syringae pv. *tomato* JL1065 has been described previously (Whalen et al. 1991). The *avrRpt2* mutant strain ML100 was constructed by marker insertion at the *avrRpt2* genomic locus. A 4.7-kb *Bgl*III/*Bam*HI fragment containing the *avrRpt2* open reading frame (ORF), flanked upstream by 1.3 kb and downstream by 0.6 kb of *P. syringae* pv. *tomato* genomic DNA sequence, was cloned from cosmid pABL18 (Whalen et al. 1991) into pBluescript-II KS+ (Stratagene, La Jolla, CA). The resulting construct was digested with *Eco*RI and *Xba*I, yielding a 4.8-kb fragment that was subcloned into suicide vector pJP5603 (kanamycin resistant) (Penfold and Pemberton 1992). A spectinomycin resistance Ω cassette (Prentki and Krisch 1984) then was inserted into the *Ec*III site located 434 nucleotides downstream of the translational start site of *avrRpt2*. The final construct (pML02) was conjugated into JL1065 by triparental mating using helper plasmid pRK2013 (Figurski and Helinski 1979). To generate the *avrRpt2* mutant ML100, strains in which pML02 had integrated in the genome via a single recombination event first were obtained by selecting for kanamycin- and spectinomycin-resistant transconjugants. Derivatives of the latter then were screened for loss of kanamycin resistance resulting from a second recombination event. Disruption of the *avrRpt2* ORF in ML100 was confirmed by polymerase chain reaction and DNA hybridization analysis. Loss of *avrRpt2* activity was confirmed further by testing for loss of the avirulence activity of *avrRpt2* on *A. thaliana* plants of ecotype Columbia (Col-0) that express a functional *RPS2* gene (Kunkel et al. 1993). Wild-type JL1065 elicited a hypersensitive response (HR) at 16–18 h postinoculation (hpi) on Col-0. ML100 failed to elicit an *RPS2*-dependent HR, even when scored at 24 hpi (data not shown). Plasmid pV288, which consists of the *avrRpt2* cloned into the freely replicating vector pVSP61 (kanamycin resistant) (Kunkel et al. 1993), was used to complement the *avrRpt2* mutation in ML100.

Plant material and infection assays.

Tomato (*L. esculentum*) cvs. Rio Grande PtoS and Pearson were used in all assays unless otherwise stated. Cvs. Pearson, Castlemart, and Moneymaker were used as isogenic control lines in experiments with the *Nr* and *jail-1* mutants and the

NahG transgenic line, respectively (Brading et al. 2000; Lanahan et al. 1994; Li et al. 2001). The *jail-1* homozygotes were identified from F2 segregating populations as described by Li and associates (2004). From October to May, tomato plants were grown in the greenhouse where conditions were adjusted to provide 16 h of light at a minimum intensity of 200 $\mu\text{E m}^{-2} \text{s}^{-1}$. During the summer (June to September), tomato plants were maintained in growth chambers under 16 h of light (200 $\mu\text{E m}^{-2} \text{s}^{-1}$) at 25°C and 8 h of dark at 20°C and 70% humidity. Four- to five-week old plants were used for bacterial inoculation. *P. syringae* strains were grown overnight on King's B (KB) plates containing rifampicin at 100 $\mu\text{g/ml}$ and kanamycin at 50 $\mu\text{g/ml}$ when appropriate. For vacuum infiltration of tomato plants, bacterial strains were resuspended in 10 mM MgCl_2 to a density of 1×10^4 CFU/ml. For measuring growth of bacteria in planta, four 0.5-cm² leaf disks were sampled from each of three to four infected plants. Leaf disks were ground in 10 mM MgCl_2 and appropriate dilutions of the resulting extracts were plated on Luria Bertani (LB) plates containing rifampicin at 50 $\mu\text{g/ml}$. *Arabidopsis* was maintained in growth chambers under an 8-h photoperiod at 24°C and 75% humidity. An HR was induced on *Arabidopsis* by infiltrating the underside of leaves with a bacterial suspension of 1×10^8 CFU/ml, using a 1-ml flat-tipped syringe (Kunkel et al. 1993). Plants were scored 16 to 24 h after inoculation.

Electrolyte leakage.

Conductivity measurements were performed using a protocol adapted from Rate and Greenberg (2001). Five leaf disks (6 mm in diameter) were sampled from each of four bacteria-inoculated or mock-inoculated tomato plants. The leaf disks were incubated for 15 min in distilled water to wash off surface contaminants. They then were floated on 5 ml of distilled water, adaxial side down, and incubated at 28°C. After 30 min, 1 ml was sampled from the bathing solution and assayed using a CDM2 conductivity meter (Radiometer, Copenhagen, Denmark). Then, 4.5 h later, a second 1-ml sample of the bathing solution was assayed for conductivity. Final conductivity values represent the difference between measurements made at 30 min and 4.5 h.

RNA blot analysis.

Approximately 10 mg of fresh tissue was harvested from each of three bacteria-inoculated or mock-inoculated plants. Pooled samples were immediately flash frozen in liquid nitrogen and stored at -80°C until extraction in RNeasy (Ambion, Austin, TX). RNA samples were run on a formaldehyde gel and then transferred onto nylon membranes according to standard procedures (Ausubel et al. 1987). DNA probes for *PR-1a1*, *PR-1b1*, *ACO1*, and the chitinase and β -1-3 glucanase genes have been described previously (Ciardi et al. 2000; Lund et al. 1998; Tornero et al. 1994).

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