Pseudomonas viridiflava* and *P. syringae*—Natural Pathogens of *Arabidopsis thaliana

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We report the isolation and identification of two natural pathogens of *Arabidopsis thaliana*, *Pseudomonas viridiflava* and *Pseudomonas syringae*, in the midwestern United States. *P. viridiflava* was found in six of seven surveyed *Arabidopsis thaliana* populations. We confirmed the presence in the isolates of the critical pathogenicity genes *hrpS* and *hrpL*. The pathogenicity of these isolates was verified by estimating in planta bacterial growth rates and by testing for disease symptoms and hypersensitive responses to *A. thaliana*. Infection of 21 *A. thaliana* ecotypes with six locally collected *P. viridiflava* isolates and with one *P. syringae* isolate showed both compatible (disease) and incompatible (resistance) responses. Significant variation in response to infection was evident among *Arabidopsis* ecotypes, both in terms of symptom development and in planta bacterial growth. The ability to grow and cause disease symptoms on particular ecotypes also varied for some *P. viridiflava* isolates. We believe that these pathogens will provide a powerful system for exploring coevolution in natural plant-pathogen interactions.

Additional keyword: 16S rDNA.

Plants are chronically attacked by pathogens and thus possess mechanisms to resist pathogen infection or disease development. In a gene-for-gene interaction, a plant resistance (*R*) gene confers resistance specific to a corresponding avirulence (*avr*) gene in a pathogen (Flor 1942, 1971). Gene-for-gene interactions have been studied in a wide variety of natural and agricultural systems, primarily through the use of phenotypic data alone (Bevan et al. 1993a, 1993b, 1993c; Burdon and Jarosz 1991; Geffroy et al. 1999; Restrepo and Verdier 1997). These studies indicate high levels of plant resistance specificities within and among plant populations.

In agricultural systems, major gene disease resistance that has been introduced into crops is often overcome rapidly by pathogens (Cruz et al. 2000; Leach et al. 2001; Mew et al. 1992). Given the lack of durable resistance, it is somewhat surprising that there are an increasing number of examples of long-lived resistance alleles; for example, *R*-gene resistance in wild relatives of tomato to *Pseudomonas syringae* pv. *tomato* (Riely and Martin 2001) and nonhost resistance in maize to

Cochliobolus carbonum (Multani et al. 1998). Polymorphisms for *R*-gene alleles in *Arabidopsis thaliana* have similarly been shown to contain both resistant and susceptible forms that have persisted for millions of years (reviewed in Bergelson et al. 2001; Bittner-Eddy et al. 2000; Caicedo et al. 1999; Holub 2001; Stahl et al. 1999).

Uncovering the mechanisms that drive the evolutionary dynamics in resistance genes, including rapid evolution and the maintenance of polymorphism, will require knowledge of the epidemiology of the disease and the fitness effects of resistance and virulence. For example, in the interaction between *Silene alba* and the anther smut *Ustilago violacea*, resistant and susceptible individuals are maintained by costs and benefits of resistance and frequent colonization and extinction events among host and pathogen populations (Alexander et al. 1996; Thrall and Antonovics 1995). With the exception of the pathogens *Peronospora parasitica* and *Albugo candida* (Holub et al. 1991), most of the pathogens used to identify functional resistance in *Arabidopsis* have been collected from other host plant species. There are two consequences of this practice. First, most evolutionary studies of *Arabidopsis* *R*-genes focus on genes that are not known to interact with natural pathogens. Second, without a natural pathogen in hand, investigation of microevolutionary and ecological dynamics that might help explain evolutionary patterns is infeasible.

In an effort to develop *Arabidopsis* as a model system for coevolutionary interactions, we report here that *Pseudomonas viridiflava* and *Pseudomonas syringae* are natural pathogens of *Arabidopsis* in the midwestern United States. We find *P. viridiflava* to be the most common pathogen collected from natural *A. thaliana* populations. We also investigate variation in the ability of independent isolates of this pathogen to infect plant leaves; we also investigate variation among *A. thaliana* ecotypes in the ability to resist infection.

RESULTS

***P. viridiflava* is a common bacterial pathogen collected from natural *Arabidopsis* populations.**

A. thaliana leaves collected from seven natural populations showed disease symptoms in the form of chlorotic, necrotic, and tan-colored lesions (Fig. 1). Of the 179 bacterial isolates obtained from these leaves, 40 developed a hypersensitive response (HR) when infiltrated into tobacco and were thus candidate pathogens of *Arabidopsis* (Klement et al. 1964) (Table 1). The majority of isolates that induced a HR on tobacco were identified as *P. viridiflava*, based on LOPAT determinative tests (Lelliott et al. 1966) and a polymerase chain reaction (PCR) assay (discussed below). *P. viridiflava* is a fluorescent, gram negative bacterium that rots potato and is negative for levan, oxidase, and arginine dihydrolase activity. Two of the HR-

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Nucleotide sequence data reported are available under the following accession numbers. *hrpS*: PNA29.1a, AF508888; RM29.1a, AF508889; PNA3.3a, AF508890; LP23.1a, AF508891; KNOX3.4a, AF508892; RMX23.1a, AF508893; ME3.1b, AF508894; and RMX3.1b, AF508895; and *hrpL*: PNA29.1a, AF508896; RM29.1a, AF508897; PNA3.3a, AF508898; and LP23.1a, AF508899.

causing isolates were identified as *P. syringae*. Five isolates tentatively identified as *P. syringae* did not develop a HR on tobacco and, together with the remaining 95 gram negative and 44 gram positive isolates, may be epiphytic or saprophytic strains from the leaf surface or dead tissue.

Six locally collected *P. viridiflava* isolates were further examined to confirm our identification based on LOPAT and PCR tests. Two isolates were selected from one Michigan site, Rmx, and one isolate was selected from each of four other sites (Knox, Lp, Me, and Pna). First, we checked for ice nucleating activity. All six isolates were ice nucleating but differed in their level of activity. The isolates RMX3.1b and RMX23.1a expressed very high levels of ice-nucleation activity, whereas the activities of the other four *P. viridiflava* isolates were much lower (data not shown).

Second, we obtained 1,500 bp of the 1,538-bp 16S gene sequence from the six selected isolates. All six showed 99% identity to the 1,492-bp 16S sequence for *P. viridiflava* strain LMG 2352 listed in GenBank (accession number Z76671). A consistent difference between the sequences of our isolates and the GenBank strain was a C to G base change at position 30. There were five additional polymorphic sites. The isolate RMX3.1b differed at positions 440 and 441 from the other local isolates and the GenBank sequence. Isolates RMX23.1a and ME3.1b shared nucleotide polymorphisms at sites 1,104 and 1,424, and KNOX3.4a, LP23.1a and PNA3.3a shared a base difference at position 1,106.

We additionally obtained 16S sequence for the two *P. syringae* isolates. A 1,529-bp sequence of the isolate PNA29.1a was 99% identical to 16S sequence of *P. syringae* pv. *glycinea*, with one base change from G to A at position 1,952 of the GenBank strain (accession number AB001443). The 1,530-bp sequence of the isolate RM29.1a matched to 99% the 16S sequence of *P. syringae* pv. *phaseolicola* with two polymorphic sites at positions 1,017 and 1,130 of the GenBank strain (accession number AB001448).

Fig. 1. Symptoms of *Pseudomonas viridiflava* on *Arabidopsis thaliana* plants in natural populations.

Arabidopsis populations differed in the extent to which they were attacked by *P. viridiflava*. A random collection of leaves from seven populations revealed that at three Michigan sites (Me, Pna, and Rmx), 3 of 6 (50%), 6 of 47 (12.7%), and 3 of 31 (9.7%) leaves contained a detectable level of *P. viridiflava*. At three of four Indiana sites sampled (Knox, Lp, and Rm), *P. viridiflava* was found in 6 of 12 (50%), 4 of 26 (15.4%), and 16 of 45 (35.5%) leaves. We found no *P. viridiflava* in 19 at a site in Indiana (Ez) in which *Arabidopsis* is sparsely distributed. The two *P. syringae* isolates causing a HR on tobacco were found at a Michigan site (Pna) and a site in Indiana (Rm), respectively.

P. viridiflava is pathogenic on *Arabidopsis*.

Arabidopsis plants infected with *P. viridiflava* at high humidity developed disease symptoms consisting of watersoaked, translucent spots that began as early as 48 h postinfection, developed into chlorotic lesions, and later became necrotic. Daily titers of bacterial growth in the local *Arabidopsis* line Pna-7 showed that all six local *P. viridiflava* isolates grew at least two orders of magnitude day 1 to 6 postinfection, with no significant variation among isolates at day 4 (Fig. 2). It is interesting to note that 6 days after infection, there is significant variation among strains in the bacterial titer ($F_{5,29} = 4.13$, $p < 0.0059$). Disease symptoms for these infections at day 4 are shown in Table 2. Both the number of diseased leaves and lesion size vary significantly among the isolates.

The HR associated with gene-for-gene resistance could not be observed under the high-humidity conditions that were most favorable for bacterial growth. Therefore, we infected the highly resistant *Arabidopsis* ecotype Wu-0 (discussed below, Table 3) with each of the six *P. viridiflava* isolates under low humidity conditions. The isolates KNOX3.4a, ME3.1b, RMX3.1b, and RMX23.1a induced clear HR symptoms in the inoculated leaves after 24 h (Table 3). The response of Wu-0 to infection with LP23.1a and PNA3.3a was less dramatic but still consistent with the role of these isolates as pathogens of *Arabidopsis*. Approximately 67% of the leaves infected with LP23.1a and PNA3.3a showed tissue collapse after 24 h, and between 93 to 100% of the leaves had HR symptoms after 48 h. The tissue remained necrotic for several days, and no chlorotic spreading appeared.

We additionally tested the pathogenicity of a field-collected *P. syringae* isolate, PNA29.1a. This was one of the two *P. syringae* isolates that caused a HR in tobacco. This pathogen also required 100% humidity and high inoculation concentration for disease development, which was characterized by chlorotic spots. Infection at a concentration of 3×10^6 CFU per ml revealed a nearly uniform response within each infected plant and within each ecotype; both infected leaves within each plant showed chlorotic lesions, and a high number of replicates within each ecotype developed disease symptoms.

Table 1. Characterization of the collected isolates from *Arabidopsis thaliana* plants in seven populations in Indiana and Michigan during Spring 2000

Collected Isolates	Sum	Location						
		Ez	Knox	Lp	Me	Pna	Rm	Rmx
Total number	179	12	12	26	6	47	45	31
Causing HR on tobacco ^y	40	—	6	4	3	7	17	3
Identified as <i>P. viridiflava</i>	38	—	6	4	3	6	16	3
Identified as <i>P. syringae</i> , HR+ ^z						1	1	
Identified as <i>P. syringae</i> , HR—						1	2	4
% <i>P. viridiflava</i> % of collection	21.2	—	50.0	15.4	50.0	12.7	35.5	9.7
Not causing HR	139	12	6	22	3	37	26	24
Gram negative	95	7	3	10	1	25	20	20
Gram positive	44	5	3	12	2	12	6	4

^y HR = Hypersensitive response on tobacco cv. Burley.

^z HR+ = positive HR, and HR— = negative HR.

To confirm the pathogenicity of our isolates, we were able to PCR-amplify and sequence *hrpS* and *hrpL* genes involved in the type III secretion system from the six *P. viridiflava* and the two local *P. syringae* isolates. First, we sequenced a 280-bp partial *hrpS* coding sequence from all the eight strains (accession numbers AF508888 to AF508895) and found that the sequences are highly diverged both within and between species. Mean peptide identity within *P. viridiflava* and within *P. syringae* (including *P. syringae* pv. *tomato* DC3000 [GenBank accession number AF232004]) were 16.3% and 81.5%, respectively, and the peptide identity between species was 16.3%. Even after excluding RMX3.1b, which is the most divergent *P. viridiflava* isolate for this region, mean peptide identity within *P. viridiflava* was 44.5%. Second, we sequenced a 555-bp coding sequence of *hrpL* from two *P. viridiflava* (LP23.1a and PNA3.3a) and the two *P. syringae* isolates (accession numbers AF508896 to AF508899), and mean peptide identity between species was 80.5%. Although we did not successfully amplify the complete *hrpL* coding sequence from RMX3.1b, RMX23.1a, KNOX3.4a, and ME3.1b, we amplified and sequenced a 335-bp partial sequence of *hrpL* from all the strains. These sequences are nearly identical within species (99%, data not shown), indicating the presence of the *hrpL* gene in all eight strains.

Genetic variation is evident in virulence of

Pseudomonas isolates and resistance of *Arabidopsis* plants.

The infection of 21 *Arabidopsis* ecotypes with each of six *P. viridiflava* isolates revealed phenotypic variation in the resistance of *A. thaliana* to infection by *P. viridiflava*. The average number of diseased leaves per plant varied among the ecotypes for each bacterial isolate (Table 4). This was also evidenced by significant differences in bacterial growth between ecotypes, which ranged from log 3.16 to log 9.06 CFU per leaf disk (Table 5). Analysis of variance reveals a significant effect of the bacterial isolate ($F = 13.53$, $df = 5,450$, $p < 0.001$), the plant ecotype ($F = 5.29$, $df = 24,450$, $p < 0.001$) and their interaction ($F = 2.35$, $df = 120,450$, $p < 0.001$). Note, also, that there is variation in the virulence of *P. viridiflava* strains from the same population; isolate RMX3.1b reveals higher virulence than isolate RMX23.1a (Tables 4 and 5).

Averaged over all six *P. viridiflava* isolates, some ecotypes appeared relatively more resistant than others (Fig. 3). The local Gr-13 plants and the ecotype Kas-1 were highly susceptible with more than 50% of infected leaves showing disease symptoms for most *P. viridiflava* isolates. Wu-0 was particularly resistant to symptom formation, while Chi-0 and Mt-0 showed low levels of bacterial growth.

The *A. thaliana* ecotypes also exhibited significant variation in resistance to the *P. syringae* isolate PNA29.1a (Tables 4 and 5). Bacterial growth ranged between log 3.42 and log 6.26 among ecotypes infected with *P. syringae* (Table 5).

Results from our surveys of plant accession and pathogen isolates reveals substantial variation among replicate leaves

and plants in their response to infection. Obtaining consistent results across replication within and between plants is inherently difficult with these pathogens. To investigate the nature of this variation, we asked how the bacterial titer within leaves of a particular accession was related to presence or absence of disease symptoms in that accession. We found that the number of diseased leaves four days after infection was positively correlated with bacterial growth for each of the six *P. viridiflava* isolates ($r = 0.59$, Fig. 4) as well as for the single *P. syringae* isolate ($r = 0.82$, Fig. 5). There does, however, appear to be variation in the threshold titer leading to disease symptoms. For example, the plant accession Wu-0 infected with LP23.1a at log 5.2 CFU per ml did not show disease symptoms, whereas Kas-1, infected with the same isolate at log 5.3 CFU per ml, was highly diseased.

DISCUSSION

We collected and characterized bacterial isolates in a field survey of natural *A. thaliana* populations and infected plants under controlled conditions in the greenhouse. Our field collections show that *P. viridiflava* is a natural pathogen of *Arabidopsis*, occurring at relatively high frequencies in the midwestern United States. We also identified *P. syringae* as a pathogen of *Arabidopsis*, although occurring at a much lower frequency than *P. viridiflava*. A sample of 17 worldwide and four local plant accessions reveals compatible as well as incompatible interactions when infected with each *P. viridiflava*, and variation among isolates reveals phenotypic differences in virulence as well.

We confirmed the identity of the six locally collected *P. viridiflava* isolates used in our experiments by sequencing the 16S ribosomal DNA gene in each strain. The identification of bacteria through comparison of 16S rDNA sequences is one of the most effective methods for species identification. The region is

Fig. 2. Growth of locally collected *Pseudomonas viridiflava* strains in the *Arabidopsis thaliana* ecotype Pna-7. Plants were infected at a concentration of 10^5 CFU per ml and kept at 100% humidity for 72 h.

Table 2. Number of diseased leaves (out of 48 infected) and lesion size for six locally collected *P. viridiflava* strains in the local *Arabidopsis thaliana* ecotype Pna-7 at day 4 postinfection

<i>P. viridiflava</i> strain	Diseased leaves		Diseased leaves/plant $F(4,115) = 7.21; p < 0.0001$	Log CFU leaf/disk $F(5,30) = 0.64; p < 0.6735$	Lesion size (1-9) $F(4,124) = 3.77; p < 0.0062$
	sum	%			
KNOX3.4a	33	68.8	1.38 ab ^z	6.44 a	3.9 a
LP23.1a	42	87.5	1.75 a	6.26 a	3.4 ab
ME3.1b	12	25.0	0.50 c	5.75 a	1.5 d
PNA3.3a	14	29.6	0.58 c	5.76 a	1.7 d
RMX3.1b	27	56.3	1.13 b	6.03 a	3.0 bc
RMX23.1a	30	62.5	1.25 b	6.44 a	2.6 c

^z Values with the same letter are not statistically different (Duncan test).

highly conserved but shows characteristic species-specific sequence differences (Laguette et al. 1994; Moore et al. 1996; Weisburg et al. 1991; Widmer et al. 1998). Our sequences showed 99% identity to the single *P. viridiflava* sequence in GenBank. Within the genus *Pseudomonas*, three hypervariable regions in the 16S gene have been used to differentiate member species (Moore et al. 1996). The nucleotide polymorphism that was unique to strain RMX3.1b among the six strains tested occurs in hypervariable region 3. Nucleotide differences in this region are currently used to distinguish among biotypes of *P. fluorescens* and pathovars of *P. marginalis* (Moore et al. 1996). The polymorphism at position 1,424 is located within helix 49 (hypervariable region 9). The sequence differences that we observed at positions 1,104 and 1,106 are not located in a designated hypervariable region, nor are they involved in protein helix formation (Van de Peer et al. 2000). The polymorphisms among *P. viridiflava* isolates in the 16S gene may distinguish among groups of strains, as in *P. fluorescens* and *P. marginalis*.

The pathogenicity of our *P. viridiflava* isolates was verified by demonstrating the presence of components of the type III secretion system. The type III (Hrp) secretion pathway is involved in the transfer of effector proteins into the plant cell, and, thus, serves a basic function needed for pathogenicity. Genes in this pathway are conserved among bacterial plant and mammalian pathogens (Cornelis and Van Gijsegem 2000;

Hueck 1998; Staskawicz et al. 2001). In *P. syringae* the transcription of *avr* genes is known to be dependent on the *hrpS*, *hrpR*, and *hrpL* genes (Gopalan et al. 1996; Innes et al. 1993; Lindgren 1997; Salmeron and Staskawicz 1993). The *hrpL* gene is the primary transcription factor for a large number of genes whose products are targeted for the surface or interior of the plant cells (Cornelis and Van Gijsegem 2000; Xiao et al. 1994). The *hrpS* and *hrpR* genes are required for the activation of *hrpL* and are located in a conserved region within a cluster of pathogenicity genes called the pathogenicity island (Hutcheson et al. 2001). The successful amplification of a portion of the *hrp* gene cluster in each *P. viridiflava* and *P. syringae* isolate suggests that these isolates have functioning type III secretion systems and thus have the necessary mechanism for pathogenicity. Charkowski and associates (1998) successfully hybridized the harpin *hrpW* in a *P. viridiflava* strain and similarly suggested that *P. viridiflava* has a type III secretion system.

We observed variation in the number of infected leaves developing symptoms among *Arabidopsis* accessions of local and worldwide origin. Many of the plant lines showed intermediate levels of resistance, although there were also highly susceptible and highly resistant plants. Consistently high levels of resistance, as was observed in Wu-0, have previously been observed in the *Arabidopsis* ecotype Sf-2 when inoculated with 30 strains of *P. syringae* pv. *tomato* (Whalen et al. 1991) and in the natural *Linum marginale*-*Melampsori lini* plant-fungus system (Burdon et al. 1999). The development of a HR in Wu-0 confirms that the resistance observed in this line can be attributed, at least in part, to an inducible defense response, presumably involving one or more *R*-genes.

Variation in response to *P. viridiflava* (as assayed through disease symptoms) was also evident on a local scale, among plants collected from Indiana and Michigan *Arabidopsis* populations (within a 70-km radius) and even between pairs of plants from the same population. For instance, the Michigan plant Pna-7 was more susceptible to the *P. viridiflava* isolates

Table 3. Leaves (%) with HR symptoms on *Arabidopsis* ecotype Wu-0 infected with six *Pseudomonas viridiflava* strains

<i>P. viridiflava</i> strain	HR after 24 h	HR after 48 h
KNOX3.4a	70.8	83.3
LP23.1a	66.6	93.8
ME3.1b	70.8	81.3
PNA3.3a	68.8	100.0
RMX3.1b	91.7	91.7
RMX23.1a	80.0	85.4

Table 4. Diseased leaves (%)^z

<i>A. thaliana</i>	Origin worldwide	<i>P. viridiflava</i>						
		KNOX3.4a	LP23.1a	ME3.1b	PNA3.3a	RMX3.1b	RMX23.1a	PNA29.1a*
Aa-0	Germany	3	34	19	0	13	6	78
Chi-0	Russia	0	41	6	0	22	3	3
Col-0	USA	19	16	44	0	34	31	100
Cvi-0	Cape Verde Island	19	22	50	0	44	6	100
Kas-1	India	63	100	31	63	94	56	78
Kz-8	Kazakhstan	19	13	28	0	34	16	9
Ler-0	Germany	22	16	6	0	53	22	53
Mt-0	Libya	25	3	6	0	9	3	6
Nd-1	Germany	19	22	6	16	6	3	88
Oy-0	Norway	28	9	0	3	9	6	78
Pu2-2	Czechoslovakia	3	44	9	0	22	0	9
Ren-20	France	19	50	0	0	13	6	16
Shakdara	Tajikistan	16	41	16	50	38	9	13
Tsu-0	Japan	6	22	13	3	28	25	0
Tul-0	USA	9	41	6	3	28	16	16
Ws-0	Russia	22	6	0	6	9	0	50
Wu-0	Germany	3	0	0	0	3	0	28
Local field populations								
Dem-2	Indiana	34	38	31	0	6	19	69
Dem-4	Indiana	28	31	25	0	0	28	56
Gr-13	Michigan	53	63	53	22	94	56	88
Gr-42	Michigan	28	22	9	0	6	44	94
Knox-3	Indiana	34	16	6	0	16	22	19
Knox-11	Indiana	9	9	0	0	9	25	3
Pna-7	Michigan	6	69	41	0	9	63	6
Pna-30	Michigan	9	9	16	0	16	13	0

^z At day 4 postinfection with *Pseudomonas viridiflava* (10⁵ CFU per ml) and ^{*}*P. syringae* (10⁶ CFU per ml) for 17 *Arabidopsis thaliana* ecotypes and pairs of plants from four local populations

than Pna-3 from the same population. These results suggest that variation in resistance to *P. viridiflava* is found within *Arabidopsis* populations, as has been found for a number of other natural plant-pathogen interactions (Burdon et al. 1999; Geffroy et al. 1999; Harry and Clarke 1987).

Although there was generally a strong correlation between the number of symptomatic leaves and the bacterial titer within a particular accession, we nevertheless found some exceptions. In particular, some accessions are relatively free of symptoms despite supporting high bacterial growth. For example, the accession Knox-3 showed symptoms in only 6% of leaves when infected with ME3.1b (associated with bacterial levels of 7.27 log CFU per leaf disk) whereas the accession Cvi-0 showed symptoms in 50% of leaves when infected with ME3.1b (associated with bacterial levels of 5.5 log CFU per leaf disk). The ability of particular accessions to tolerate bacterial growth without symptom development is not well studied, although at least one published example exists. Although *Xanthomonas campestris* pv. *campestris* achieves the same titer in leaves of two *A. thaliana* ecotypes, Columbia and Pr-0, Columbia remains asymptomatic whereas Pr-0 develops spreading chlorosis 3 to 4 days after infection (Buell and Somerville 1995).

Kas-1 provides another interesting exception in that it appears to show heightened susceptibility; clear disease symptoms appear at only moderate bacterial titers (for example, log 5.3 to log 5.9 for five bacterial isolates). Parker and associates (1993) have also observed enhanced susceptibility in Kas-1; in their work this susceptibility was to a virulent strain of *X. campestris* pv. *campestris* carrying the *avrXca* gene from *X. campestris* pv. *raphani*. On the other hand, Kas-1 is known to be highly resistant to infection by the fungal pathogen *Erysiphe chioracearum* (Adam and Somerville 1996), suggesting that its enhanced susceptibility is not broad spectrum.

P. viridiflava was first isolated as a plant pathogen from bean pods in Switzerland in 1927 (Billing 1970) and reportedly causes disease on a variety of commercial crops damaging roots, stems, foliage, buds, and flowers (Alippi and Wolcan 2000; Caruso and Catara 1996; Conn et al. 1993; Engelhard

and Jones 1990; Gitaitis et al. 1991; Goumas and Chatzaki 1998; Leath et al. 1989; Lukezic et al. 1983; Wilkie et al. 1973; Young et al. 1988, 1997). It additionally appears as a postharvest pathogen (Morris et al. 1992) and is known to colonize leaves of crop plants and weed species at high population levels without causing disease symptoms (Gitaitis et al. 1998; Goumas and Chatzaki 1998; Mariano and Carter 1993). In at least six systems, *P. viridiflava* is thought to reside as an epiphyte and opportunistically cause plant damage under certain environmental conditions (Alippi et al. 2000; Gitaitis et al. 1998; Goumas and Chatzaki 1998; Mariano and Carter 1993; Pennycook et al. 1991; Young et al. 1988). In such cases, disease symptoms are more frequently observed when the plants are under stressful conditions, including low temperatures, high altitudes, or high levels of rainfall, or when the plants have been wounded prior to infection. Ice nucleation activity, as observed in the six tested *P. viridiflava* isolates, may promote infection by wounding the plant tissue and contributing to overall plant stress (Lindow 1983; Lindow et al. 1982).

Soft rot is the most commonly reported disease symptom of *P. viridiflava*. Characteristic of infection with *P. viridiflava* was some inconsistency in disease development, both within the plant and within the plant lines. As shown for other soft rot pathogens, 100% humidity is necessary to observe disease under greenhouse conditions (Ren et al. 2001). A typical infection protocol is to cover plants with plastic bags or to keep plants in mist chambers (Caruso and Catara 1996; Goumas and Chatzaki 1998; Hu et al. 1999; Jones et al. 1984; Young et al. 1997). *P. viridiflava* is very sensitive to environmental conditions, and we tested a large number of infection techniques before reliable results could be obtained. Our infections of *Arabidopsis* with *P. viridiflava* in controlled growth chamber conditions were most successful when the plants were kept at 100% humidity for 24 to 72 hours. Lower humidity decreased the virulence of the tested bacteria or resulted in no visible disease symptoms. The humidity likely creates favorable conditions for pathogen development and may increase the susceptibility of the plant to pathogen attack by creating suboptimal growth conditions. This extreme sensitivity on

Table 5. Number of bacteria (log CFU/leaf disk) at day 4 postinfection with *Pseudomonas viridiflava* (10^5 CFU/ml) and *P. syringae* (10^6 CFU/ml) for 17 *Arabidopsis thaliana* ecotypes and pairs of plants from four local populations

<i>P. viridiflava</i>	KNOX3.4a	LP23.1a	ME3.1b	PNA3.3a	RMX3.1b	RMX23.1a	PNA29.1a*
<i>A. thaliana</i>	F(24,71) = 2.93; p < 0.0002	F(24,73) = 3.26; p < 0.0001	F(24,64) = 3.32; p < 0.0001	F(24,75) = 5.70; p < 0.0001	F(24,66) = 2.92; p < 0.0003	F(24,68) = 6.39; p < 0.0001	F(24,72) = 4.24; p < 0.0001
Aa-0	4.58	5.58	5.59	3.59	5.04	4.07	6.14
Chi-0	3.46	6.39	3.43	3.52	4.45	4.04	4.02
Col-0	6.84	5.63	5.97	4.42	7.29	4.04	5.89
Cvi-0	5.18	5.97	5.50	4.57	7.79	4.26	5.57
Kas-1	5.73	5.35	5.74	5.84	5.79	7.55	4.94
Kz-8	4.29	4.59	5.95	4.08	4.93	3.82	3.41
Ler-0	6.85	5.38	4.31	4.92	7.38	4.56	3.68
Mt-0	5.00	3.72	3.15	4.72	4.14	3.16	3.96
Nd-1	5.52	5.10	4.62	5.06	5.37	3.26	6.04
Oy-0	8.10	6.55	3.86	4.88	6.73	4.30	5.54
Pu2-2	4.27	6.69	3.67	4.59	6.08	3.72	4.10
Ren-20	4.80	8.19	4.44	4.80	4.93	4.46	4.64
Shakdara	5.28	9.06	3.61	6.42	6.68	4.58	4.65
Tsu-0	4.60	6.81	4.22	4.78	3.82	4.21	4.16
Tul-0	3.36	6.54	5.10	4.86	5.85	5.52	4.33
Ws-0	5.88	6.23	3.88	5.09	5.31	4.92	5.32
Wu-0	4.14	5.24	4.29	4.98	4.69	5.03	4.29
Dem-2	5.58	8.26	6.01	4.72	4.85	6.28	4.66
Dem-4	3.78	7.57	7.70	4.67	5.35	6.92	5.89
Gr-13	6.36	8.19	7.77	4.83	7.43	6.68	5.24
Gr-42	6.01	7.99	6.60	4.45	5.06	8.98	6.26
Knox-3	4.81	5.98	7.27	4.38	4.82	6.37	3.82
Knox-11	4.47	6.60	4.63	4.67	4.80	8.25	3.42
Pna-7	4.33	7.46	7.05	4.58	7.08	8.62	4.62
Pna-30	5.27	5.23	4.92	4.44	4.65	6.39	3.45

environmental conditions was not observed in our infections with the locally collected *P. syringae* strain, which exhibited more uniform disease symptoms. A higher cell concentration was used in the infections with *P. syringae*, but preliminary experiments varying the infection concentration of *P. viridiflava* did not reveal higher uniformity in disease symptoms with higher infection concentration.

In summary, our experiments revealed variation in virulence among *P. viridiflava* isolates originating from several *Arabidopsis* populations and variation in the resistance and tolerance of *Arabidopsis* lines to these isolates. These patterns are suggestive of polymorphism in the genes involved in resistance in the plant and virulence in the pathogen and are consistent with the high level of specificity in the gene-for-gene relationship, the diversity observed in other plant-pathogen systems, and a dynamic relationship between host and pathogen. *P. viridiflava* and *P. syringae* belong to a group of bacterial pathogens that has been characterized extensively at the molecular level and are as suitable for genetic studies as *Arabidopsis*. The relationship between these *Pseudomonas* pathogens and Midwestern *Arabidopsis* populations will provide the natural system necessary for further study of the coevolution of plant-pathogen interactions.

MATERIALS AND METHODS

Field collection of bacterial isolates.

Diseased leaves were collected from plants in seven Indiana and Michigan *Arabidopsis* populations in the spring of 1999 and 2000. Four of the sites are agricultural fields, three of which (Lp and Rm in Indiana and Pna in Michigan) were culti-

vated with corn in the year preceding our collections and one of which (Me in Michigan) was cultivated with soybeans in the preceding year. Sites Rmx (Michigan), Knox (Indiana), and Ez (Indiana) are fallow fields.

The leaves were surface-sterilized with 70% ethanol, ground, and resuspended in 10mM MgSO₄. Aliquots from this solution were plated onto King's medium B (KB). Single bacterial colonies were selected from these plates following 48 h. of incubation at 28°C. Colonies were streaked on KB, grown overnight in liquid KB, and stored at -80°C in 15% glycerol.

In order to estimate rates of infection, we collected one leaf per plant from 26 to 60 *Arabidopsis* plants, chosen at random along two to four transects in each population, between April 14 and 26, 2000. The leaves were collected directly into sterile Eppendorf tubes, and later, bacteria were isolated as described above.

Bacterial identification.

The LOPAT determinative tests were performed according to standard methods (Lelliott et al. 1966; Schaad 1980). The protocol of Suslow and associates (1982) was used for the gram test, and ice-nucleating activity was tested as described by Klement and associates (1990).

The LOPAT test differentiates *P. syringae* and *P. viridiflava* only by the potato test. Since the results of the potato test were not always definitive, we additionally confirmed these results by developing a PCR assay to distinguish the *P. syringae* from the *P. viridiflava* isolates. This assay was based on a diagnostic restriction site in a 414-bp portion of the 16S rRNA. Bacterial genomic DNA was extracted following the protocol of Ausubel and associates (1996). The lower primer and conditions used

Fig. 3. Number of bacteria and diseased leaves for the tested *Arabidopsis thaliana* ecotypes at day 4 postinfection at a concentration of 10⁵ CFU per ml averaged over the six locally collected *Pseudomonas viridiflava* strains.

for the amplification of the 414-bp fragment of the 16S gene were as described by Jones and Bej (1994). The upper primer was created according to the 16S *P. viridiflava* GenBank sequence (16R874: 5'-CCGTCAATTCATTTGAGTTT-3'). These primers are located at positions 482 and 874 of the *P. viridiflava* 16S sequence (GenBank accession number Z76671). One unit of Ban II (New England Biotech) was added to 4 µl PCR product and incubated for 3 h at 37°C. The total reaction volume was analyzed on a 2% agarose gel. In *P. viridiflava*, Ban II cuts this fragment at nucleotide 712 of the *P. viridiflava* 16S sequence, whereas in *P. syringae*, it cuts at nucleotide 816 of the *P. viridiflava* 16S sequence.

Sequencing.

Bacterial strains for sequencing were streaked onto KB and incubated for 48 h at 28°C. Colony PCR was performed using 1.875 µM MgCl₂, 0.25 µM dNTPs, 100 nM primer, and 2.5 units of Taq polymerase. The primers and conditions for the amplification of the 16S gene were from Moore and associates (1996). Four additional sequencing primers were constructed in accordance with the published *P. viridiflava* 16S sequence (GenBank accession number Z76671). These four primers are as follows: 16F407 5'-AAGTTGGGAGGAAGGCAGTA-3'; 16R1037 5'-ACCCAACATCTCACGACACGA-3'; 16F839 5'-AAGTTGACCGCTGGGGAGTA-3'; 16R620 5'-CACCAC-CCTCTGCCCTACTCT-3'.

The 280-bp partial *hrpS* coding sequence, corresponding to positions 30,031 through 30,310 in *P. syringae* pv. *tomato* DC 3000 (Deng et al. 1998 [accession number AF232004]), was amplified using combinations of the following primers: (forward primer) *hrpS*-f 5'-CTCCAGGCCAAGCTGCTGAGG-GTG C-3'; *hrpS*-f2 5'-CGGAACGCTGTATCTGGATGA-3'; (reverse primer) *hrpS*-r 5'-TTGAGCTCACGGATATTGCCG-GCCC-3'. The 555-bp complete coding region of the *hrpL* gene was amplified using the following primers based on Alfano and associates (2000) [accession number AF232004]: *hrpL*-f 5'-TTTTGGCTGGCACGGTTATCGCTAT-3'; *hrpL*-r 5'-TGTTGGTTTGGCTGCGAGTTGGTTC-3'. Additional internal primer sets, *HrpL*82f 5'-GACCAGATCCARATGCT-CAG-3' and *HrpL*439r 5'-TCCTGATAGTTGCCRTCCAT-3', were used for PCR and sequencing of the *hrpL* in four *P. viridiflava* strains for which we were unable to amplify PC products of the entire *hrpL* gene. PCR amplification was performed under the following conditions. After initial denaturation at 95°C for 2 min 30 s, 36 cycles of the following sequence were run: 30 s denaturation at 95°C, 40 s annealing at 51°C, 1 min 15 s extension at 72°C. A final extension for 7 min at 72°C completed the reaction. The samples were stored at 4°C.

Fig. 4. Correlation between number of bacteria in the leaf (log CFU/leaf disc) and diseased leaves per plant (number) for the infection of 17 *Arabidopsis thaliana* ecotypes and four pairs of plants of local ecotypes with six locally collected *Pseudomonas viridiflava* strains at a concentration of 10⁵ CFU per ml.

Plant materials.

The local plant lines used in the infection experiments originated from single *Arabidopsis* plants in disturbed or agricultural fields in Michigan (Gr-13, Gr-42, Pna-7, Pna-30) or Indiana (Dem-4, Dem-4, Knox-3, Knox-11). The seeds from worldwide populations were obtained from the Nottingham *Arabidopsis* Stock Center. Seeds were germinated after a 2-day cold treatment and grown in the greenhouse under short day (12 h) conditions at 20°C for 19 days prior to infection.

Infection procedure.

We tested a number of methods for infection of *Arabidopsis* with *P. viridiflava*, including wounding with Carborundum or cutting with a scalpel at the time of infection and dipping the plant leaves into a suspension of Silwett and bacteria. We tested concentrations between 10⁴ and 10⁸ CFU per ml, and varied postinfection humidity conditions from 70 to 100%. We found that the infiltration of a cell suspension of 3 × 10⁵ CFU per ml with a blunt-end syringe and 100% humidity for 72 h after infection gave the most consistent disease symptoms among leaves. We used this infection method for all of the infection experiments.

The *P. viridiflava* isolates used for infection were incubated on KB for 48 h at 28°C. Single colonies were grown overnight in liquid KB media at 28°C, diluted 1:10 the following morning, and grown to an optical density at 600 nm of 0.7 to 0.9. The bacteria were resuspended in 10 mM MgSO₄ prior to infection and diluted to the required concentration (10⁵, 10⁶, or 10⁸, depending on the experiment). Two randomly selected leaves per plant were infiltrated with approximately 25 µl of bacterial suspension using a blunt-end syringe. Negative control plants were inoculated only with 10mM MgSO₄.

Bacterial growth curves.

Two leaves of 36 plants of the Pna-7 ecotype were infected with each of the six *P. viridiflava* isolates at 3 × 10⁵ CFU per ml. One leaf per isolate was harvested from a randomly selected plant at each of six time points: 0, 1, 2, 3, 4, and 6 days postinfection. The plants were kept at 100% humidity under closed lids for 72 h. Lids were then removed, and the infected plants were exposed to the 50% humidity of the growth chamber. Homogenized hole-punches from sampled leaves were serial-diluted and plated onto KB. Colony number was determined following a 48-h incubation at 28°C. The colonies were counted by eye, with the ProtoCol Colony Counting system (Don Whitley Scientific Limited, Shipley, U.K.), or both. The 48 leaves that remained on day 4 in each experiment (72 were originally infected, 24 were harvested on days 0 to 3) were

Fig. 5. Correlation between diseased leaves and number of bacteria in the leaf at day 4 after infection of *Arabidopsis thaliana* ecotypes with the field-collected *Pseudomonas syringae* strain PNA29.1a at a concentration of 10⁶ CFU per ml.

scored for lesion size on day 4 (on a scale of 1 to 9 in which 1 = no lesion and 9 = complete leaf surface diseased).

Ice-nucleating activity.

Ice-nucleating activity was determined following the protocol of Schaad (1980). We used aluminum weighing dishes (Fisher Scientific, Pittsburgh) as a test surface rather than spraying aluminum foil with paraffin.

HR test.

The Wu-0 ecotype was used to test for a HR against each of the six *P. viridiflava* isolates. For each isolate, 16 plants were infected at a concentration of 2.5×10^8 CFU per ml. The plants were kept at low humidity (approximately 50%), and scored for possible HR symptoms at 24 and 48 h postinfection.

Ecotype experiment.

Ecotypes (21) representing 4 local and 17 worldwide samples were infected at a concentration of 3×10^5 CFU per ml with each of six *P. viridiflava* isolates. There were 16 replicates of each ecotype for infection by each isolate. Following this inoculation, the plants were kept at 100% humidity for 72 h and then were kept at 50% humidity. Plants were scored at the fourth day postinfection. Leaf disks were harvested for bacterial count as described above. Colony identity was checked using the LOPAT scheme. For this set of isolates, the potato test gave a clear result, and thus, the PCR assay was not necessary.

Statistics.

The statistical tests were performed with the software package Statistica 5.7 (Statsoft, Inc., Tulsa, OK, U.S.A.). A two-way analysis of variance was used to compare the effects of the ecotypes, isolates, and their interactions, treating the ecotype and isolates as fixed effects. For the daily growth curve experiment, growth after 4 days was analyzed with a one-way analysis of variance considering isolate as a fixed effect, followed by a Duncans test.

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