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VARIATION IN RESISTANCE AND VIRULENCE IN THE INTERACTION BETWEEN *ARABIDOPSIS THALIANA* AND A BACTERIAL PATHOGEN

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Abstract.—We examined patterns of variation and the extent of local adaptation in the interaction between the highly selfing annual weed *Arabidopsis thaliana* and its foliar bacterial pathogen *Pseudomonas viridiflava* by cross-infecting 23 bacterial isolates with 35 plant lines collected from six fallow or cultivated fields in the Midwest, USA. We used two measures of resistance and virulence: bacterial count in the leaf and symptom development four days after infection. We found variation in resistance in *A. thaliana* and virulence in *P. viridiflava*, as well as a significant difference in symptoms between two distinct genetic clades within *P. viridiflava*. We also observed that both resistance and plant development rate varied with field type of origin (cultivated or fallow), possibly through age-related resistance, a developmentally regulated general form of resistance. Finally, we did not observe local adaptation by host or pathogen, rather we found patterns of variation across populations that depended in part on *P. viridiflava* clade. These data suggest that the interaction between *A. thaliana* and *P. viridiflava* varies across space and is mediated by the selection regime of the host populations and differential performance of the *P. viridiflava* clades. This is one of a very limited number of studies examining a bacterial pathogen of wild plant populations and one of a few studies to examine patterns of variation in a plant-pathogen association that is not a highly specialized gene-for-gene interaction.

Key words.—Coevolution, flowering time, geographic mosaic, host-pathogen interaction, local adaptation, plant-pathogen interaction, *Pseudomonas viridiflava*.

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Parasite local adaptation (or maladaptation) has been looked to by researchers as an indicator of coevolutionary dynamics between parasites and their hosts (Kaltz and Shykoff 1998). To achieve coevolutionary cycling and a pattern of parasite local adaptation, a genotype-specific interaction is required between host and parasite (e.g., Parker 1994; Kaltz and Shykoff 1998; Sasaki and Godfray 1999). In plant-pathogen interactions, this specific interaction generally takes the form of the gene-for-gene recognition system, in which products of resistance genes in the plant recognize the presence of specific pathogen proteins (effectors) and trigger a rapid resistance response (Staskawicz et al. 1995; Hammond-Kosack and Jones 1996). The gene-for-gene mechanism can be the predominant factor determining the response of the plant to encounters with the pathogen and vice versa. Yet, only a few wild pathosystems have shown clear evidence of local adaptation by plant or pathogen (Parker 1985; Kaltz et al. 1999; Thrall et al. 2002). Effects of gene flow and random processes, such as genetic drift and bottlenecks, are often invoked to explain the failure to see local adaptation in gene-for-gene systems.

The expectations regarding whether or not local adaptation should be observed are less clear in systems in which gene-for-gene resistance does not dominate the interaction. This resistance is often described as quantitative rather than qualitative. The mechanisms maintaining variation in quantitative interactions are thought to include fitness trade-offs (Frank 1993; Thrall et al. 2005) together with temporal and spatial heterogeneity in the environment (Ennos and McConnell 1995). For example, there could be a competition versus transmission trade-off among pathogen genotypes (Levin and Pimentel 1981; Nowak and May 1994), or certain forms of metabolic costs of virulence and resistance that create diversifying selection (Frank 1993). There is also increasing evidence that quantitative interactions may include underlying polygenic genotype-specific resistance (Leonards-Schippers et al. 1994; Caranta et al. 1997; Geffroy et al. 2000; Talukder et al. 2004).

Virulence factors found in some phytopathogenic bacteria may affect interactions with plant hosts in a quantitative rather than qualitative manner (Abramovitch and Martin 2004). For example, some strains of the foliar bacterial pathogen *Pseudomonas syringae* contain the toxin coronatine, which suppresses targeted defense responses by activating an alternate antagonistic pathway (Zhao et al. 2003; Brooks et al. 2005). A more dramatic example may be the pectate lyases used by soft-rotting bacteria to break down plant cell walls and gain direct access to nutrients (Collmer and Keen 1986). Here, underlying specific recognition of these bacteria may only be apparent when the soft-rotting activity is weakened or experimentally knocked out (Bauer et al. 1994). Coevolution between these pathogens and their plant hosts may be driven more by the costs and benefits of, or trade-offs between, different strategies for resistance or virulence than by specific recognition. In a survey of *P. syringae* strains, isolates with more than one of four tested toxins (tabtoxin, phaseolotoxin, syringomycin, and coronatine) were rare, suggesting that they may indeed confer a metabolic cost (Hwang et al. 2005).

Expectations for a particular host-parasite interaction may additionally be complicated by other mediating factors. For example, it is not clear whether one should expect parasite local adaptation when parasites have broad host ranges, whatever the genetic mechanism underlying the interaction. An analysis of the parasite local adaptation literature comparing parasites with potentially broad versus narrow host ranges found that studies examining parasites with broad host ranges were less likely to find local adaptation by the parasite (La-

jeunesse and Forbes 2002). The spatial scale at which to study an interaction can also be unclear a priori. For example, high levels of gene flow or random processes can obscure coevolutionary patterns at small scales (Gandon et al. 1996; Thrall and Burdon 2000), but adaptation could then be observed at a larger spatial scale (e.g., Thrall et al. 2002; Laine 2005).

Factors determining the evolutionary dynamics of a plant-pathogen interaction should be highly dependent on the life history of the pathogen (Thrall and Burdon 1997). However, we currently have little data with which to explore variation in patterns of evolution across the spectrum of plant-pathogen associations. For example, bacteria, viruses and nematodes have been so understudied in natural settings, compared to fungal pathogens, that literature reviews of natural plant-pathogen interactions tend to not cover diseases caused by these types of pathogens (e.g., Alexander 1990; Burdon 1991; Jarosz and Davelos 1995). For bacteria in particular, this is likely related to the difficulty in studying these pathogens. Bacterial pathogens do not produce reproductive structures on the plant surface that can be used for rapid identification, and the yellowing and necrosis that they cause is difficult to distinguish from damage due to other causes. Furthermore, infected plants may be entirely asymptomatic. As a consequence, the simple identification of infected plants is an intensive process usually requiring the culturing and subsequent identification of bacteria from each plant of interest. Another complication is that species designations in bacterial pathogens are unclear, such that even if the bacteria are identified to species, different isolates may represent distinct genetic subspecies. For example, the common bacterial plant pathogen *Pseudomonas syringae* is in fact a large species complex, composed of numerous pathovars that can themselves be polyphyletic (Sarkar and Guttman 2004).

Bacterial plant pathogens have been studied almost entirely in association with agricultural crops, consequently there is little knowledge of the host range of these populations. Few studies of bacterial pathogens have explicitly examined host range, and because of the problems in identifying taxonomic units, we would argue that very little is really known about the host breadth of single isolates or lineages. Foliar bacterial pathogens are thought to be poor dispersers, relying primarily on rain splash for transmission between plants, a much less efficient method than viruses that use insect vectors or fungal pathogens adapted for wind dispersal. As a result, a single species host range could be a risky strategy for a bacterial pathogen, except perhaps when its host is an agricultural crop planted in monoculture. Many bacterial phytopathogens are thought to be able to survive epiphytically on the outer surfaces of leaves. It is not clear whether this behavior expands their effective host range or transmission efficiency.

Here we examine the extent to which the soft-rotting foliar bacterial pathogen *Pseudomonas viridiflava* is locally adapted to the annual weed *Arabidopsis thaliana*. In doing so, we are interested in the amount and patterns of variation in virulence in *P. viridiflava* and resistance in *A. thaliana*, because so little data is available on interactions involving wild plant populations and foliar bacterial pathogens. We further wished to investigate whether phenotypic variation in virulence corresponded to the genetic variation we observed in *P. viridiflava* in a previous study (Goss et al. 2005). In particular, we were interested in whether there is a detectable functional difference between two genetically distinct clades found within *P. viridiflava*. To examine these questions, we cross-inoculated plant lines and pathogen isolates collected from fallow and cultivated fields in northwest Indiana and southwest Michigan, USA, and assessed resistance and virulence using symptom development and bacterial growth four days after inoculation.

Materials and Methods

Study systems

Pseudomonas viridiflava is a soft-rotting, gram-negative bacterial pathogen of a number of commercial crops, causing lesions on foliage and rotting fleshy material such as fruits and tubers (Billing 1970; Wilkie et al. 1973; Liao et al. 1988). It causes localized infections and is not systemic. Many consider it to be an opportunistic pathogen, only causing severe disease when conditions are favorable (e.g., Jones et al. 1984; Young et al. 1988; Pennycook and Triggs 1991; Mariano and McCarter 1993). Its host range is notably wide given that *P. viridiflava* is not subdivided into host-specific pathovars, as is *P. syringae*. It is possible that the species is a composite of subspecies with restricted host ranges, yet *P. viridiflava* strains collected from one host have been reported to cause disease in other host species (Leben 1981). *Pseudomonas viridiflava* can be epiphytic and has been observed at high densities on leaves with no associated disease symptoms (Young et al. 1988; Mariano and McCarter 1993; Gitaitis et al. 1998).

In the Midwest, USA, *P. viridiflava* is a common bacterial pathogen of the annual weed *Arabidopsis thaliana* (Brassicaceae). It is one of a few known natural pathogens of *A. thaliana* (Jakob et al. 2002), a plant that has become an important genetic model system and a focus of molecular genetic research on plant response to pathogen infection. *Arabidopsis thaliana* contains numerous polymorphic resistance genes (Bergelson et al. 2001) and the pathways by which it responds to infection are being increasingly well characterized (Glazebrook 2005). There is a growing body of work on the evolution of resistance genes in *A. thaliana*, several of which are known to recognize bacterial effectors, yet the majority of this work uses bacterial isolates collected from other host species.

In the Midwest, *A. thaliana* is a winter annual; seeds germinate in the late fall and plants bolt in April, setting seed by June. It is a highly inbred plant, with a selfing rate of roughly 99% (Abbot and Gomes 1989; Bergelson et al. 1998a, 1998b). Midwest *A. thaliana* populations can have limited genetic variation within populations due to its high degree of self-fertilization, yet some population samples have been surprisingly variable and neighboring populations may be distinct in their genetic composition (Bergelson et al. 1998b; Bakker et al. 2006). The relatively recent colonization of the Midwest by *A. thaliana* is evident in shared genotypes across Midwest populations and extensive linkage disequilibrium among Midwest genotypes, likely the result of a genetic bottleneck upon introduction (Nordborg et al. 2005; Bakker et al. 2006). Nevertheless, there is evidence of var-

TABLE 1. Collection sites of *Arabidopsis thaliana* lines and *Pseudomonas viridiflava* isolates used in inoculations.

Site	Location (GPS coordinates)	Field type	No. plant lines	No. bacterial isolates	
				Clade A	Clade B
Knox	Knox, IN (−86.62, 41.265)	fallow	9	0	4
Rm	La Porte County, IN (−86.74, 41.36)	cultivated	3	2	3
Rt	La Porte, IN (−86.76, 41.59)	fallow	2	2	3
Lh	Bridgman, MI (−86.51, 41.96)	cultivated	5	3	1
Rmx	St. Joseph, MI (−86.51, 42.03)	fallow	9	—	—
Me	Benton Harbor, MI (−86.36, 42.09)	cultivated	7	5	0

iation in disease resistance in and among Midwest populations (Stahl 2000; Jakob et al. 2002).

A study of population genetic variation in *P. viridiflava* showed two distinct, genetically diverse clades that can be found within meters of each other (Goss et al. 2005). Synonymous site divergence (K_s) between clades averaged 33% based on 2.3 kb of DNA sequence over five loci, while within clades there was evidence of frequent recombination. *Pseudomonas viridiflava* showed significant genetic variation within populations but little to no population structure when the two clades were taken into account.

In gram-negative bacteria, pathogenicity is conferred by the Type III secretion system, which delivers pathogenicity and virulence factors into host cells, including effectors involved in gene-for-gene recognition (Staskawicz et al. 1995; Hammond-Kosack and Jones 1996). The components of this system are often located in a physical gene cluster called a pathogenicity island (PAI). As a species, *P. viridiflava* appears to contain two alternate types of PAI with different structures, designated the S-PAI and the T-PAI (Araki et al. 2006). *Pseudomonas viridiflava* isolates containing the more common S-PAI caused larger lesions on average than isolates with the relatively rare T-PAI on *A. thaliana*, but isolates with the T-PAI elicited a more rapid hypersensitive (resistance) response in tobacco. In relation to the two *P. viridiflava* clades, isolates with the T-PAI were only found in clade A, whereas the S-PAI has been observed in both clades. It is possible that the two distinct clades and the two types of PAIs may be indicative of host specialization within *P. viridiflava*.

Both *P. viridiflava* and *A. thaliana* have the genetic machinery required for a gene-for-gene interaction (The Arabidopsis Genome Initiative 2000; Araki et al. 2006). Jakob et al. (2002) observed specificity in inoculations with Midwest *P. viridiflava* isolates and a worldwide sample of *A. thaliana* genotypes suggestive of a gene-for-gene interaction. Yet, resistance to *P. viridiflava* in *A. thaliana* appears to primarily be a function of the jasmonic acid pathway, a defense pathway used to fight infection by necrotrophs, in contrast to the salicylic acid-dependent resistance observed in classic gene-for-gene interactions (K. Jakob, J. Kniskern, and J. Bergelson, unpubl. ms.). The importance of the jasmonic acid pathway suggests that the pectate lyase activity of *P. viridiflava* may play an important virulence function in this interaction.

Pseudomonas viridiflava may use multiple host species (Gitaitis et al. 1998; Goumans and Chatzaki 1998). In the Midwest, *A. thaliana* population infection rates can reach nearly 50% (Goss 2005), but we have collected genetically similar isolates from other common weed species (Goss et al. 2005). *Pseudomonas viridiflava* is not known to infect corn or soybean, which are the dominant crops in and around the particular fields used in this study, and is therefore unlikely to be primarily associated with crop plants. If *P. viridiflava* does use multiple hosts, this could be a major factor contributing to the genetic variation observed in these populations.

Collection of material

Six sites in which *P. viridiflava* was found to infect *A. thaliana* were chosen (Table 1). Three of the sites were located in Berrien County in southwest Michigan and three in Starke and LaPorte Counties in northwest Indiana. One Michigan and two Indiana sites were fallow or old fields. The remaining sites were agricultural fields; Rm was planted with corn in the years immediately prior to seed collection, whereas Me and Lh were cultivated with soybean, which is typically planted after herbicide treatment rather than plowing.

To guard against the possibility of sampling multiple demes and mistakenly designating them as a single population, we took a conservative approach of sampling a small area within each site. In April 2001, leaves exhibiting necrosis and/or chlorosis were collected from 50 plants within a single 10-m diameter circle at each site. Leaves were collected into a sterile 1.6 ml microcentrifuge tube and kept on ice during transport. In the laboratory, leaves were surface sterilized with 70% ethanol, ground in sterile buffered water (10mM $MgSO_4$), and plated on King's Medium B (KB). After incubation at 28°C for two days, colonies were picked from these plates, reisolated if necessary, and a glycerol stock of each colony was prepared for indefinite storage at −80°C. Colonies were later screened and *P. viridiflava* isolates were identified (Jakob et al. 2002; Goss et al. 2005).

Throughout May 2001 plant seed was collected from the same locations as plants matured and without regard to infection status. Each plant was selfed for one generation in the greenhouse to obtain the quantity of seed required for the following experiments. *A. thaliana* shows a high level of homozygosity (Nordborg et al. 2005), therefore this selfing should produce identical siblings in most or all cases.

To distinguish plant lines from bacterial isolates, the names of *P. viridiflava* populations are given in all caps (e.g., KNOX), whereas sites or *A. thaliana* populations are referred to with only the first letter capitalized (e.g., Knox).

Selection of A. thaliana lines and P. viridiflava isolates

Inoculation of *A. thaliana* with *P. viridiflava* does not produce a clear hypersensitive response, diagnostic of gene-for-

gene recognition, and must be evaluated in a quantitative manner. Symptomatic leaf area is a measure often used to evaluate quantitative interactions, but infection by bacterial pathogens is also usually evaluated by measuring bacterial growth in the leaf. Although these measures are generally correlated (Jakob et al. 2002; Kover and Schaal 2002), *in planta* bacterial growth and lesion development may make different contributions to fitness (Wichmann and Bergelson 2004). We therefore thought it important to measure both bacterial growth and symptoms. Bacterial titers are time consuming and intensive and thus to maximize the information obtained from the inoculations, we chose to exclude potentially identical genotypes from both pathogen and plant populations. This decision was informed by previous studies indicating the potential for clones to be included in random samples of individuals of *P. viridiflava* (Goss et al. 2005) and *A. thaliana* (Bergelson et al. 1998b; Bakker et al. 2006) from Midwest populations. This approach makes no assumptions about resistance or virulence of each genotype. This method further allowed us to evaluate phenotypic variation given genetic variation, because genetic variation does not ensure variation in resistance or virulence.

Genomic DNA sequence from five loci in *P. viridiflava* was generated as part of another study using a random sample of isolates from each site (Goss et al. 2005) and these isolates were later typed for PAI by Araki et al. (2006). Plant lines were typed at three microsatellite loci, nga106, nga111, and nga168 (Bell and Ecker 1994), shown to be polymorphic in Midwest populations (Bakker et al. 2006). Plant total genomic DNA was extracted using the Wizard Magnetic 96 DNA Plant System (Promega Corp., Madison, WI). Primers labeled with fluorescent dyes (Genset) were used to amplify each locus by PCR using the following conditions: 95°C for 2.5 min, 36 cycles of 95°C for 30 sec, annealing for 40 sec and 72°C for 90 sec, finishing with 7 min at 72°C; annealing temperature was 50°C for nga106 and nga111 and 55°C for nga168 (Bakker et al. 2006). Fragment length was measured with the fragment analysis module of a CEQ 8000 sequencer (Beckman Coulter, Fullerton, CA).

Inoculations

Arabidopsis thaliana lines were inoculated with each of 23 *P. viridiflava* isolates, replicated five times. Inoculations were replicated over days, with only one replicate of each plant line inoculated with a particular bacterial isolate on any given day.

For each inoculation, all 35 plant genotypes were sown in a single 48-cell 1020 flat in 1:1 Promix:Metro soil and cold treated at 5°C for three days. Flats were then placed in a growth room at 20°C under short day (12 h) light conditions. After 21 days in the growth chamber (approximately 19 days after germination), the 3rd and 4th leaves of each plant line were inoculated using a single bacterial isolate per flat. The inoculation solution was prepared as follows. Three days prior to inoculation, the bacterial isolate was streaked out on a KB plate from the glycerol stock and incubated at 28°C for two days. One day prior to inoculation, one colony was picked from this plate and suspended in 100 μl 10mM $MgSO_4$. From this suspension, 50 μl was plated on a new KB plate and incubated overnight. The following morning, the bacteria were resuspended in 5 μl of 10mM $MgSO_4$, then spun down and resuspended again to remove any residual growth media. The optical density at 600 nm (OD_{600}) of the suspension was adjusted to 0.02 and then the suspension was diluted 1:100, which is equivalent to about 2×10^5 colony-forming units (cfu) per ml. Entire leaves were infiltrated with this cell suspension using a blunt-end syringe from the underside of the leaf. To promote bacterial growth, flats were placed in a humidity controlled growth chamber at 20°C and 100% relative humidity for 24 h following inoculation, and then kept at approximately 85% humidity for three days.

Four days after inoculation, diseased leaf area was scored by eye (estimated to 0, 1, 5, 10, 20, 30, . . . up to 100% of leaf area) on both inoculated leaves on each plant. One randomly selected inoculated leaf was clipped from each plant to measure bacterial growth in the leaf. A hole punch (1.2 cm^2) was taken from the center of each clipped leaf, ground in 200 μl 10mM $MgSO_4$, and serial dilutions of the leaf homogenate were plated on KB.

Developmental measurements of plant lines

To follow up on results of the cross-inoculation experiment, we investigated the possibility that the observed variation in resistance was due to developmental differences. In particular, *A. thaliana* is known to exhibit age-related general resistance to *P. syringae* and other pathogens (Kus et al. 2002). This resistance is observed in older plants as compared to younger plants, and in older leaves relative to younger leaves on the same plant. If development rates vary between field types, then variation in developmental age at the time of inoculation could contribute to the observed variation in resistance among populations.

We examined variation in developmental timing as measured by days from germination to the appearance of the flowering stem (bolting). After three days of cold treatment at 5°C, four plants of each of the 35 lines were germinated in four-inch pots in 1:1 mix of Promix:Metro potting soil under 20°C and 12-h daylight. Pots were randomized in and across flats, which were rotated twice per week. Dates of germination and bolting were recorded for each plant. Time to bolting was adjusted by date of germination. All replicates of the plant line Me-330 had failed to bolt after more than 140 days from germination. At this time they were in very poor condition and were abandoned because it was clear that they would not survive to bolt.

We then tested for age-related resistance to *P. viridiflava* in three *A. thaliana* lines varying in resistance and bolting time. The lines Lh-304, Rt-313, and Rm-305 were sowed at one-week intervals so that at the time of inoculation they were approximately 18, 25, 32, or 39 days old. One leaf on each of 15 replicates of each plant line and age was inoculated with *P. viridiflava* isolate LH206.1a (Clade A with S-PAI) in the same manner as the above inoculations except with a slightly higher concentration of inoculum. The 4th leaf was inoculated on 18- and 25-day old plants. On the 32- and 39-day old plants, we inoculated the 4th leaf or, if this leaf had senesced, the oldest available leaf. Five replicates were harvested several hours after inoculation for a day 0 bacterial

titer and the remaining 10 replicates of each line and age were harvested four days after inoculation. Due to the age differences in the inoculated leaves, we could not evaluate symptoms in a manner that would be consistent across treatments.

Analysis

For the cross-inoculation experiment, symptomatic leaf area was averaged across the two inoculated leaves on each plant. For analysis of variance and regression, these values were ln-transformed [ln(average symptoms per plant + 1)] to conform to the assumptions of these analyses. Bacterial count (cfu) per leaf disc was log-transformed for all analyses. If no colonies were observed, the leaf disc was assumed to have contained a single colony for ease of transformation.

Nested analysis of variance was used to assess variation within and between field type (cultivated or fallow), pathogen clades, plant and pathogen populations, plant lines, and pathogen isolates. Plant lines were nested within plant population and plant populations were nested within field type. Pathogen isolates were nested within both pathogen population and pathogen clade. The field type term was meant to reflect the obvious differences between plants in cultivated versus fallow fields and there was little reason to suspect that the cultivation regime of a field would influence the pathogen population beyond the indirect effect through the plant community, therefore we did not nest pathogen population in field type.

The decision to treat factors as fixed or random effects is often quite subjective and open to debate (Newman et al. 1997). In analyzing our experiment, we treated plant and pathogen populations as fixed effects. Our rationale was that our field sites were not randomly selected from Midwestern populations, but instead were chosen to represent fallow or agricultural fields for which we had background data. We treated plant lines and pathogen isolates as random effects, because this is typically done when individuals are selected at large from a population. Note, however, that because we prescreened our individuals and selected particular genotypes, it could be argued that plant lines and pathogen isolates are fixed effects. We chose not to do that here, and instead report results of a mixed model treating plant lines and pathogen isolates as random factors, because the mixed model was conservative in the sense that it resulted in fewer significant terms. Importantly, all terms that were significant in the mixed model were also significant under a fixed effect model.

The MIXED procedure in SAS version 9.1 (SAS Institute Inc., Cary, NC) was used to analyze the nested model treating plant lines, pathogen isolates, and all interaction terms including either of these effects as random. We used REML estimation of covariance parameters, Satterthwaite approximation for the denominator degrees of freedom, and Type III tests of fixed effects.

Local adaptation across populations has previously been tested using a means contrast of sympatric versus allopatric inoculations within the overall model (e.g., Ahmed et al. 1995; Kaltz et al. 1999; Thrall et al. 2002). Our complex unbalanced model did not allow for this comparison in SAS. As an alternative approach, we tested for a difference between sympatric versus allopatric inoculations in ANOVAs on both symptoms and bacterial count, including *P. viridiflava* clade as a main effect and in interaction with the sympatric/allopatric term. Pathogen local adaptation can also be observed by examining the performance of a pathogen population on its sympatric host population compared to other host populations or by comparing sympatric and allopatric pathogen populations on a given host population (Gandon and Van Zandt 1998). We therefore tested for adaptation in each host and pathogen population by separately comparing the performance of pathogen populations on each host population and the performance of host populations inoculated with each pathogen population (as in Thrall et al. 2002). Separate ANOVAs were conducted for each host population and each pathogen population, using the Dunn-Sidak correction of *P*-values for multiple comparisons.

Analysis of variance in bolting time similarly used a nested model with plant line nested within plant population nested within field type with plant line treated as a random effect and all other factors treated as fixed. The model was analyzed using the MIXED procedure in SAS, as above. The days to bolting values were inverse-transformed to meet the assumptions of analysis of variance. For analysis of inoculations examining age-related resistance, ANOVAs were performed on log-transformed day 0 and day 4 titers with plant line and age as fixed main effects. These analyses were conducted using JMP IN version 5.1.2 (SAS Institute Inc., Cary, NC). Linear regressions were also performed using JMP.

RESULTS

Plant lines and bacterial isolates

Samples from two populations contained isolates from only one of the two *P. viridiflava* clades: KNOX contained all clade B isolates and ME contained entirely clade A isolates. The RM and RT population samples were a mix of both genetic groups (Table 1). The *P. viridiflava* sample from RMX contained only two genotypes and, due to time and space constraints, was not included in the experiment in favor of including more genotypes from the other pathogen populations. Only one isolate in the entire sample, ME210b, contained the T-PAI rather than the S-PAI. Of all the isolates that were sequenced from these sites, there were no isolates from different sites that shared haplotypes across five genomic loci. Therefore, each isolate selected for the inoculations, both within and between populations, is a distinct genotype.

In *A. thaliana*, microsatellites were genotyped at three loci as a coarse method for excluding potentially identical genotypes from population samples in order to avoid redundant inoculations. Therefore, all genotypes within each population sample are distinct. The Rm and Rt *A. thaliana* populations contained few three-locus haplotypes. Three three-locus haplotypes were identified from 18 typed plants from Rm and two from 17 Rt plants. Rmx plants were included even though the corresponding *P. viridiflava* population was not, because of the genetic diversity of the plant sample. Most three-locus haplotypes were found in only a single population, but one haplotype was found at almost all sites and was included in

TABLE 2. Analysis of variance in symptoms and bacterial count in inoculated leaves four days after inoculation. Pathogen isolate is nested within pathogen clade and pathogen population. Plant line is nested within plant population, which is nested within field type. Significance of random effects was tested using Wald *Z*-tests rather than *F*-tests. Significant *P*-values are bolded.

	Average symptoms (ln-transformed)		Bacterial count (log cfu/leaf disc)	
Source of variation: fixed effects	*F*	*P*	*F*	*P*
Pathogen clade	10.14	**0.0057**	0.17	0.6899
Pathogen population	0.62	0.6515	0.19	0.939
Field type	7.39	**0.0106**	5.51	**0.0257**
Plant population	1.59	0.2018	0.76	0.562
Clade × pathogen popn.	1.12	0.3496	0.15	0.8589
Clade × field type	0.96	0.3368	3.04	0.0831
Clade × plant popn.	2.98	**0.0182**	0.02	0.9993
Field type × pathogen popn.	0.81	0.5329	0.56	0.6897
Pathogen popn. × plant popn.	1.63	0.0677	0.78	0.7076
Clade × pathogen popn. × field type	0.12	0.8831	0.57	0.5688
Clade × pathogen popn. × plant popn.	1.97	**0.0459**	0.78	0.6199
Source of variation: random effects[1]	*Z*	*P*	*Z*	*P*
Plant line	3.37	**0.0004**	3.36	**0.0004**
Pathogen isolate	2.19	**0.0144**	2.42	**0.0078**
Plant line × pathogen isolate	—	—	—	—
Plant line × pathogen population	0.001	0.4544	—	—
Plant line × clade × pathogen popn.	—	—	—	—
Pathogen isolate × field type	0.01	0.1713	—	—
Pathogen isolate × plant popn.	—	—	0.26	.3956

[1] Terms shown with dashes had zero variance.

four population samples. While these four lines (Knox-325, Rt-302, Lh-311, and Me-306) share a three-locus haplotype, the average bolting time and resistance of these plant lines differed substantially. We believe that these lines are genetically distinct and we opted to retain all four lines in our experiments. Perhaps not surprisingly, typing only three microsatellite loci appears to underestimate the genetic variation in *A. thaliana* in the Midwest, USA.

Variation in resistance and virulence

There was significant intrapopulation variation among *A. thaliana* lines and *P. viridiflava* isolates in both bacterial count and symptom development four days after inoculation (Table 2, Fig. 1). We did not observe variation in the interaction between plant line and pathogen isolate, as would be expected under genotype-specific (i.e., gene-for-gene) resistance. There was no significant effect of pathogen or plant population of origin when the variation within populations was taken into account in the mixed model ANOVA. Yet, whether the plant line originated from a fallow or agricultural field had a significant effect on both measures of resistance (Table 2). Average resistance was significantly greater for plants from cultivated sites than for plants from fallow fields both in terms of symptoms (mean ± SE: 7.26 ± 0.37 vs. 10.04 ± 0.39) and bacterial count (mean ± SE: 4.20 ± 0.02 vs. 4.36 ± 0.02).

We also found significant variation in symptoms between the two genetic groups within *P. viridiflava* (Table 2). Clade B isolates caused significantly greater symptom development upon inoculation, on average, than clade A isolates (mean ± SE: 11.08 ± 0.45 vs. 6.80 ± 0.32). There was no difference in average bacterial count between clades. We also observed a significant interaction between clade and plant population, such that the effect of clade on symptom development varied by host population (Fig. 2). Clade B isolates exhibited relatively greater symptom development on plants from certain populations, in particular, the fallow fields Knox, Rt, and Rmx. Greater virulence of clade B isolates on plants from fallow fields was also suggested in the analysis of bacterial titer, although this result failed to reach statistical significance (clade × field type $P = 0.08$). None of these results are sensitive to the inclusion of the single T-PAI isolate in Clade A.

Adaptation to local genotypes

The interaction between *A. thaliana* population and *P. viridiflava* population was not significant in our model indicating that there were no statistically significant specific interactions between populations, a requirement for a pattern of local adaptation. However, we did find a statistically significant interaction between *A. thaliana* population, *P. viridiflava* population, and *P. viridiflava* clade on symptom development (Table 2). Yet, when we compared sympatric inoculations versus allopatric inoculations, they were not significantly different for symptoms or bacterial count. We also examined each population in turn, comparing performance of pathogen populations on each host population and performance of host populations inoculated with each pathogen population, and found that the RT pathogen population produced significantly more symptoms on sympatric hosts before but not after significance thresholds were adjusted for multiple tests. Tests for all other populations were non-significant even before correction. Therefore, although there may be some specific interactions between populations when *P. viridiflava* clade is taken into account, there is no indication of pathogen local adaptation or maladaptation.

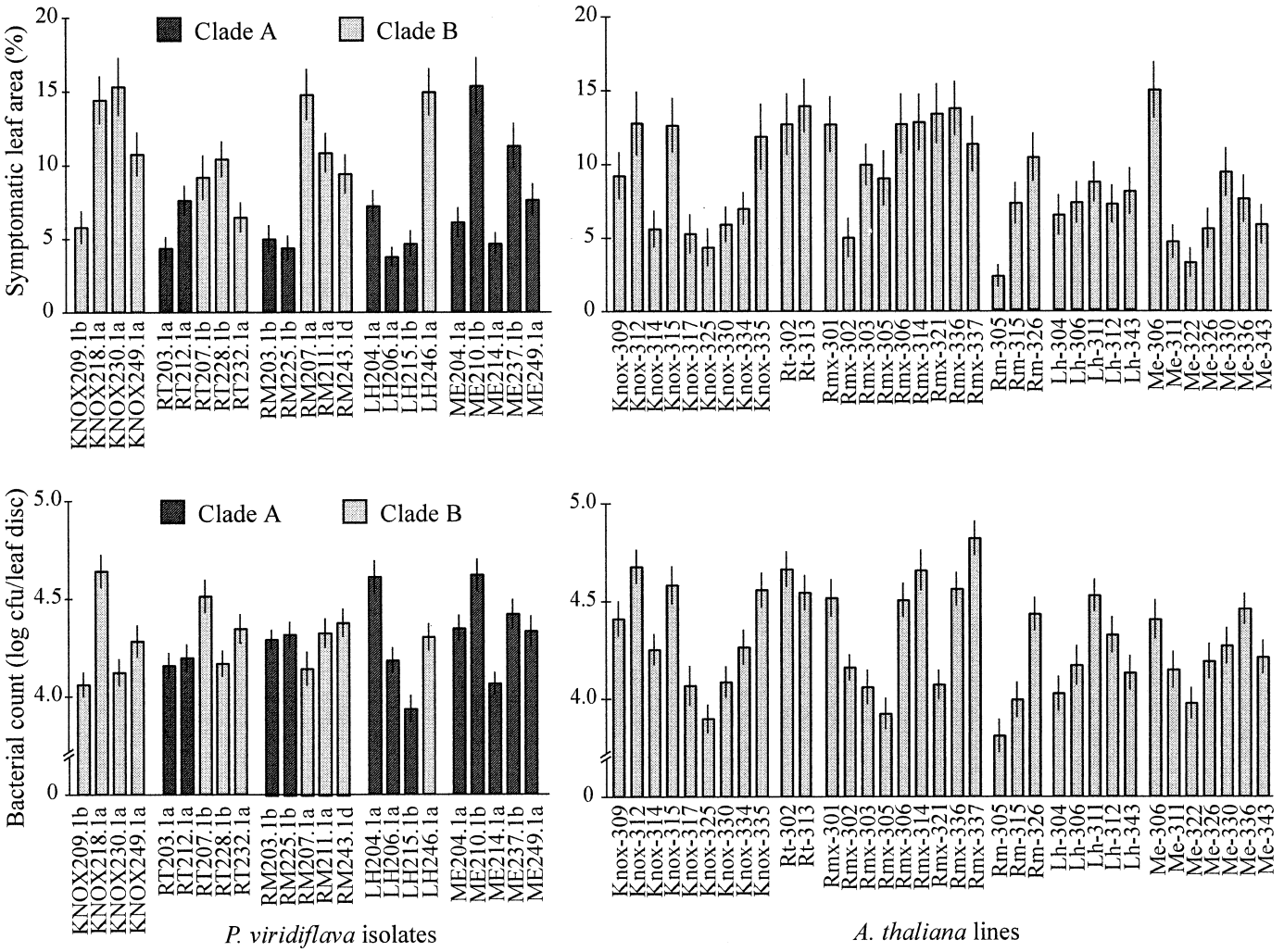

FIG. 1. Variation within and among populations in virulence of *Pseudomonas viridiflava* isolates (left panels) and in resistance of *Arabidopsis thaliana* lines (right panels), expressed in terms of symptom development (top panels), and bacterial count in the leaf (bottom panels) four days after inoculation. Note that *A. thaliana* susceptibility increases with increasing symptoms and bacterial count; resistance is associated with lower values of these two measures. Values are mean (± standard error) for each pathogen isolate averaged across all plant lines (left panels) and for each plant line averaged across all pathogen isolates (right panels). Symptoms were generally but not strongly correlated with bacterial count over all sampled leaves ($R^2 = 0.14$, $P < 0.001$).

Developmental variation and resistance to P. viridiflava

We found that our plant lines varied significantly in time to bolting (Table 3, Fig. 3), which was positively correlated with symptom development ($R^2 = 0.15$, $F_{1,32} = 5.54$, $P = 0.025$), but not bacterial count ($R^2 = 0.00$, $F_{1,32} = 0.005$, $P = 0.94$). Plant populations were also significantly different in bolting time (Table 3) with a nonsignificant pattern towards increasing susceptibility to *P. viridiflava* infection with later flowering (symptoms: $R^2 = 0.37$, $F_{1,4} = 2.36$, $P = 0.20$; bacterial count: $R^2 = 0.25$, $F_{1,4} = 1.34$, $P = 0.31$). Finally, whether plant lines originated from cultivated or fallow fields had a large effect on bolting time (Table 3); plants from fallow fields bolted an average of three weeks later than plants from cultivated fields (69.6 ± 2.15 vs. 48.4 ± 3.0 days). As reported above, plants from cultivated fields showed significantly greater resistance to *P. viridiflava* in both measures.

We tested for age-related resistance in three *A. thaliana* lines: one fast flowering and relatively resistant (Lh-304), one slow flowering and relatively susceptible (Rt-313), and one from Rm (Rm-305) that was exceptional in its long bolting time and relative resistance. Analysis of variance on bacterial count 4 days after inoculation indicated significant effects of plant line ($F_{2,84} = 3.99$, $P = 0.02$) and plant age ($F_{3,84} = 7.97$, $P < 0.0001$), as well as a significant interaction between line and age ($F_{6,84} = 2.64$, $P = 0.02$) on plant resistance to *P. viridiflava* growth. We observed decreasing growth by *P. viridiflava* with increasing age for the Lh and Rt *A. thaliana* lines (Fig. 4), but Rm-305 showed no evidence of reduced bacterial growth with increasing age. Average bacterial counts were higher in the 18 day old plants in this experiment than observed in the cross-inoculations, but the rank order of lines was the same. Analysis of variance on day 0 counts indicated a marginally significant effect of plant age ($F_{3,45} = 2.68$, $P = 0.058$), therefore these values are also shown in Figure 4.

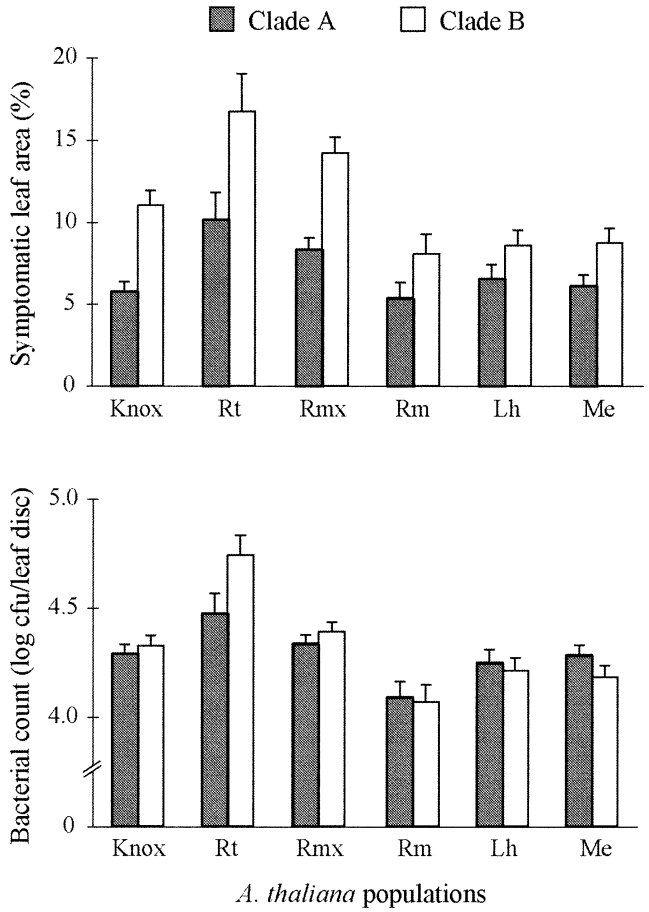

FIG. 2. Mean resistance of *Arabidopsis thaliana* populations to each *Pseudomonas viridiflava* clade (± standard error) as measured by symptom development (top panel) and bacterial count in the leaf (bottom panel) across all isolates in each clade. Resistance is associated with lower values of these two measures. The three left-hand populations (Knox, Rt, and Rmx) are fallow fields and the three right-hand populations (Rm, Lh, and Me) are cultivated fields.

TABLE 3. Analysis of variance in bolting time of *Arabidopsis thaliana* lines used in inoculations. Plant line is nested within plant population, which is nested within field type. Significance of random effects was tested using Wald *Z*-tests rather than *F*-tests. Significant *P*-values are bolded.

Days to bolting (inverse-transformed)		
Source of variation: fixed effects	*F*	*P*
Field type	10.65	**0.0029**
Plant population	2.85	**0.0426**
Source of variation: random effects	*Z*	*P*
Plant line	3.69	**0.0001**

DISCUSSION

We found variation for resistance and virulence in the interaction between the annual weed *A. thaliana* and the foliar bacterial pathogen *P. viridiflava* in the Midwest, USA. Within populations, we observed significant variation among *A. thaliana* lines and *P. viridiflava* isolates, even though our collection area from each site was small. We also found a significant effect of the two genetic clades within *P. viridiflava* on symptom development and significant effects of the cultivation regime of the field of origin on both measures of plant resistance. We further observed that the symptom development of the two genetic clades of *P. viridiflava* depended on the plant population of origin. Examination of time to bolting of these plants showed that plants from cultivated fields tended to be faster developers and more resistant to infection by *P. viridiflava* than plants from fallow fields. We assume in this study that the variation in pathogen aggressiveness that we measured correlates with pathogen fitness, as have other studies of plant-pathogen interactions. One would expect some degree of correlation since *in planta* growth and surface lesions are thought to contribute to pathogen transmission by allowing high densities of cells to reach the outer surface of the leaf (Beattie and Lindow 1999), thus allowing *P. viridiflava* to spread and infect other hosts.

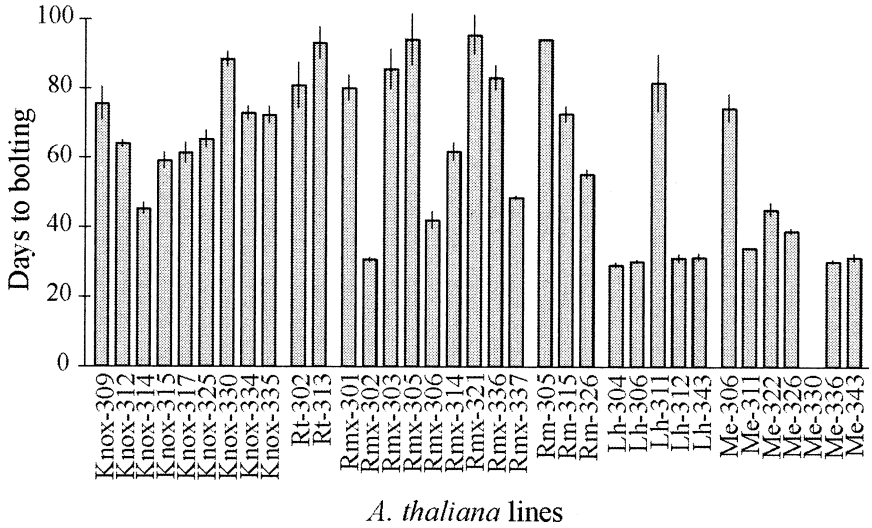

FIG. 3. Variation in developmental rate of *Arabidopsis thaliana* lines within and among populations, as measured by days from germination to bolting (mean ± standard error). Plant lines are ordered as in Figure 1.

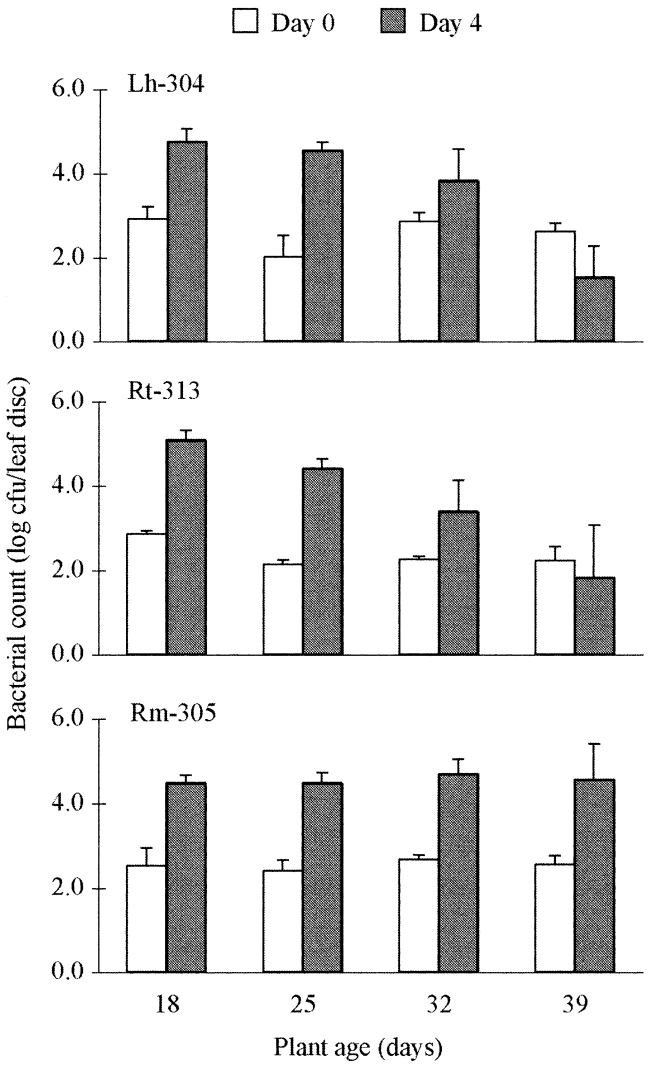

FIG. 4. Resistance of three *Arabidopsis thaliana* lines to *Pseudomonas viridiflava* isolate LH206.1a by plant age. Resistance was measured in terms of bacterial count in the leaf, shown for the day of inoculation and four days after inoculation (mean ± standard error). Lh-304 (fast-developing) and Rt-313 (slow-developing) showed increasing resistance to bacterial growth with age. Rm-305 (slow-developing but relatively resistant) showed no such pattern. For comparison, average bacterial counts in the cross-inoculation experiment for Lh-304, Rt-313, and Rm-305 inoculated with LH206.1a were 3.99, 4.31, and 3.80 log cfu/leaf disc, respectively.

We found differences in virulence between the two *P. viridiflava* clades in the symptoms that they produced but not bacterial count. A study that knocked out known effector genes in another foliar bacterial pathogen, *Xanthomonas axonopodis* pv. *vesicatoria*, also found different effects on lesion development and bacterial growth in the greenhouse; knockouts that showed no difference from the wild-type strain in bacterial growth nevertheless exhibited reduced lesion formation, which translated into reduced fitness in the field (Wichmann and Bergelson 2004). Pectate lyase, the enzyme responsible for the soft-rotting activity of *P. viridiflava*, shows significantly higher activity in clade B isolates than clade A isolates [diameter of pitting ring produced by 10 μl of OD_{600} 0.1 cell suspension on pH 8.3–8.5 Hildebrand's media (Schaad et al. 2001) after four days incubation at 28°C; clade A: $n = 29$, 3.18 ± 0.16 (mean ± SE); clade B: $n = 9$, 4.70 ± 0.41; $F_{1,38} = 8.43$, $P = .006$; K. Jakob, pers. comm.]. Pectate lyase activity may therefore be responsible for the greater symptom development by clade B isolates in *A. thaliana*. It has also been shown that *in planta* growth of clade B isolate RMX3.1b (not used in this study) was adversely affected by neither the salicylic acid nor jasmonic acid defense pathways in contrast to the three clade A isolates tested, all three of which responded to jasmonic acid-based defense (K. Jakob, J. Kniskern, and J. Bergelson, unpubl. ms.). These data, although preliminary, suggest that clade B isolates could respond to host defenses differently than clade A isolates.

We did not find evidence of local adaptation by *P. viridiflava* genotypes to *A. thaliana* genotypes, or vice versa, in the sampled Midwest populations. There are several possible explanations for this result. First, we did not observe a specific interaction between *P. viridiflava* and *A. thaliana*, which is likely required for specific adaptation. Yet, Jakob et al. (2002) did observe this specificity between *P. viridiflava* and *A. thaliana* genotypes. They had a much larger sample size (16 replicates of each combination of genotypes); it is possible that the variation among our five replicates may have been too great to detect a plant line by pathogen isolate interaction. Second, in some cases local adaptation may be completely a function of gene frequencies. It is possible that extensive samples from each population would reveal that genotype frequencies are such that there is effective local adaptation in these populations, due to the dominance of genotypes that favor host or pathogen. In a quantitative interaction such as this, we cannot construct virulence or resistance profiles and are therefore unable to quantify gene frequencies as can be done so nicely in classic gene-for-gene interactions. We did find high levels of phenotypic variation within *A. thaliana* and *P. viridiflava* populations, together with a complex interaction between plant and pathogen populations and *P. viridiflava* clade affecting symptom development, suggesting that there is the potential for adaptation in these populations. Finally, it is possible that *P. viridiflava* is adapted to *A. thaliana* at a different spatial scale than what was sampled in this study or that *P. viridiflava*'s use of multiple host species prevents specific adaptation to *A. thaliana*.

Clade B isolates averaged greater symptom development than clade A isolates overall, but the difference in virulence between the clades varied with *A. thaliana* population. In particular, clade B isolates appeared to have a greater advantage over clade A isolates in the fallow field *A. thaliana* populations. The clades may respond differently to host defenses that are themselves varying among populations. A contingency test on the original random sample of isolates from these five *P. viridiflava* populations (Goss et al. 2005) shows an excess of clade A isolates collected from cultivated fields compared to fallow fields and an excess of clade B isolates collected from fallow compared to cultivated fields ($\chi^2 = 10.66$, $P = 0.001$). Because clade A isolates still did worse than clade B on plants from cultivated sites, there must be some other factor contributing to the maintenance of clade A isolates in cultivated field *A. thaliana* populations. Genetic

data on *P. viridiflava* indicates that it is unlikely that dispersal limitation is preventing clade B isolates from dominating cultivated fields (Goss et al. 2005). Clade A isolates may transmit, overwinter, or compete better than clade B isolates, or if these *P. viridiflava* populations use multiple hosts, there could be a trade-off in virulence between host species such that clade A is superior to clade B on another common host. Similar sorts of trade-offs have been suggested as having a role in maintaining variation in the necrotrophic pathogens *Crumenulopsis sororia* (Ennos and McConnell 1995) and *Alternaria brassicicola* (Thrall et al. 2005).

As part of this study, we found that plants from the cultivated fields generally developed more rapidly than plants from the fallow fields. Faster flowering of weed populations from cultivated or weeded sites as compared to ruderal sites has been documented before, both for *A. thaliana* (Jones 1971) and other weed species (e.g., Kadereit and Briggs 1985; Miura and Kusanagi 2001). Weeds are widely known to adapt to agricultural practices in general and even to mimic particular crop species (Barrett 1983; Jordan 1989; Clements et al. 2004). Lh and Me were both soybean fields, in which the plants experienced strong selection for rapid development in order to mature before herbicide treatment. Both of these population samples showed rapid bolting times with the exception of one line each, perhaps recent migrants from the field margins. Rm was a cornfield that was plowed rather than treated with herbicide, which allows some fraction of plants to survive and may be one explanation for the slower development rates of Rm plants relative to the majority of Lh and Me plants. We also know that Rm was fallow in 1999 (although we do not know about years prior), so this population may not have experienced consistent selection for fast flowering.

These differences in developmental timing between fallow and cultivated fields corresponded to differences in plant resistance to *P. viridiflava* between these field types. Thus, cultivation practices appear to select faster flowering genotypes that also tend to be more resistant to inoculation with *P. viridiflava*. This is consistent with age-related resistance contributing to the variation in resistance observed within these *A. thaliana* populations and between the two field types. Our limited followup experiment suggested that age-related resistance to *P. viridiflava* may indeed contribute to this pattern. Plants from the cultivated fields, forced to develop faster than those in fallow fields, may have shown increased resistance in our experimental inoculation due to greater developmentally regulated resistance when the plants were inoculated. Interestingly, in contrast to the overall pattern of increasing resistance with faster bolting, resistance in the three Rm plants decreased with faster bolting. When we tested the most resistant of these lines for age-related resistance, we did not find increasing resistance with plant age.

A more dynamic and diverse view of coevolutionary interactions has emerged that recognizes that they are variable in space, time, and players (Thompson 1994, 1999). Extensive study of the host-specific, gene-for-gene interaction between the wild flax *Linum marginale* and its rust pathogen *Melampsora lini* has shown that investigation at different spatial scales can reveal different patterns of adaptation (Burdon and Thrall 2000; Burdon et al. 2002). Here, the quantitative, nonspecialized interaction between *P. viridiflava* and *A. thaliana* varied across a relatively small geographic region. Coevolutionary dynamics may be expected to vary across the range of this association, including regions as diverse as England, Spain, Sweden, and North Carolina, as evolutionary trajectories are likely to depend on local selection regimes, host availability, and other external factors that affect the relative success of the *P. viridiflava* clades and patterns of variation in plant and pathogen. In fact, a virulent form of *P. viridiflava* was recently found infecting crops on the north coast of Spain, where it was previously rarely found to be associated with disease symptoms (Gonzalez et al. 2003), thus showing *P. viridiflava*'s potential as an emergent pathogen.

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