Comparative Large-Scale Analysis of Interactions between Several Crop Species and the Effector Repertoires from Multiple Pathovars of *Pseudomonas* and *Ralstonia*[1][W][OA]

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Bacterial plant pathogens manipulate their hosts by injection of numerous effector proteins into host cells via type III secretion systems. Recognition of these effectors by the host plant leads to the induction of a defense reaction that often culminates in a hypersensitive response manifested as cell death. Genes encoding effector proteins can be exchanged between different strains of bacteria via horizontal transfer, and often individual strains are capable of infecting multiple hosts. Host plant species express diverse repertoires of resistance proteins that mediate direct or indirect recognition of bacterial effectors. As a result, plants and their bacterial pathogens should be considered as two extensive coevolving groups rather than as individual host species coevolving with single pathovars. To dissect the complexity of this coevolution, we cloned 171 effector-encoding genes from several pathovars of *Pseudomonas* and *Ralstonia.* We used *Agrobacterium tumefaciens*-mediated transient assays to test the ability of each effector to induce a necrotic phenotype on 59 plant genotypes belonging to four plant families, including numerous diverse accessions of lettuce (*Lactuca sativa*) and tomato (*Solanum lycopersicum*). Known defense-inducing effectors (avirulence factors) and their homologs commonly induced extensive necrosis in many different plant species. Nonhost species reacted to multiple effector proteins from an individual pathovar more frequently and more intensely than host species. Both homologous and sequence-unrelated effectors could elicit necrosis in a similar spectrum of plants, suggesting common effector targets or targeting of the same pathways in the plant cell.

Plants and potential pathogens are locked in continual antagonism involving alternating cycles of selection to increase resistance and virulence, respectively. There are many biochemical exchanges between plants and pathogens, and selection can act at multiple points in the host-pathogen interaction. Several overlapping mechanisms of resistance in plants and strategies of pathogens to interdict these resistance responses are being elucidated (Jones and Dangl, 2006). The combined abilities of a pathogen to overcome host resistance mechanisms are major determinants of its host range and success as a pathogen.

Plants possess elaborate mechanisms for detecting the presence of potential pathogens. Basal defenses are triggered by microbe (or pathogen)-associated molecular patterns (MAMPs), ubiquitous components of microbes such as flagellin, lipopolysaccharide, and bacterial translation factor EF-Tu (for review, see Grant et al., 2006; Jones and Dangl, 2006). Detection of MAMPs by extracellular receptor-like kinases results in signal transduction cascades and the elicitation of basal defense or MAMP-triggered immunity, which includes production of active oxygen species and antimicrobial compounds as well as modification of cell walls, including callose deposition (for review, see Bent and Mackey, 2007; McDowell and Simon, 2008).

Pathogens have evolved effector molecules that are translocated into their hosts and often interfere with one or more steps in the induction of resistance (Alfano and Collmer, 2004; Grant et al., 2006; Gohre and Robatzek, 2008; Hogenhout et al., 2009). This has

[1] This work was supported by the National Science Foundation Plant Genome Program (grant no. 0211923 to R.W.M. and J.T.G.) and by a postdoctoral fellowship award from the National Institutes of Health (grant no. 1 F32 G066606–02 to B.A.V.).

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[W] The online version of this article contains Web-only data.

[OA] Open Access articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.109.140251

been best characterized for effectors from gram-negative bacterial pathogens. *Pseudomonas*, *Xanthomonas*, and *Ralstonia* species and other bacterial pathogens cause disease by injecting 20 or more effector proteins into host cells via the type III secretion system (Lindeberg et al., 2006; for review, see Mudgett, 2005). Multiple effector proteins have been shown to function as virulence factors in the absence of the cognate resistance (R) protein; the type III secretion system is essential for pathogenicity, and strains defective in the secretion apparatus are nonpathogenic (Lindgren et al., 1986, 1988; Ashfield et al., 1995). While the precise activity of the majority of secreted proteins is currently unknown, there is increasing evidence that the function of many, but not all, effectors is to inhibit plant defenses, including MAMP-triggered immunity (DebRoy et al., 2004; Block et al., 2008; Boller and He, 2009; Cunnac et al., 2009; Jeong et al., 2009).

Plants have countered the pathogen's virulence actions by evolving the ability to directly or indirectly detect the activities of effectors (for review, see Chisholm et al., 2006; Bent and Mackey, 2007). Such recognition results in effector-triggered immunity (ETI) and is mediated predominantly by intracellular nucleotide-binding site-Leu-rich repeat (NBS-LRR) proteins that have often been identified as monogenic *R* genes. ETI involves signal transduction and a resistance response that overlaps qualitatively with basal defenses but differs in its timing and amplitude, often resulting in a more extreme resistance phenotype. *R* genes correspond in a gene-for-gene manner to genetically defined avirulence (*avr*) genes in pathogens (Flor, 1971). In addition to inhibiting basal defense responses, pathogens have in turn evolved effectors that abrogate ETI and that plants have subsequently evolved to detect (Jones and Dangl, 2006). The cycle of plants developing novel recognition specificities and pathogens evolving to overcome them is a continuous process the molecular diversity of which remains to be resolved. Most known gene-for-gene interactions have been characterized in fairly homogenous germplasm of cultivated crop species or in Arabidopsis (*Arabidopsis thaliana*). There are few data currently available that document resistance mediated by recognition of individual effectors among diverse plant species (e.g. Ashfield et al., 2004; Kuang et al., 2006).

The recognition of effectors by the plant's receptor proteins may occur through direct or indirect interaction (for review, see Jones and Dangl, 2006; Bent and Mackey, 2007). Direct recognition of effectors involves protein-protein interactions between the R protein and the effector. This has been demonstrated for the AvrPita/Pi-ta, PopP2-RRS1, and AvrL576-L pairs of avirulence factors and corresponding R proteins (Jia et al., 2000; Deslandes et al., 2003; Dodds et al., 2006). Indirect recognition involves R proteins acting as "guards" that monitor the status of plant proteins targeted by pathogen effectors (Dangl and Jones, 2001). The best understood example of the "target and guard" situation is recognition by the R proteins RPM1 and RPS2 of the effects of three effectors from *Pseudomonas syringae* (*Ps*), AvrRpm1, AvrB, and AvrRpt2, on the RIN4 protein in Arabidopsis (Mackey et al., 2002, 2003; Axtell and Staskawicz, 2003).

Therefore, there are at least three broad classes of coevolving molecules involved in plant-bacterial pathogen interactions: the pathogen effectors, the plant targets of these effectors (either as the virulence target or decoys), and the plant receptor proteins that recognize the presence of effectors on their targets (Jones and Dangl, 2006; Van der Hoorn and Kamoun, 2008; Caldwell and Michelmore, 2009). Bacterial pathogens contain clusters of genes encoding effector proteins that exhibit elevated levels of sequence variation consistent with diversifying selection acting on these genes (Rohmer et al., 2004; Ma et al., 2006). Because of the selective advantage conferred by such genes, there has been extensive horizontal transfer among microbial pathogens of genes that encode effectors (Lindgren et al., 1988; Hueck, 1998; Deng et al., 2003; Rohmer et al., 2004; Ma et al., 2006; Lindeberg et al., 2008). As a result, diverse pathogens express overlapping sets of effectors (Lindeberg et al., 2006; Sarkar et al., 2006; Stavrinides et al., 2006). On the plant side, *R* genes, particularly those encoding NBS-LRR proteins and receptor-like kinases, are among the largest and most diverse classes of plant proteins (Clark et al., 2007). Genes encoding R proteins are often clustered in plant genomes, which helps to maintain existing resistance specificities and generate new ones (Halterman et al., 2001; Meyers et al., 2003; Kuang et al., 2004; McHale et al., 2006).

There are several implications arising from the coevolution of these three classes of molecules. Pathogens, particularly bacterial species that infect multiple hosts, should be considered as coevolving with a broad range of plant species rather than individual pathovars coevolving with a limited number of plant hosts. This paradigm leads to several testable hypotheses. One is that plant populations have been exposed to overlapping subsets of pathogen effectors and, consequently, individual plant species have evolved the ability to recognize numerous effectors. Second, nonhosts will react to effectors from nonpathogens either as a consequence of direct recognition or due to the detection of similar effector activities. Recognition of some effectors will be fixed in the species, while recognition of others will display intraspecific variation, especially if there is a fitness cost associated with the expression of the cognate resistance gene (Tian et al., 2003; Bomblies et al., 2007). Screens of plant germplasm, therefore, will reveal intraspecific variation for the reaction to effectors, and the detection of orthologous effectors will vary within and between species. Furthermore, there may be a limited number of points of vulnerability in plants that are targeted by multiple effectors from diverse pathogens. Consequently, host and nonhost plants may exhibit parallel reactions to nonhomologous effectors.

To test these hypotheses, we conducted a large-scale comparative analysis of the reactions of germplasm of several crop species to a library of effector proteins representing nearly the entire secretomes of five bacterial plant pathogens. The reactions of 59 plant genotypes representing 13 species from four dicotyledonous families were tested for reactions to over 171 bacterial effector proteins. *Agrobacterium tumefaciens*-mediated transient expression was used to provide isogenic delivery of individual effectors into a broad range of species to avoid the confounding effects of multiple effectors secreted by a pathogen and to overcome the limited host ranges of individual pathogens. Variation in effector-elicited chlorotic and necrotic phenotypes that resulted from cell death was observed both within and between species. Several lines of evidence indicated that necrotic phenotypes were often the result of effector recognition rather than the result of their overexpression or enzymatic activity related to their virulence function. Effectors from incompatible pathovars induced necrosis substantially more frequently in nonhosts compared with host species. Twenty-seven known avirulence factors and their homologs frequently induced a necrotic phenotype in multiple taxonomically unrelated species. An additional 32 novel putative avirulence determinants were identified. Common patterns of reaction were identified for homologous as well as sequence-unrelated effectors, implying that multiple effectors targeted the same host proteins or pathways. Finally, we identified several potential new sources of resistance to bacterial plant pathogens.

RESULTS

Confirmed and Putative Effector Proteins Induced Necrotic Responses across Diverse Plant Species

Reactions to 171 effector and other pathogenicity-related proteins were tested in 59 plant accessions to assay interspecific and intraspecific diversity for the elicitation of a phenotypic response, particularly necrosis. We used previously published data and our own sequence searches to identify genes encoding confirmed and putative effectors representing nearly the entire secretomes of four pathovars of *Ps* and one strain of *Ralstonia solanacearum* (*Rs*; Supplemental Table S1). Specifically, we cloned 42 of 54 genes encoding effectors and related proteins from *Ps* pv *tomato* DC3000 (*Pto* DC3000), 24 of 36 from *Ps* pv *phaseolicola* 1448A (*Pph* 1448A), and 22 of 27 from *Ps* pv *syringae* B728a (*Psy* B728a; Table I; Guttman et al., 2002; Greenberg and Vinatzer, 2003; Chang et al., 2004; Schechter et al., 2004, 2006; Joardar et al., 2005; Vinatzer et al., 2005, 2006; Lindeberg et al., 2006; http://pseudomonas-syringae.org/). The sequence of the *Ps* pv *maculicola* strain ES4326 (*Pma* ES4326) genome was not available to us when we initiated these studies; therefore, we could not determine the exact number of effector genes in this strain. Nevertheless, genes encoding 14 of 16 effectors previously identified in *Pma* ES4226 (Guttman et al., 2002; Vinatzer et al., 2005) were cloned and utilized (Table I). The genome of *Rs* strain BS048 also had not been sequenced, but it is known to be similar to the recently sequenced *Rs* strain UW551 (Gabriel et al., 2006; Castillo and Greenberg, 2007); 41 putative effector genes were successfully amplified from *Rs* BS048 using primers based on the genomic sequence of *Rs* UW551. In addition to the 143 effector-encoding and pathogenicity-related genes from the five pathogens mentioned above, we cloned genes encoding several effectors from other bacterial strains, including five from the biocontrol *Pseudomonas fluorescens* strain SBW25 (Rainey, 1999), seven from *Xanthomonas campestris* strain ATCC33913, and five from *Rs* strain GMI1000. We also cloned a limited number of genes encoding confirmed and putative effector proteins from other strains of *Pseudomonas* and *Ralstonia* (Table I; Supplemental Table S1). Results are presented for putative effectors and their homologs as well as for so-called helper proteins with predicted activity outside the plant cell, regardless of whether secretion or translocation of each protein into or out of the plant cell had been previously confirmed (Table I; Supplemental Table S1; Lindeberg et al., 2006; http://pseudomonas-syringae.org/).

To provide transient expression of effectors in planta, genes encoding putative effectors were cloned behind the cauliflower mosaic virus 35S promoter in the binary vector pBAV139 (Vinatzer et al., 2006) and subsequently transformed into two strains of *A. tumefaciens*. Transient expression experiments were performed by infiltrating suspensions of *A. tumefaciens* into leaves as described previously (Wroblewski et al., 2005). A wide range of interaction phenotypes were observed that varied from no visible macroscopic symptoms through various degrees of chlorosis to extensive tissue damage and cell death in the infiltrated area, as evidenced by extensive macroscopic necrosis (Fig. 1). Infiltration with *A. tumefaciens* harboring a vector expressing GFP produced no reaction or, occasionally, mild chlorosis. For several effectors, the reaction was weak and manifested as slight discoloration or chlorosis of the infiltrated area. The most severe phenotypes involved extensive cell death accompanied by a brown or black color and collapse of the leaf tissue. The severity of the reactions increased with time, so the reactions were recorded at multiple time points; phenotypes scored 4 to 5 d post infiltration (dpi) were usually more definitive compared with the first scorings at 2 to 3 dpi. Occasionally, necrosis occurred slowly and was only observed at the second scoring (4–5 dpi) with no reaction visible earlier. We generated over 25,000 data points, including replications, that have been archived in a publicly accessible database (http://charge.ucdavis.edu). The consensus result for each interaction was calculated automatically as described in "Materials and Methods" and displayed using a color code to facilitate the visual identification

Table I. *Genes encoding effectors and related proteins assayed for their ability to elicit a macroscopic reaction in planta following A. tumefaciens-mediated transient expression*

Cloned and tested effectors are indicated in boldface. Superior letters are defined as follows: a, known avirulence factor; h, identifier of the closest known homolog in *Ps* or other *Rs* strain; t, truncated; ta, truncated to the first 2,000 bp of the N terminus to make amplification possible; tr, transposon. *, Identifiers of effectors from *Rs* BS048 were created by adding "BS0" to the last four digits of identifiers assigned to putative effectors identified in *Rs* strain UW551 by Gabriel et al. (2006).

Pathogen	Effector (Locus)		
P. fluorescens SBW25	***avrA***	***OspC2***	***ExoY***
	lpa	***PopC***	
Pto DC3000	***avrE1***^{t} (PSPTO_1377)	***hopP1*** (PSPTO_2678)	*hopAG*::*ISPssy*^{tr} (PSPTO_0901)
	avrPto1 (PSPTO_4001)	***hopQ1-1***^{a} (PSPTO_0877)	***hopAH1*** (PSPTO_0905)
	hopA1 (PSPTO_5354)	***hopQ1-2*** (PSPTO_4732)	***hopAH2-1*** (PSPTO_3292)
	hopB1 (PSPTO_1406)	***hopR1***^{t} (PSPTO_0883)	*hopAH2-2* (PSPTO_3293)
	hopC1 (PSPTO_0589)	***hopS1*****::*****ISPssy***^{tr} (PSPTO_4597)	***hopAI1*** (PSPTO_0906)
	hopD1 (PSPTO_0876)	***hopS2*** (PSPTO_4588)	*hopAJ1* (PSPTO_0852)
	hopE1 (PSPTO_4331)	***hopT1-1*** (PSPTO_A0019)	***hopAK1*** (PSPTO_4101)
	hopF2 (PSPTO_0502)	*hopT1-2* (PSPTO_4593)	***hopAM1-1*** (PSPTO_1022)
	hopG1 (PSPTO_4727)	*hopT2* (PSPTO_4590)	*hopAN1* (PSPTO_5061)
	hopH1 (PSPTO_0588)	***hopU1*** (PSPTO_0501)	***hopAO1*** (PSPTO_4722)
	hopI1 (PSPTO_4776)	*hopV1* (PSPTO_4720)	***hopAQ1*** (PSPTO_4703)
	hopJ1 (PSPTO_1179)	***hopX1*** (PSPTO_A0012)	*hopAS1* (PSPTO_0474)
	hopK1 (PSPTO_0044)	***hopY1*** (PSPTO_0061)	*hopAT1′* (PSPTO_5618)
	hopL1 (PSPTO_2872)	***hopAA1-1*** (PSPTO_1372)	***hrpA1*** (PSPTO_1381)
	hopM1 (PSPTO_1375)	***hopAA1-2*** (PSPTO_4718)	***hrpK1*** (PSPTO_1405)
	hopN1 (PSPTO_1370)	***hopAB2***^{a} (PSPTO_3087)	*hrpW1* (PSPTO_1373)
	hopO1-1 (PSPTO_A0018)	*hopAD1* (PSPTO_4691)	***hrpZ1*** (PSPTO_1382)
	hopO1-2 (PSPTO_4594)	***hopAF1*** (PSPTO_1568)	
	hopO1-3 (PSPTO_4592)		
Ps pv *tomato* JL1065	***avrPto1***^{a}	***avrRpt2***^{a}	
Ps pv *tomato* T1	***hopAB2***		
Pma ES4326	*avrE1*	***hopX1***	***hopW1-1***^{a}
	hopAA1	***hopX2***	***hopO1-1***
	hopAA1-2	***hopZ1***	***hopT1-1***
	hopAB3	***hopAJ1***	***hrpW1***
	hopAK1	***hopAL1***	
	hopJ1	***hopI1***	
Ps pv maculicola M2	***avrRpm1***^{a}		
Psy B728a	***avrB3***^{a} (Psyr_1219)	***hopX1*** (Psyr_1220)	***hopAI1*** (Psyr_0780)
	avrE1 (Psyr_1188)	***hopZ3***^{a} (Psyr_1224)	***hopAJ2*** (Psyr_4357)
	avrPto1 (Psyr_4919)	***hopAA1***^{a} (Psyr_1183)	***hopAK1*** (Psyr_3839)
	avrRpm1 (Psyr_0738)	***hopAB1***^{a} (Psyr_4659)	*hopAN1* (Psyr_0465)
	hopH1 (Psyr_1889)	***hopAE1*** (Psyr_4269)	*hopAP1* (Psyr_1890)
	hopI1 (Psyr_4326)	***hopAF1*** (Psyr_3813)	*hrpA2* (Psyr_1192)
	hopJ1(Psyr_1017)	***hopAG1*** (Psyr_0778)	***hrpK1*** (Psyr_1218)
	hopL1 (Psyr_2631)	***hopAH1*** (Psyr_0779)	***hrpW1*** (Psyr_1184)
	hopM1 (Psyr_1186)	***hopAH2*** (Psyr_3123)	***hrpZ1*** (Psyr_1193)
Ps pv syringae Cit7	***hopAE1***	***hopI1***	
Pph 1448A	***avrB2*** (PSPPH_A0120)	***hopQ1-1*** (PSPPH_A0012)	*hopAJ1* (PSPPH_0763)
	avrB4-1 (PSPPH_3028)	*hopR1* (PSPPH_0171)	***hopAJ2*** (PSPPH_4398)
	avrB4-2 (PSPPH_0784)	*hopV1* (PSPPH_2351)	*hopAK1* (PSPPH_1424)
	avrD1 (PSPPH_A0113)	***hopW1-1*** (PSPPH_A0009)	*hopAN1* (PSPPH_0456)
	avrE1^{t} (PSPPH_1268)	*hopW1-2* (PSPPH_A075)	*hopAS1* (PSPPH_4736)
	avrRps4 (PSPPH_A0087)	***hopX1*** (PSPPH_1296)	*hopAU1* (PSPPH_A0031)
	hopD1 (PSPPH_A0010)	*hopAA1* (PSPPH_1263)	***hopAV1***^{t} (PSPPH_A0056)
	hopF3 (PSPPH_3498)	***hopAB1*** (PSPPH_A0127)	***hopAW1*** (PSPPH_A0122)
	hopG1 (PSPPH_0767)	***hopAB3***′ (PSPPH_2294)	*hrpA2* (PSPPH_1272)
	hopI1 (PSPPH_4366)	***hopAE1***^{t} (PSPPH_4326)	*hrpK* (PSPPH_1295)
	hopJ1 (PSPPH_1068)	***hopAF1*** (PSPPH_1443)	***hrpW1*** (PSPPH_1264)
	hopM1 (PSPPH_1266)	*hopAH2* (PSPPH_3036)	***hrpZ1*** (PSPPH_1273)
Ps pv *phaseolicola*	***hopAR1***^{a}		
Ps pv *pisi*	***avrRps4***^{a}	***hopAB1***	
Ps pv *glycinea* race 4	***avrB1***^{a}		
Ps pv *glycinea* 49a/90	***hopAB1***		

(*Table continues on following page.*)

Table I. (*Continued from previous page.*)

Pathogen	Effector (Locus)		
Rs BS048*	**BS00326**	**BS01019**	**BS03105**
	BS00508 [*hrpY*]^{h}	**BS01066**	**BS03109**
	BS00515 [*hpaP*]^{h}	**BS01071**^{ta}	**BS03113**
	BS00531	**BS01260**	**BS03375**^{ta} [*hopR1*]^{h}
	BS00532 [*Gala 4*]^{h}	**BS01554**	**BS03418**^{ta}
	BS00546^{ta}	**BS01561** [*Gala 4*]^{h}	**BS03559**
	BS00571	**BS01562** [*Gala 5*]^{h}	**BS03923** [*hopX1*]^{h}
	BS00576	**BS01581** [*AvrA*]^{h}	**BS04655**
	BS00703	**BS02213** [*hopAV1*]^{h}	**BS04736**
	BS00752 [*Gala 3*]^{h}	**BS02264**^{ta} [*Gala*]^{h}	**BS04744** [*hopAJ1*]^{h}
	BS00852 [*hopX1*]^{h}	**BS02442** [*popB*]^{h}	**BS04764** [*popF1*]^{h}
	BS00926 [*hopG1*]^{h}	**BS02443** [*popA*]^{h}	**BS10001**
	BS00947 [*hopD1*]^{h}	**BS02573**	**BS10010** [*hopH1*]^{h}
	BS01016 [*popC*]^{h}	**BS02682**	
Rs GMI1000	***AvrPphF***	***AvrD***	***ORF5CEL***
	RS04833 (RSc0608)	***hopQ1-1***	
X. campestris ATCC33913	***hopB***	***XopD***	***hopH1***
	Hpa2	***hopG1***	***hopQ1-1***
	HrpB2		

of trends and patterns. Although chlorotic phenotypes were often highly reproducible, we primarily focused on the phenotypes exhibiting stronger reactions involving at least some level of macroscopic necrosis (Fig. 1).

No effector induced a response in all plants tested, but many induced responses in multiple accessions. More than one-third (66 of 171 tested) of the effectors elicited a reaction (chlorosis or necrosis) in at least one genotype (Fig. 2). Four of 14 effectors from *Pma* ES4326, nine of 24 from *Pph* 1448A, 11 of 22 from *Psy* B728a, 19 of 42 from *Pto* DC3000, and 11 of 41 from *Rs* BS048 induced a reaction in at least one accession. Of the 22 effectors from other strains of *Pseudomonas*, *Ralstonia*, and *Xanthomonas*, 15 induced reactions on at least one plant genotype. All of the known avirulence determinants tested induced a necrotic response in at least one genotype, and most of them elicited a reaction in many accessions. Several homologs of known avirulence determinants also induced necrotic phenotypes in one or more accessions (Fig. 2).

All accessions reacted to multiple effectors from multiple pathogens. The average number of reactions per accession was 19. Lettuce (*Lactuca sativa*), tomato (*Solanum lycopersicum*), and pepper (*Capsicum annuum*), each represented by multiple accessions, were able to react to at least one effector from each of the five major bacterial pathogens. The severity of the reactions was highly variable but consistent across the plant accessions tested. Variability in the severity of reactions to individual effectors was observed at the family, genus, and species levels.

The Frequency of the Determinants of the Interaction Phenotypes Varied within Cultivated Lettuce and Tomato

We sampled the diversity of interaction phenotypes within cultivated germplasm of lettuce and tomato. Nineteen cultivars of lettuce and 19 cultivars of tomato were selected as representing a large number of known resistance specificities, many of which had been introgressed from wild species (Farrara et al., 1987; Laterrot, 1987; Williams and St. Clair, 1993; Grube et al., 2000; Van Deynze et al., 2007; Michelmore and Wong, 2008). These accessions, therefore, were expected to express diverse clusters of *R* genes. Fifty-four and 32 effectors induced strong reactions in at least one genotype of lettuce and tomato, respectively. Some effectors (16 in lettuce and nine in tomato) elicited a necrotic reaction in most or even all genotypes tested (Fig. 3). For example, $HopAA1_{PsyB728A}$ induced a strong reaction in all of the lettuce and tomato genotypes tested. Expression of $HopM1_{PsyB728a}$ caused strong reaction in most of the lettuce genotypes tested, and expression of $HopAM1\text{-}1_{PtoDC3000}$ caused strong necrosis in all tomato accessions (Fig. 3). Other effectors, 23 in lettuce and seven in tomato, elicited reactions in only a subset of the accessions tested (e.g. $HopC1_{PtoDC3000}$ and $HopT1\text{-}1_{PmaES4326}$ in lettuce and $HopG1_{Pph1448A}$ and $HopM1_{PsyB728a}$ in tomato; Fig. 3). Yet other effectors, five in lettuce and 10 in tomato, rarely induced necrosis (i.e. reactions were observed in three or fewer cultivars within a species). For example, only lettuce cultivars Mariska and Salad Bowl reacted to $HopH1_{PtoDC3000}$ and $HopX1_{PsyB728A}$, respectively (Fig. 3). Among the tomato accessions tested, only cv Rio Grande and LA1800 responded with necrosis to $HopAE1_{Pph1448A}$; cv Rio Grande was also the sole tomato accession showing a necrotic reaction to $HopAB3_{PmaM2}$ (Fig. 3). These rare reactions to effectors were distributed among various cultivars rather than being restricted to one or a few specific accessions. Therefore, the frequency of plant determinants responsible for reactions to effectors varied considerably within cultivated lettuce and tomato. Some were pres-

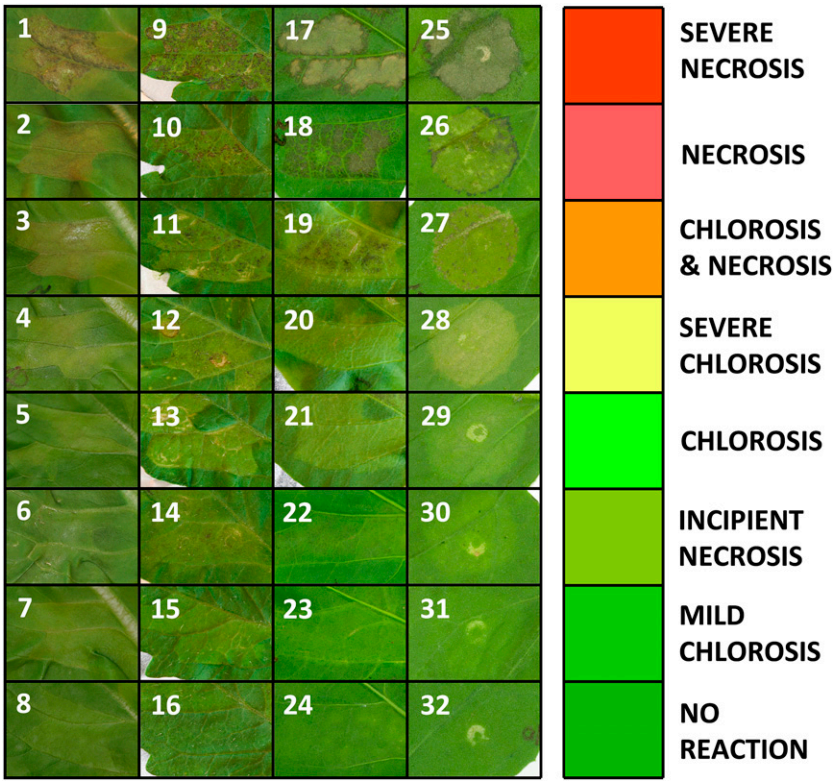

Figure 1. Interaction phenotypes resulting from transient expression of effectors at 4 to 5 dpi. Each interaction phenotype was assigned a color to represent the reactions in the database. Panels 1 to 8, Lettuce cv Ninja: 1, $HopAB2_{PtoDC3000}$; 2, $HopM1_{PtoDC3000}$; 3, $AvrRpt2_{PtoJL1065}$; 4, $AvrB4\text{-}2_{Pph1448A}$; 5, $HopM1_{PsyB728a}$; 6, $HopZ3_{PsyB728a}$; 7, $HopT1\text{-}1_{PmaES4326}$; 8, vector control. Panels 9 to 16, Tomato cv Mocimor: 9, $HopAA1_{PsyB728A}$; 10, $HopAR1_{Pph}$; 11, $HopW1\text{-}1_{PmaES4326}$; 12, $HopG1_{PtoDC3000}$; 13, $HopM1_{PtoDC3000}$; 14, $HopZ3_{PsyB728a}$; 15, $HopT1\text{-}1_{PtoDC3000}$; 16, vector control. Panels 17 to 24, Pepper line RNaKy: 17, $HopH1_{PsyB728a}$; 18, $HopW1\text{-}1_{PmaES4326}$; 19, $AvrB2_{Pph1448A}$; 20, $AvrPto1_{PtoJL1065}$; 21, $AvrB3_{PsyB728A}$; 22, $HopAE1_{PsyCit7}$; 23, $AvrRps4_{Pph1448A}$; 24, vector control. Panels 25 to 32, *N. benthamiana*: 25, $HopT1\text{-}1_{PtoDC3000}$; 26, $AvrB1_{Pgyrace4}$; 27, $HopQ1\text{-}1_{PtoDC3000}$; 28, $HopQ1\text{-}1_{RalGMI1000}$; 29, $HopC1_{PtoDC3000}$; 30, $HopAA1_{PsyB728A}$; 31, $HopX1_{PmaM2}$; 32, vector control. Enlarged, high-resolution versions of all images in Figure 1 can be found at http://charge.ucdavis.edu/supdata/effectors_in_planta_2009.html.

ent in multiple or even all accessions, several had intermediate frequency, while others were rare and present in only one or a few accessions.

Reactions to Individual Effectors Were Similar among, But Highly Polymorphic within, Taxonomically Distinct Groups of Plants

The 59 plant accessions tested represented four families, seven genera, and 14 species (Fig. 4). This allowed us to investigate the specificity of interaction phenotypes relative to different taxonomic groups. Within the Compositae, in addition to the 19 accessions of cultivated *L. sativa*, we tested five lines of *L. serriola* (the wild progenitor of *L. sativa*) and one line each of *L. saligna* and the more distantly related Mexican sunflower (*Tithonia rotundiflora*). Within the Solanaceae, in addition to the 19 cultivars of tomato, we tested three species of wild tomato (*S. pimpinellifolium*, *S. cheesmanii*, and *S. pennellii*), four lines of pepper from three different species (*C. annuum*, *C. chinense*, and *C. frutescens*), as well as one line of *Nicotiana benthamiana*. Within the Malvaceae, we tested three lines of cotton from two different species (*Gossypium hirsutum* and *G. barbadense*). The Brassicaceae were represented by three accessions of Arabidopsis.

Thirty-nine of the 66 effectors capable of eliciting necrotic reactions listed in Figure 3 did so in more than one family. Three effectors, $HopM1_{PsyB728a}$ (Fig. 4), $HopAA1_{PmaM2}$, and $HopAE1_{PsyCit7}$, induced necrotic reactions across all four families. None of the effectors induced necrosis in all of the accessions tested; however, three effectors, $AvrB3_{PsyB728A}$ and two HopAA1 homologs from *Pma* ES4326 and *Psy* B728a, elicited necrosis in more than 90% of the genotypes tested.

Generally, there was no clear relationship between the distribution of necrotic reactions and the taxonomic affinity of the plant genotypes tested. For example, most of the 23 effectors that induced necrosis in lettuce but not in tomato were able to elicit reactions in pepper or *N. benthamiana*, indicating that these responses were not specific to lettuce or the Compositae family. Similarly, *T. rotundifolia* did not respond to 12 effectors that elicited strong reactions in most lettuce accessions; however, five of those induced necrosis in tomato. A few reactions were specific to particular taxonomic groups and present in all or nearly all accessions within that group. For example, multiple accessions of *L. sativa* and *L. serriola* reacted to AvrRps4 homologs, including $HopK1_{PtoDC3000}$ or $HopAB1_{Pph1448A}$ (Fig. 4). However, none of the other plants tested (including *T. rotundiflora*, another member of the Compositae) responded with necrosis to these effectors, making the reaction specific to the genus *Lactuca* (Fig. 4). Reactions to $HopAI1_{PtoDC3000}$ and $HopE1_{PtoDC3000}$ were observed exclusively among several *Lactuca* lines and in *T. rotundiflora* and, therefore, were family specific among the accessions tested (Fig. 4). In contrast, determinants of responses to $HopH1_{PsyB728a}$ were specific to *L. serriola* (LSE18) and two accessions of pepper (CW300 and RNaKy). Similarly, determinants of responses to $HopT1\text{-}1_{PmaES4326}$

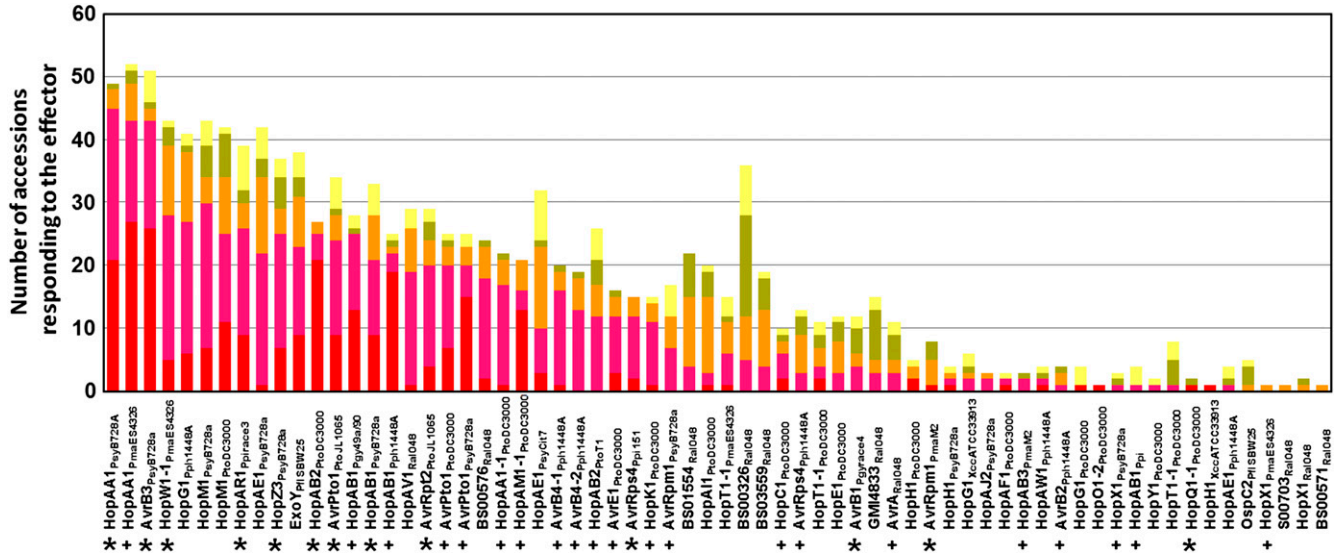

Figure 2. Frequency of chlorotic or necrotic phenotypes of reaction to 66 effectors in 59 plant accessions and genotypes. Color coding corresponds to the severity of the different reactions as shown in Figure 1. Known avirulence determinants are indicated by asterisks; homologs of known avirulence determinants are indicated by plus signs.

were present among accessions of lettuce and Arabidopsis belonging to the Compositae and Brassicaceae families, respectively. Substantially fewer effectors (13) induced reactions in *T. rotundiflora* as compared with lettuce, in which each accession responded to an average of 29 effectors, ranging from 19 in cv Pennlake to 33 in cv Salad Bowl. The only effector that elicited a reaction in *T. rotundiflora* but not in lettuce was HopQ1-1$_{PtoDC3000}$ (data not shown). Within the Solanaceae, tomato, pepper, and *N. benthamiana* genotypes responded to similar numbers of effectors on average (12, 12, and 13, respectively); even though the subset of

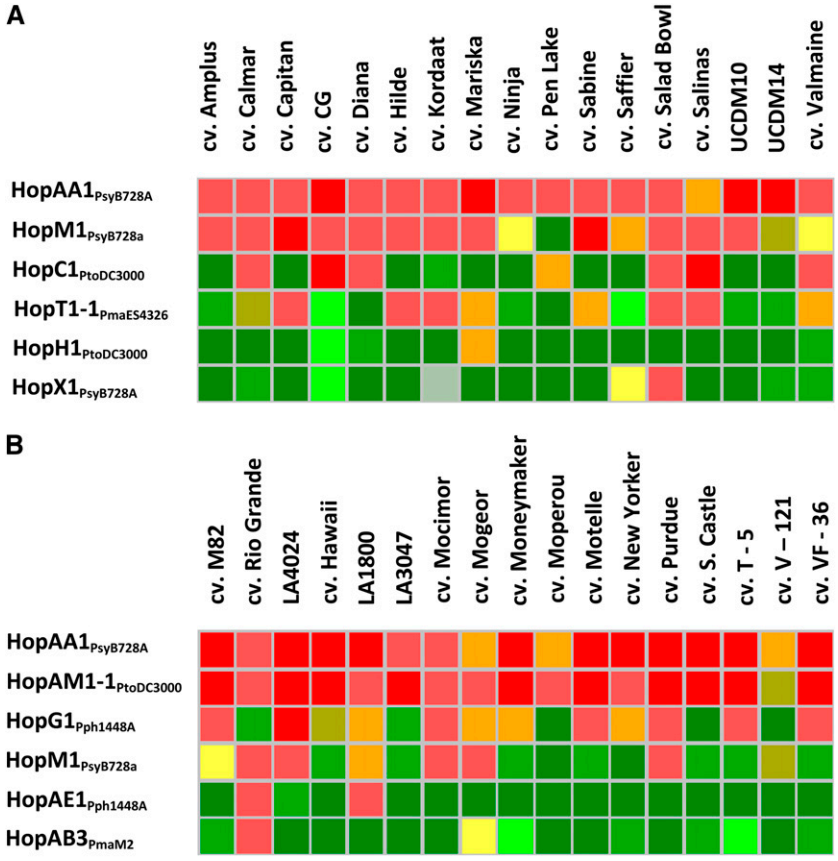

Figure 3. Examples of different patterns of interaction phenotypes observed among cultivars of lettuce (A) and tomato (B). Color coding is as described in Figure 1; gray indicates inconsistent reaction.

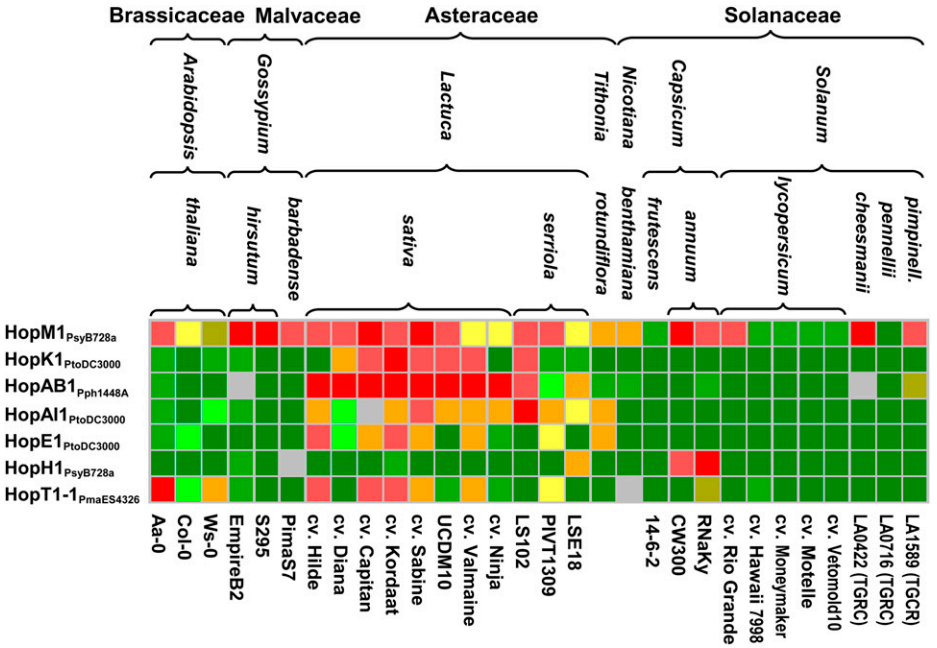

Figure 4. Interspecific differences in reactions to transient expression of effectors in 13 species from seven genera belonging to four plant families: Brassicaceae, Malvaceae, Asteraceae (referred to as Compositae in the text), and Solanaceae. Color coding is as described in Figure 1; gray indicates inconsistent reaction.

effectors that induced necrosis in each genus overlapped, several effectors only induced necrosis in accessions from one or two genera and elicited no responses in the other(s). After several repetitions using all 171 effectors, we were able to identify 12 effectors that induced necrosis at least once. Despite efforts to optimize assays in Arabidopsis (Wroblewski et al., 2005), this species remained recalcitrant to reliable *A. tumefaciens*-mediated transient assays; therefore, while observations of necrotic reactions are informative, the lack of a reaction was not.

Overall, almost two-thirds of effectors that induced a response in at least one accession were able to elicit a reaction in more than one family.

Effectors from *Pto* DC3000 and *Rs* BS048 Elicited More Frequent and Stronger Responses in Lettuce Than in Tomato

Lettuce is a nonhost for *Pto* DC3000 and *Rs* BS048, while tomato is a good host for both of these pathogens. Of 42 effectors from *Pto* DC3000, 13 elicited necrosis in one or more accessions of lettuce but only six did so in tomato (Fig. 5). Furthermore, six of these effectors (AvrPto1, HopAI1, HopM1, HopAB2, HopAA1-1, and HopE1) elicited reactions in all or nearly all of the lettuce genotypes tested, whereas only two effectors (HopM1 and HopAM1-1) elicited reactions in more than one accession of tomato, and only one of them (HopAM1-1) elicited a reaction in multiple tomato lines (Fig. 5). Interestingly, the avirulence determinants detected by the product of the *Pto* gene in tomato and that provide resistance against *Pto* DC3000, AvrPto1, and HopAB2 rapidly elicited severe necrosis in nearly all of the lettuce genotypes tested as well as in *L. serriola* UC96US23 (Fig. 5). Of 41 effectors from *Rs* BS048 (Table I), seven induced necrotic reactions in at least one lettuce genotype and six did so in tomato accessions (Supplemental Fig. S1). Similar to the effectors from *Pto* DC3000, the reactions to the *Rs* BS048 effectors in lettuce were stronger and more frequent than those observed among the tomato genotypes. For example, Ral028 and Ral033 induced necrosis in all lettuce accessions tested. In contrast, responses in tomato were rare: five effectors elicited necrosis in only one or two accessions, and a sixth effector, Ral040, elicited necrotic reactions in approximately half of the genotypes tested (Supplemental Fig. S1). Therefore, effectors from *Pto* DC3000 and *Rs* BS048 elicited stronger responses more frequently in the nonhost lettuce as compared with their tomato host.

Effectors Known to be Avirulence Determinants Induced Necrosis at High Frequency, and Several New Putative Avirulence Determinants Were Identified

Several effector proteins were historically identified through their avirulence activities and are known to trigger necrotic responses when the corresponding *R* gene is present in the host plant (Yu et al., 1993; Van den Ackerveken et al., 1996). Our library of effectors contained 13 genes that had been previously described as determining avirulence in various pathosystems (Fig. 6). All of them induced necrosis in at least one among the plants tested, and notably, many did so in species in addition to the ones in which their avirulence function had been initially identified (Fig. 6). Therefore, effectors originally identified as avirulence factors seem to maintain this activity across multiple plant species. The products of most of the known avirulence genes induced strong necrotic reactions in

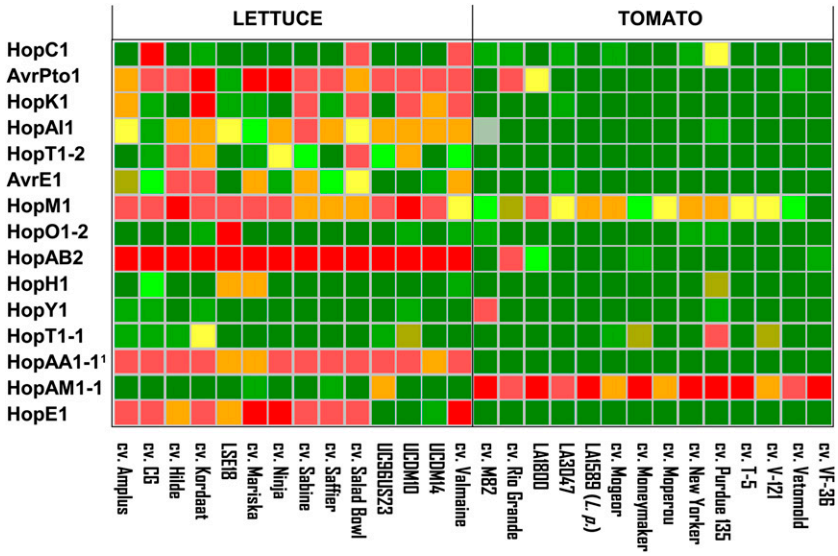

Figure 5. The differences between lettuce and tomato in their reactions to the effector repertoire from *Pto* DC3000. Only a representative subset of plants and the effectors that elicited reactions are shown. [1] The reaction of tomato to HopAA1-1 differs from results reported by Munkvold et al. (2008). Color coding corresponds to that in Figure 1; gray indicates inconsistent reaction.

at least one lettuce accession, even though none of the genes had been isolated from a bacterial strain that is pathogenic on this species.

In addition to the known avirulence genes, our library contained 39 homologs of known avirulence genes. These homologs induced necrosis more frequently than did other putative or confirmed effectors, and 21 of them induced a necrotic response in at least one genotype (Fig. 2); 63% of the known avirulence factors and their homologs induced necrotic response in at least one genotype tested. Conversely, among the 105 effectors and related proteins that did not elicit a reaction, only 18 were homologs of known avirulence determinants. The fraction of known avirulence factors and their homologs that elicited necrosis in at least one accession (63%) was significantly higher ($P < 0.05$) than the 22% of all the effectors tested that showed such activity. Therefore, the ability to elicit necrotic response was strongly biased toward effectors with known avirulence activity and their homologs.

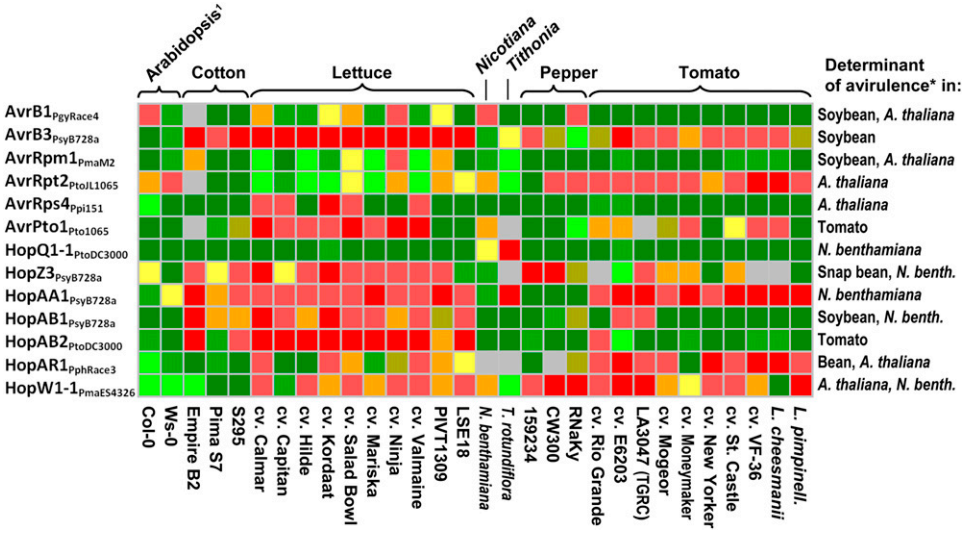

Figure 6. Reactions of different plant accessions to known avirulence determinants. Avirulence activity of $AvrB1_{Pgyrace4}$ was reported by Tamaki et al. (1988) and Wanner et al. (1993), $AvrB3_{PsyB728a}$ by Alfano et al. (2000), $AvrRpm1_{PmaM2}$ by Ashfield et al. (1995) and Ritter and Dangl (1995), $AvrRpt2_{PtoJL1065}$ by Innes et al. (1993), $AvrRps4_{Ppi151}$ by Hinsch and Staskawicz (1996), $AvrPto1_{Pto1065}$ by Ronald et al. (1992), $HopQ1\text{-}1_{PtoDC3000}$ by Wei et al. (2007), $HopZ3_{PsyB728a}$ and $HopAA1_{PsyB728a}$ by Vinatzer et al. (2006), $HopAB1_{PsyB728a}$ by Jackson et al. (1999) and Vinatzer et al. (2006), $HopAB2_{PtoDC3000}$ by Kim et al. (2002), $HopAR1_{Pphrace3}$ by Puri et al. (1997) and Simonich and Innes (1995), and $HopW1\text{-}1_{PmaES4326}$ by Lee et al. (2008). Color coding corresponds to that in Figure 1; gray indicates inconsistent reaction. [1], Ecotype Columbia (Col-0) did not respond to $AvrRpm1_{PmaM2}$ and ecotype Wassilewskija (Ws-0) did not respond to $AvrRps4_{Ppi151}$, probably due to insufficient effector expression. *, The pathosystem in which the avirulence activity for a particular effector was determined.

As a result of this study, we identified 32 effectors capable of eliciting necrosis that, to our knowledge, had not previously been reported to be avirulence factors. These effectors, including HopG1$_{Pph1448A}$, HopM1 homologs from *Psy* B728a and *Pto* DC3000, ExoY$_{PflSBW25}$, HopAE1$_{PsyCit7}$, HopAV1$_{Ral048}$, and several other effectors from *Rs*, were capable of eliciting strong reactions in multiple accessions (Fig. 2). Therefore, these 32 effectors could potentially be novel avirulence determinants (see "Discussion").

Homologous as Well as Sequence-Unrelated Effectors Elicited Similar Patterns of Reactions

To search for similarities in patterns of reactions produced by homologous and sequence-unrelated effectors across our plant collection, we performed extensive visual inspection as well as cluster analysis of the entire database or of data for subsets of accessions (Supplemental Fig. S2). Our library of effectors included 33 series of homologs containing two or more paralogs from the same pathogen or more often orthologs from different pathogens (Supplemental Table S2). Some of these series, such as those constituting AvrB, HopAB1, or HopAH1, could be divided into subsets based on sequence similarities (Lindeberg et al., 2005; Fig. 7; Supplemental Table S2). For 12 of the 33 series, no reaction to any member was observed among the plants tested. For 21 series, at least one member induced a necrotic reaction in at least one plant genotype; 10 of these reactions were elicited in similar groups of plants by at least some, but not all, of the homologs in the series. For example, the pattern of reactions produced by three AvrRps4 homologs (HopK1$_{DC3000,}$ AvrRps4$_{Ppi151}$, and AvrRps4$_{Pph1448A}$) were nearly identical across all accessions tested (Fig. 7); HopK1$_{DC3000}$ homology (84% similarity) is restricted to the first two-thirds of the protein and, therefore, is considered to be a chimera. For this reason, the N-terminal region of the protein is most likely responsible for triggering plant response. The patterns produced by AvrB4-1$_{Pph1448A}$ and AvrB4-2$_{Pph1448A}$ paralogs (99% protein identity) were also nearly identical; however, the reaction elicited by the other AvrB paralog (29% and 47% protein identity and similarity, respectively), AvrB2$_{Pph1448A}$, was very different (Fig. 7). Patterns of reaction to RS04833$_{RalGMI1000}$ and BS01581$_{RalBS048}$ were similar among tomato and pepper accessions, but among lettuce accessions cv Ninja responded only to BS01581$_{RalBS048}$ (Fig. 7) but not to RS04833$_{RalGMI1000}$. Three HopAB1 homologs from *Ps* pv *glycinea* 49a/90, *Pph* 1448A, and *Psy* B728a elicited

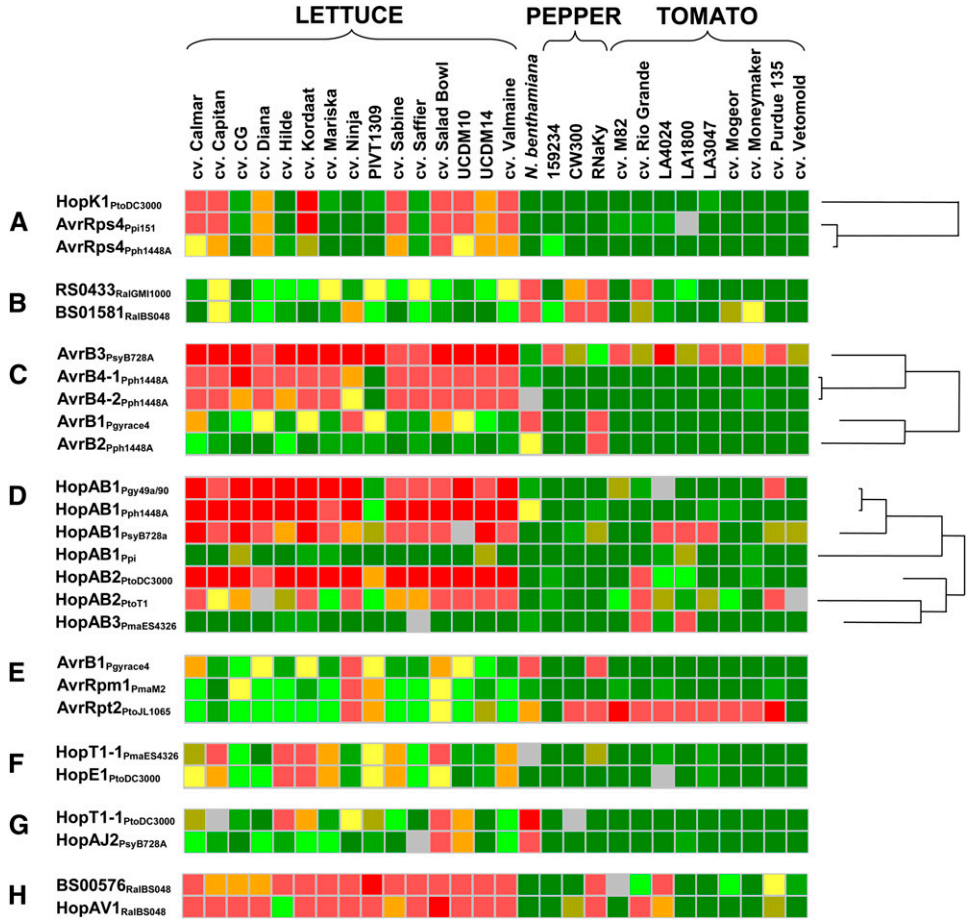

Figure 7. Common patterns induced among tested plant accessions by homologous (A–D) and sequence-unrelated (E–H) effectors. Homologous effectors often produced identical or nearly identical patterns (A and B), although differences within orthologous series were also observed (C and D). The patterns of reaction produced by three sequence-unrelated effectors, AvrB1$_{Pgyrace4}$, AvrRpm1$_{PmaM2}$, and AvrRpt2$_{PtoJL1065}$ (E), were similar among lettuce accessions but different among other plants tested. Similarly, the patterns of reaction elicited by other sequence-unrelated effectors, HopT1-1$_{PmaES4326}$ and HopE1$_{PtoDC3000}$ (F), HopT1-1$_{PtoDC3000}$ and HopAJ2$_{PsyB728a}$ (G), and BS00756$_{RalBS048}$ and HopAV1$_{RalBS048}$ (H), were common at least in the subset of accessions tested and detectable by cluster analysis (Supplemental Fig. S2).

similar patterns of reactions among lettuce accessions but different ones among tomato accessions (Fig. 7). In summary, although there were several examples of differences in the patterns of reactions produced by homologous effectors, the majority of homologs elicited similar reaction patterns among the accessions tested.

Cluster analysis using just lettuce accessions identified some similarities between patterns elicited by sequence-unrelated effectors (Supplemental Fig. S2). $AvrB1_{Pgyrace4}$, $AvrRpm1_{PmaM2}$, and $AvrRpt2_{PtoJL1065}$, each of which targets the RIN4 protein in Arabidopsis, all induced strong reactions in lettuce cv Ninja, milder reactions in cv Salad Bowl and PIVT1309, but no reaction in any other lettuce accession tested. Two other sequence-unrelated effectors, $HopT1\text{-}1_{PmaES4326}$ and $HopE1_{PtoDC3000}$, showed similar patterns of necrotic elicitation across all of the lettuce accessions. Two accessions of lettuce, cv Salad Bowl and line UCDM10, and *N. benthamiana* displayed parallel reactions to $HopT1\text{-}1_{PtoDC3000}$ and $HopAJ2_{PsyB728A}$. Finally, the reactions to $BS00576_{RalBS048}$ and $HopAV1_{RalBS048}$ were similar among lettuce, pepper, and tomato accessions (Fig. 7). These parallel patterns suggest that these effectors may be interacting with the same plant target(s) or affecting the same plant pathway(s).

The Genetic Determinants of Necrotic Reactions Mapped to *R* Gene Candidates in Lettuce

Intraspecific polymorphism in the reactions elicited by several effectors allowed the determinants of the necrotic response to be mapped in lettuce. The determinants of the reactions to six effectors, $AvrRps4_{Ppi151}$, $HopK1_{PtoDC3000}$, $AvrRpm1_{PmaM2}$, $AvrB1_{Pgyrace4}$, $AvrRpt2_{PtoJL1065}$, and $HopC1_{PtoDC3000}$, were mapped using 106 F2 individuals from a cross between *L. sativa* 'Valmaine' and *L. sativa* 'Ninja', and the reactions to $AvrRps4_{Ppi151}$, $AvrRps4_{Pph1448A}$, and $HopK1_{PtoDC3000}$ were mapped using 113 recombinant inbred lines derived from *L. sativa* 'Salinas' × *L. serriola* UC96US23 (Supplemental Table S3). In addition, the determinants of the reaction to $AvrPto1_{PtoJL1065}$ and $AvrRps4_{Ppi151}$ were mapped using 107 F3 families derived from a cross between *L. sativa* 'Valmaine' and *L. serriola* LSE18. The determinants of the reaction to each effector segregated as a single dominant locus (Supplemental Table S3).

Determinants of the reaction to two homologous effectors, $AvrRps4_{Ppi151}$ and $HopK1_{PtoDC3000}$, cosegregated and mapped in each of three populations to the same locus on linkage group 8 that contained multiple NBS-LRR-encoding *RGC4* (for *Resistance Gene Candidate4*) genes. No disease resistance phenotypes have been mapped to this locus to date, although based on the number of NBS-LRR-encoding sequences present at the locus, it is one of the larger RGC-encoding loci in lettuce (McHale et al., 2009).

The determinants of the reactions to all three of the sequence-unrelated effectors, $AvrRpm1_{PmaM2}$, $AvrB1_{Pgyrace4}$, and $AvrRpt2_{PtoJL1065}$, that target RIN4 in Arabidopsis cosegregated in lettuce and mapped to a region coincident with many *RGC* genes and resistance phenotypes on linkage group 1, including race-specific resistances to lettuce downy mildew caused by *Bremia lactucae* as well as *Turnip mosaic virus* (McHale et al., 2009). This locus, referred to as the *Dm5/8* locus, is unlinked to *LsRIN4* and the closest homologs of the Arabidopsis *RPS2* and *RPM1* genes in lettuce (McHale et al., 2009).

The determinant of the responses to $HopC1_{PtoDC3000}$ mapped to the terminal region of linkage group 8, a region that contains three NBS-LRR-encoding sequences and a gene that confers race-specific resistance to anthracnose, *Ant3*. Finally, the determinant of reactions to $AvrPto1_{PtoJL1065}$ mapped to the bottom of linkage group 9 in the region containing a single NBS-LRR-encoding RGC sequence.

Overall, these data indicate that the plant determinants of the necrotic responses to these bacterial effectors are genetically linked to confirmed and putative disease resistance genes. This is consistent with the necrotic reaction elicited by transient expression of effectors being analogous to the hypersensitive response (HR) elicited in gene-for-gene interactions following pathogen challenge.

DISCUSSION

Bacterial plant pathogens such as *Pseudomonas* and *Ralstonia* species secrete repertoires of effector proteins into the extracellular spaces and cells of their hosts (Hueck, 1998). Extensive research is revealing the roles of an increasing number of these effectors in pathogen virulence and/or avirulence (for review, see Grant et al., 2006; Jeong et al., 2009). Multiple effectors have been shown to interfere with plant defense responses in compatible interactions (for review, see Jeong et al., 2009) and to elicit an HR when a cognate gene is present (Van den Ackerveken et al., 1996). Much of our current understanding has been generated using somewhat artificial yet highly informative experimental systems, particularly Arabidopsis and $AvrB1_{Pgyrace4}$, $AvrRps4_{Ppi151}$, or AvrPphB ($HopAR1_{Pph}$). These effectors originated from the pathogens of legumes, *Ps* pv *glycinea*, *pisi*, and *phaseolicola*, respectively, and were introduced to pathogens of Arabidopsis, *Pma* ES4326 and *Pto* DC3000, to study their activity in plant cells. It should be noted that *Pma* ES4326 and *Pto* DC3000 do not even contain homologs of AvrB1.

This paper describes a comparative approach to assess natural variation in reactions to bacterial effectors by host and nonhost species. We used *A. tumefaciens* to deliver genes encoding effector proteins into plant cells to overcome several constraints associated with using the donor pathogens, *Pseudomonas*, *Xanthomonas*, and *Ralstonia*. This strategy allowed isogenic assays of a large number of effectors in a wide range of plants. Restrictions associated with the specificity of

individual pathovars to particular hosts were avoided, and each effector could be assessed without the confounding effects of the activities of other secreted effectors. Even though *A. tumefaciens*-mediated transient expression of an effector inside of the host cell may result in protein levels different from those occurring during infection and infiltration with *A. tumefaciens* is not completely benign (it may trigger ETI or interfere with salicylic acid-mediated defense signaling; Zipfel et al., 2006; Rico and Preston, 2008), our observations recapitulated previously reported phenotypes for known avirulence proteins and therefore is an informative method of analysis.

We observed a wide range of macroscopic phenotypes, from mild chlorosis to severe necrosis, in response to transient expression of various effectors. Necrotic phenotypes can be triggered in plant cells in response to a variety of signals, including molecular components of pathogens. In some cases, cell death may be triggered by activity of an effector that is associated with its virulence function, particularly if the level of expression after agroinfiltration was substantially higher than following infection with *Pseudomonas*. However, the best characterized necrosis is programmed cell death resulting in the HR elicited by avirulence factors and mediated by intracellular NBS-LRR proteins, several of which have been identified as the products of *R* genes. The HR is thought to constrain pathogen proliferation, particularly in the case of biotrophs (Goodman and Novacky, 1994; Greenberg and Yao, 2004). Partial inhibition of the HR can result in increased growth of *Ps* (Yao and Greenberg, 2006). In several cases, HR has been shown to be sufficient, although not always required, for resistance (Yu et al., 1998; Gassmann et al., 1999; Gassmann, 2005; Römer et al., 2007). Several lines of evidence suggested that many, although not necessarily all, of the necrotic responses elicited by transient expression of effectors reported here reflect the elicitation of the HR mediated by *R* genes. First, approximately 80% of the necrotic reactions were induced by previously identified avirulence factors or their homologs; the remaining 20% of reactions were induced by effectors whose avirulence status has not been determined. Second, reaction to the effectors was frequent and often stronger in nonhost compared with host species, consistent with some effectors being involved in nonhost resistance. Third, the determinants of reactions to all seven effectors mapped to clusters of NBS-LRR-encoding genes in lettuce, which is a nonhost for all of the bacterial strains used as sources of effector genes in this study. Fourth, the reaction was polymorphic even in otherwise relatively monomorphic germplasm of cultivated species; this is consistent with the selection of the lines screened for their diversity of *R* genes, many of which had been introgressed as part of breeding programs. Finally, necrosis caused by two effectors for which the avirulence functions had not been determined, $HopAE1_{PsyB728a}$ and $HopM1_{PsyB728a}$, was SGT1 dependent in *N. benthamiana* (Vinatzer et al., 2006); SGT1 is a well-characterized mediator of R-triggered defense responses (Azevedo et al., 2006).

The ability to elicit necrosis in our transient assays does not preclude a role for an effector in virulence when secreted by a pathogen. In some cases, we observed a necrotic reaction elicited by effectors from virulent pathogens. For example, $HopAM1\text{-}1_{PtoDC3000}$ elicited necrosis in all genotypes of tomato tested, which is a good host for *Pto* DC3000. There are several potential explanations of this result. First, other effectors secreted by the pathogen may block recognition or the subsequent elicitation of HR (Abramovitch et al., 2003; Kim et al., 2005; Cunnac et al., 2009). Second, not all cases of the elicitation of necrosis may be detrimental to the pathogen; in some cases, it may be critical to the pathogenesis and triggering cell death may actually benefit the pathogen. Some necrotrophs have host-specific toxins that enhance virulence by inducing programmed cell death in host cells (Sweat and Wolpert, 2007; Sweat et al., 2008). Similarly, some hemibiotrophic oomycetes such as *Phytophthora* benefit from killing host cells via the activity of necrosis-inducing proteins such as Nep1-like proteins (Pemberton and Salmond, 2004) at the later stages of infection (Kanneganti et al., 2006). Interestingly, these necrotrophic or hemibiotrophic pathogens induce programmed cell death by triggering the plant's defense response (Kanneganti et al., 2006; Sweat and Wolpert, 2007; Sweat et al., 2008). Necrosis may also help to release nutrients or aid in the liberation and dissemination of biotrophic bacteria from infected tissue (Liang et al., 2003; Abramovitch and Martin, 2004; Greenberg and Yao, 2004), or may simply contribute to disease symptoms (Badel et al., 2003, 2005). Alternatively, effectors capable of inducing HR may not be secreted from compatible strains or do not accumulate to a level required to trigger necrosis. $HopAJ2_{PsyB728a}$, for example, induces a necrotic response in multiple plant accessions (Fig. 7) but lacks a Hrp box and is not translocated from its source strain (Lindeberg et al., 2005; Oh et al., 2007); however, they may be secreted from other strains, and it is interesting that these genes still encode proteins that elicit necrosis in multiple plant genotypes. The presence of these proteins in the effector repertoires of their source strains may be a relic of virulence on other hosts, and the fact that they are not secreted may be the result of adaptation to species that can react to them.

The significance of the chlorotic phenotypes is less obvious than the consequences of necrosis. The degree of chlorosis varied from barely discernible to extensive and accompanied by limited necrosis, particularly several days after infiltration. The chlorosis observed in these studies possibly had a variety of causes. In some cases, it may have been symptomatic of a partial resistance response, as was recently described for $AvrB1_{Pgyrace4}$, which elicits TAO1-dependent chlorosis in plants lacking RPM1 (Eitas et al., 2008). Therefore, TAO1, a NBS-LRR protein, is a second, minor recognition determinant of $AvrB1_{Pgyrace4}$ in addition to

RPM1. The appearance of minor recognition determinants or quantitative avirulence factors has previously been reported for a few other effectors (Chang et al., 2002; Vinatzer et al., 2006). In other cases, chlorosis may have resulted from the activity of the effector in the plant cell; however, there is no published evidence clearly correlating chlorosis with an effector's virulence-enhancing activity.

The taxonomic distribution of necrotic reactions to individual avirulence factors among the plant genotypes tested is consistent with either convergent evolution or the maintenance of ancient recognition specificities. Known avirulence determinants often retain their activity in other pathosystems (e.g. $AvrRps4_{Ppi151}$, $AvrB1_{Pgyrace4}$, and $HopAR1_{Pph}$ in Arabidopsis and $HopZ3_{PsyB728a}$ in *N. benthamiana*; Wanner et al., 1993; Simonich and Innes, 1995; Hinsch and Staskawicz, 1996; Vinatzer et al., 2006). However, even if the determinants of a reaction were present in all or nearly all of the accessions belonging to a particular genus in our study, they were often absent in related species in the same family. Nonetheless, they were frequently present in more distantly related taxa. This is consistent with convergent evolution of recognition specificities in different families or loss during the evolution of particular species. Convergent evolution of resistance specificities has been reported for the NBS-LRR-encoding genes determining recognition of $AvrB1_{Pgyrace4}$ and AvrRpm1 in soybean (*Glycine max*) and Arabidopsis (Grant et al., 1995; Ashfield et al., 2004). Similarly, the closest homologs of *RPM1* and *RPS2* in lettuce map to a different position than the *Dm5/8* locus that determines the reaction to $AvrRpm1_{PmaM2}$, $AvrB1_{Pgyrace4}$, and $AvrRpt2_{PtoJL1065}$, and none of the NBS-LRR-encoding genes at the *Dm5/8* locus are similar in sequence to *RPM1* or *RPS2*. Not all of the taxonomically widespread recognition specificities observed in this study may be the result of convergent evolution; resistance genes exhibit heterogeneous rates of evolution, and some resistance specificities may persist over long periods of evolutionary time (Kuang et al., 2004). The loss of resistance specificities in a particular lineage could be the consequence of a lack of selection due to the absence of pathogens with cognate avirulence genes or a selective disadvantage due to the presence of a resistance gene, possibly due to the presence of necrotrophic pathogens that exploit the resistance specificity to elicit the HR to their benefit (Govrin and Levine, 2000; Kanneganti et al., 2006; Sweat and Wolpert, 2007; Sweat et al., 2008).

The repertoire of effectors in bacterial pathogens is a major factor determining host specificity. This could be due to either host-specific virulence activities or the detection of effectors by the plant and elicitation of the resistance response. A previous study failed to identify host-specific virulence determinants (Sarkar et al., 2006). In our study, nonhosts responded to more effectors than hosts. Therefore, host specificity may be determined by lack of (many) avirulence determinants and nonhost resistance may be, at least in part, the result of the recognition of multiple effectors. Of the homologous effectors that elicited necrosis in one or more accessions, approximately half exhibited patterns of reactions similar to those of another homolog. However, patterns of reactions for homologous effectors were rarely identical among accessions. This implies extensive coevolution of effectors and their plant targets and/or the corresponding plant resistance genes. This conclusion is consistent with convergent evolution of resistance specificities, particularly when patterns of recognition were similar in one family but different in others.

Patterns of reaction to sequence-related effectors do not imply similarities or differences in their virulence functions or targets. Sequence-related effectors are presumably functional orthologs and have similar targets, although this has to be verified. The variation in reactions to effectors observed in this study, therefore, probably reflects evolution in pathogens to avoid recognition. It has yet to be demonstrated that the effectors that did not elicit a reaction in this study retain virulence activity; however, they originated from virulent pathogens and often elicited a reaction in some accessions (Fig. 7). Therefore, particularly if recognition is indirect, they must have some activity. Because the number of points of vulnerability in a host may be limited (Caldwell and Michelmore, 2009), sequence-unrelated effectors may have redundant functions or common targets; for example, RIN4 in Arabidopsis is targeted by at least three effectors, $AvrRpm1_{PmaM2}$, $AvrB1_{Pgyrace4}$, and $AvrRpt2_{PtoJL1065}$ (Mackey et al., 2002, 2003; Axtell and Staskawicz, 2003). This may be recapitulated in lettuce; the determinant of reactions to the same three effectors maps to one locus. In addition, similarities in the patterns of reactions to three other pairs of sequence-unrelated effectors suggest that they may modify the same target or pathway (Fig. 7). In future studies, we will investigate the virulence activities associated with various alleles encoding several effectors as well as the ability of those effectors to interact with target proteins using the yeast two-hybrid system. The necrotic and chlorotic phenotypes associated with the expression of bacterial effector proteins may enable the cloning of the cognate determinants of the reaction from multiple plant species. Such determinants could be useful as disease resistance genes. This strategy had been successfully employed using effectors of the oomycete pathogen *Phythophthora infestans* and potato (*Solanum tuberosum*; Vleeshouwers et al., 2008).

In summary, our data are consistent with the hypotheses proposed at the outset of this paper. Individual plant species have evolved the ability to recognize, either directly or indirectly, numerous effectors, suggesting that most plants have been exposed to overlapping subsets of pathogen effectors. Nonhosts react to effectors from nonpathogens either as a consequence of direct recognition or due to similarity of the effector's activity to the activity of an analogous

effector in a pathogen of the plant. Screens of germplasm revealed intraspecific variation in reactions to individual effectors, while recognition of other effectors was constant within a species. Reaction to orthologous effectors varied within and between species. Host and nonhost plants sometimes exhibited parallel reactions to nonhomologous effectors, indicating that there may be a limited number of points of vulnerability in plants that can be targeted by multiple effectors from diverse pathogens. Therefore, bacterial pathogens should be considered as coevolving with a broad range of potential plant hosts rather than as individual pathogen species coevolving with a limited number of plant species. It will be interesting to see to what extent this paradigm can be extended to other classes of pathogens, such as oomycetes and fungi, that do not have such well-documented evidence for horizontal transfer of virulence genes.

MATERIALS AND METHODS

Identification and Cloning of Confirmed and Putative Effector-Encoding Genes

A three-tiered approach was used to mine the genomic sequence of *Pseudomonas syringae* pv *phaseolicola* 1448A. First, BLASTX analysis was used to identify homologs of previously identified effectors. Second, all open reading frames located within 300 bp downstream of sequences resembling the conserved hrp-promoter element were translated and analyzed for characteristics common to known effectors, including a high Ser or Pro bias in the first 50 amino acids, an aliphatic amino acid at the third or fourth position, and the lack of negatively charged residues in the first 12 positions (Guttman et al., 2002; Petnicki-Ocwieja et al., 2002; Schechter et al., 2004). Finally, open reading frames immediately downstream of putative effector genes identified above were also analyzed because genes encoding effectors are often clustered in the genome and can exist in operons.

Sequences encoding known and putative effectors were amplified by PCR and cloned into Gateway shuttle vector pDONR207 (Invitrogen; http://www.invitrogen.com). For five large effector-encoding genes (those encoding $AvrE1_{PtoDC3000}$, $HopR1_{PtoDC3000}$, $AvrE1_{Pph1448A}$, $HopAE1_{Pph1448A}$, and $HopAV1_{Pph1448A}$), only the N-terminal portion of the gene was cloned. After sequence validation, genes were transferred to the Gateway-compatible binary vector pBAV139 containing in its T-DNA the cauliflower mosaic virus 35S promoter to drive their expression in planta and a His tag to produce a C-terminal fusion with the protein as it is expressed (Vinatzer et al., 2006). Details of all effectors and clones are available from http://charge.ucdavis.edu.

Plant Material and Growth Conditions

All lettuce (*Lactuca sativa*) accessions used in these experiments were obtained from the collection at the University of California, Davis. Most of them had been used previously in genetic studies of resistance to lettuce downy mildew and contain diverse resistance loci introgressed from wild species (Farrara et al., 1987; Ilott et al., 1987; Maisonneuve et al., 1994). Tomato (*Solanum lycopersicum*) cultivars and genetic stocks were similarly selected to ensure high diversity in resistance to various pathogens and were obtained from the Tomato Genetic Resource Center (http://tgrc.ucdavis.edu). Pepper (*Capsicum annuum*) and cotton (*Gossypium hirsutum*) accessions had been used in previous studies involving genetic mapping (Paran et al., 2004; Gingle et al., 2006). *Tithonia rotundiflora* was selected as an additional member of the Compositae family (in addition to lettuce) because of its high compatibility with *Agrobacterium tumefaciens* and strong transient expression following agroinfiltration. Similarly, the Arabidopsis (*Arabidopsis thaliana*) ecotypes used were selected based on high expression following agroinfiltration as reported previously (Wroblewski et al., 2005). Lettuce, tomato, *T. rotundiflora*, *Nicotiana benthamiana*, and Arabidopsis plants were grown under conditions described previously (Wroblewski et al., 2005). Cotton plants were grown in the growth chamber at 26°C during the day and 22°C during the night, with a 14-h-light/10-h-dark photoperiod, and light intensity of approximately 300 μE generated by high-pressure sodium lamps.

Transient Expression Assay and Scoring Criteria

Transient expression assays were performed as described previously (Schob et al., 1997) by infusing a suspension of *A. tumefaciens* in water (optical density at 600 nm = 0.4–0.5) into the leaf lamina using a blunt-ended 1-mL syringe. To facilitate high-throughput analyses, our collection of effector genes in *A. tumefaciens* was cultured and manipulated using 48-cell deep-well polypropylene plates. To induce transient expression in tomato and pepper, and to avoid nonspecific necrosis, *A. tumefaciens* strain 1D1249 was used (Wroblewski et al., 2005); for all other plants tested, *A. tumefaciens* strain C58 was used. The consistency in reactions induced by effectors delivered using strains 1D1249 and C58 was confirmed in *N. benthamiana*. The onset of reaction varied among the plants and was usually quicker in lettuce and *T. rotundiflora* as compared with the other species tested. Reactions were scored two and 4 dpi in lettuce and *T. rotundiflora* and 3 and 5 dpi in all other species. Eight types of reaction phenotypes could be distinguished in the infiltrated areas (Fig. 1), and they were assigned numeric values for computational analyses and colors for visualization as follows: 0 (dark green), no reaction or minor discoloration comparable to "no-effector" control; 1 (green), mild discoloration, a reaction often observed as a result of plant response to the presence of *A. tumefaciens* even if no effector was delivered; 2 (light green), chlorosis manifested as discoloration of the infiltrated area; 3 (yellow), extensive chlorosis manifested as severe discoloration of the infiltrated area but not accompanied by necrosis; 4 (orange), chlorosis accompanied by necrosis, a reaction similar to chlorosis but with stronger discoloration and some browning; 5 (brown-green), incipient necrosis, occasional small necrotic spots within the infiltrated area considered to be indicative of necrosis having been induced in only a subset of the plant cells (because this phenotype sometimes appeared to be the plant's response to clumps of *A. tumefaciens* cells, "potential necrosis" was considered a significant reaction only if the response was consistent from experiment to experiment); 6 (magenta), necrosis manifested as browning and often tissue collapse within the infiltrated tissue; and 7 (red), severe necrosis of the infiltrated area. In most cases, each effector was tested in each genotype three times. All numerical values, summary scores, and other supplementary information are available at http://charge.ucdavis.edu.

Data Management and in Silico Analyses

The Comparative Analyses of Resistance Gene Evolution database (http://charge.ucdavis.edu) was designed to implement, store, and display information about these pathogen-plant interactions. We developed a Web interface to progressively enter the data from different experiments in the form of the numerical values described directly above. All interaction data are stored in linear tables that contain the name of the effector, plant genotype, number of replications of the assay, and experimental conditions. A summary reaction was automatically generated for each interaction based on reproducibility of the replications. The summary scores are stored in both a linear table and a matrix table for easy query. The database is fully searchable using the Web interface by effector, pathogen, pathovar, plant species, or particular genotype. The cluster analysis to search for common patterns of reactions among the summarized reaction data was performed using a modified version of MadMapper (http://cgpdb.ucdavis.edu/XLinkage/MadMapper/). The grouping was performed using Python_UniCluster_V014.py, and the order was refined using Python_MadMapper_V248_XDELTA_119.py (http://cgpdb.ucdavis.edu/scripts_and_tools/). For further manual inspection, alignment was visualized using Pixelirator (http://cgpdb.ucdavis.edu/data_pixelirator/; Supplemental Fig. S2).

Genetic Analysis

Phenotypic data were collected for 106 F2 plants descendent from an *L. sativa* 'Ninja' × *L. sativa* 'Valmaine' cross segregating in response to $AvrRps4_{Ppi151}$, $HopK1_{PtoDC3000}$, $AvrRpm1_{PmaM2}$, $AvrB1_{Pgyrace4}$, $AvrRpt2_{PtoJL1065}$, and $HopC1_{PtoDC3000}$, for 113 recombinant inbred lines (F7 generation) descendent from an *L. sativa* 'Salinas' × *L. serriola* (UC96US23) cross segregating in response to $AvrRps4_{Ppi151}$ and $HopK1_{DC3000}$, and for 107 F3 families descendent from an *L. sativa* 'Valmaine' × *L. serriola* (LSE18) cross segregating in

response to AvrPto1$_{PtoJL1065}$. Initially, the mapping was done by bulk segregant analysis (Michelmore et al., 1991) using previously developed molecular markers derived from *RGC* sequences (http://cgpdb.ucdavis.edu; McHale et al., 2009). Ninety-six PCR-based markers were run on contrasting pairs of bulks of genomic DNA from 10 individuals that produced necrotic responses and from 10 individuals that did not show the reaction. Separate pairs of bulks were made for responses to AvrPto1$_{PtoJL1065}$ and HopC1$_{PtoDC3000}$. Additionally, a single pair of bulks each was made for the responses to AvrRps4$_{Ppi151}$ and HopK1$_{PtoDC3000}$ and to AvrRpm1$_{PmaM2}$, AvrB1$_{Pgyrace4}$, and AvrRpt2$_{PtoJL1065}$, as these phenotypes cosegregated. Markers that distinguished the bulks as well as additional markers used for fine-mapping were then analyzed on all phenotyped individuals in the population (McHale et al., 2009). Genetic linkage was determined using MapMaker version 3.0 as described previously (Lander et al., 1987).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. The differences between lettuce and tomato in their reactions to the effector repertoire from *Rs* BS048.

Supplemental Figure S2. Example of a two-dimensional diagonal CheckMatrix heat plot after cluster analysis of the reactions to effectors in the 59 plant accessions tested.

Supplemental Table S1. Genes encoding effectors and related proteins.

Supplemental Table S2. Orthologous series of effectors used in the experiments.

Supplemental Table S3. Segregation of the reaction determinants to seven effector proteins in three lettuce mapping populations.

ACKNOWLEDGMENTS

We thank multiple laboratories for providing DNA of effector-encoding genes. We thank Pauline Sanders (University of California, Davis) for greenhouse assistance, Luis Williams (University of California, Davis) and Daniel Coerper (University of Chicago) for technical help, members of the Michelmore laboratory for helpful discussions, and Belinda Martineau (University of California, Davis) for editing the manuscript. Seeds of pepper, cotton, and *T. rotundiflora* were provided by M. Jahn (Cornell University), A. Paterson, and S. Knapp (both at University of Georgia), respectively. Seeds of tomato and Arabidopsis were obtained from the Tomato Genetic Resource Center (http://tgrc.ucdavis.edu) and the Arabidopsis Biological Resource Center (http://arabidopsis.org).

Received April 21, 2009; accepted June 23, 2009; published July 1, 2009.

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