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Bacterial Effectors in Plant Disease and Defense: Keys to Durable Resistance?

To develop effective strategies to protect crops from diseases, it is important to understand the molecular basis of the pathogen and host plant interactions that result in disease or resistance. Much past research emphasis was placed on identifying pathogen genes and proteins involved in triggering defense, i.e., the avirulence genes that interact, in a gene-for-gene manner, with plant disease resistance (R) genes (22). Mutational analysis of some avirulence genes demonstrated that many encoded essential components of pathogenicity or provided a selective (fitness) advantage to the pathogen in the absence of a corresponding plant resistance gene (52,84). Thus, the effectors function in both avirulence and pathogenicity, with their primary function likely being the promotion of disease (Table 1). Research is now directed at identifying and understanding the functions of bacterial pathogen effectors, which are defined as bacterial-produced proteins or compounds that contribute to an induced plant response, either disease or resistance.

Our understanding of effector function has advanced rapidly with the identification of interacting partners from the host plants. With the cloning of both bacterial and plant gene partners, we have benefited from increasing information on key structural features of the genes and their products, the locations of the proteins during critical phases, and most recently, on the intermolecular interactions in which these proteins are involved. Functional analyses

of the pathogen effector genes and their products have provided new insights into both how bacteria cause disease and induce resistance. One of the many lessons learned from these advances is that the early models predicting that gene-for-gene interactions involve elicitor-receptor interactions between the bacterial effector and plant resistance gene products don't adequately address the nuances and complexities now being revealed.

The demonstration of roles for effector genes in both avirulence and pathogenicity also has renewed interest in the relationship between importance of effector genes in pathogen fitness and durability of plant R genes (52). Gene-for-gene resistance governed by R genes is frequently rendered ineffective through the mutation or loss of effector gene avirulence function. Flor (26,27) first suggested that easily mutated avirulence genes in pathogen populations are less critical to pathogenic fitness than rarely mutated genes. The logical extension of this is that the R gene recognizing these critical avirulence effector genes might be more durable because the pathogen harboring these changes may suffer reduced competitive or fitness advantage in the plant and in the field (27,52). The ability to predict durability of R genes would be of tremendous use to breeding programs, but until recently, our understanding of effector genes and the tools to monitor changes in these genes in field populations were insufficient to evaluate the concept. With the identification and molecular characterization of effector genes with fitness value, experimental studies to evaluate this concept are now emerging and are driving new strategies to create plant varieties with longer lasting resistance.

In this treatise, we first discuss the current models describing how bacterial effectors are delivered to the host plant cell and

the structural features and activities that are important to induction of disease and resistance. We focus on the effectors delivered by the type III secretion system (TTSS) since most of the known avirulence effectors are secreted through this pathway. In the latter sections, we discuss how analysis of these effectors has provided insights into their evolution in field populations and has led to an understanding of their relationships to plant R gene durability.

Mechanisms to Deliver Bacterial Effectors to Plant Cells

For a pathogen to survive and multiply, it produces effector molecules to obtain nutrients from its host plant and cultivate the right environment in which to establish infection. Phytopathogenic bacteria use a number of secretion pathways to deliver effector molecules, either into the intercellular spaces or even directly into the host cells. These pathways vary in their complexity for delivery of the effectors (68). The complexity of the pathways is based on the number of proteins involved in the assembly of a channel or pore formed between the bacteria inner and outer membranes and through which the effectors are transported from the cytosol to the outside of the bacterium. There are four basic types of secretion pathways. Type I and II pathways secrete proteins to the supernatant or host intercellular spaces, whereas type III and IV pathways can deliver proteins or nucleic acids directly into the host cell (Fig. 1). The type I pathway is structurally the simplest; it allows direct secretion of effectors from the bacteria cytosol to the external environment. Examples of plant pathogen effectors secreted via the type I pathway are proteases and lipases from the soft-rot pathogen *Erwinia chrysanthemi* (62,89).

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Table 1. Pathogenicity functions of effector genes from some plant pathogenic bacteria

Pathogen	Gene	Pathogenicity function	Reference
<i>Erwinia amylovora</i>	<i>dspEF</i>	Fire blight symptom expression in pear, apple, and cotoneaster; sequence similar to <i>avrE</i> (53) and <i>wtSE</i> (29)	(11)
<i>Pseudomonas syringae</i> pv. <i>maculicola</i>	<i>avrRpm1</i>	Water-soaked symptom expression and bacterial multiplication	(66)
<i>P. syringae</i> pv. <i>phaseolicola</i>	<i>avrPphF</i>	Water-soaked symptom expression and bacterial multiplication in bean and soybean	(80)
	<i>virPphA</i>	Water-soaked symptom expression in bean	(44)
	<i>avrPphC</i>	Blocks avirulence function of <i>avrPphF</i> on Canadian Wonder bean	(44,88)
<i>P. syringae</i> pv. <i>tomato</i>	<i>avrA</i>	Symptom expression and bacterial multiplication	(54)
	<i>avrE</i>	Symptom expression and bacterial multiplication	(54)
	<i>avrPto</i>	Aggressiveness and bacterial multiplication in tomato	(17,70)
	<i>avrRpt2</i>	Aggressiveness and bacterial multiplication in tomato	(19)
<i>Xanthomonas axonopodis</i> pv. <i>citri</i>	<i>pthA</i>	Intercellular growth and induction of cankers in citrus	(74)
<i>X. campestris</i> pv. <i>malvacearum</i>	<i>avrB6</i>	Water-soaked symptom expression in cotton	(87)
	<i>pthN</i>	Water-soaked symptom expression in cotton	(16)
<i>X. campestris</i> pv. <i>vesicatoria</i>	<i>avrBs1</i>	Survival in soil and dead leaf tissue	(59)
	<i>avrBs2</i>	Aggressiveness and bacterial multiplication in pepper	(47)
	<i>avrBs3</i>	Mesophyll cell hypertrophy in different solanaceous plants	(57)
<i>X. oryzae</i> pv. <i>oryzae</i>	<i>avrxa5</i>	Lesion length and bacterial multiplication in rice	(5)
	<i>avrXa7</i>	Aggressiveness, lesion length, and bacterial multiplication in rice	(5)

The type II pathway is composed of a more complex secretion structure, and two steps are required for secretion of an effector: (i) transport to the periplasm (the space between the inner and outer bacterial membranes), and (ii) secretion across the outer membrane. Transport into periplasm requires an N-terminal signal sequence, and during transfer to the periplasm, the protein is processed by a signal peptidase. The intermediate location in the periplasm allows proper folding of the effector before it is secreted (18). Pathogen effectors involved in cell wall degradation, such as pectate lyase, polygalacturonase, and cellulase from *Erwinia* and *Xanthomonas* species, are secreted by the type II pathway (7,64,68).

The type III (also known as TTSS for type three secretion system) and type IV secretion pathways use complex structures similar to flagella (type III; 9) and conjugation structures (type IV; 20) to interact with the eukaryotic host cells and deliver their effectors. The type IV pathway is best known from studies on *Agrobacterium tumefaciens*. This pathway is the only secretion pathway described to translocate both proteins and nucleic acids. For example, the VirD2/T-DNA nucleoprotein complex is delivered through the type IV pilus from *A. tumefaciens* directly into the plant cell (21).

The TTSS has been widely studied because it is present in disease-causing bacteria of mammals and plants and is generally not found in their nonpathogenic counterparts (14,15,24,43). The genes encoding the TTSS are called the *hrp* (hypersensitive response and pathogenicity) genes in phytopathogenic bacteria. The *hrp* genes are usually arranged in clusters and are located in pathogenicity islands (PAIs), which are discrete regions that vary in G+C content from the overall genome and are flanked

Fig. 1. Gram-negative phytopathogenic bacteria use four major pathways to secrete effectors (type I and II) to the intercellular spaces, or directly translocate effectors (type III and IV) into the plant cell. The effectors are pathogen-produced proteins that alter the plant cell or its environment to cause disease or to trigger defense. Specific examples of effectors transported by each pathway are indicated.

by insertion sequences, bacteriophage genes, and transposable elements (33). These features suggest that PAIs originated from other species, and that they were acquired by horizontal gene transfer. The newly acquired genetic material may confer new pathogenic and fitness traits to the bacteria (37). The *hrp* genes encode proteins that either regulate synthesis or assembly of the TTSS, or are extracellular proteins (e.g., harpins) secreted by the TTSS (33). The hallmark characteristic of the TTSS structure, a needle-like protruding structure with a channel along which proteins travel, is its resemblance to bacteria flagella (9), both at the structural and functional level. Like the type I pathway, secretion of effector proteins via the TTSS is a one-step process with no intermediates in the periplasm. Some effectors require small

acidic proteins called chaperones to stabilize the effector protein or aid in its secretion through the TTSS (23,61). Unlike type IV secretion, TTSS effector proteins can be delivered either directly into the plant cell or into the extracellular spaces (39,45).

The TTSS effectors described so far are structurally very diverse, suggesting bacteria may target multiple host functions to cause disease. Most of these effectors were detected by using screens for avirulence functions. Now, more and more candidate TTSS effectors are being discovered through bioinformatic analyses of bacterial genome sequences (28,36). These screens make use of conserved sequences, such as conserved regulatory domains or the signal sequence, to target these proteins through the TTSS that reside in the extreme N-terminus of the protein and are rich in polar amino acids (36,63).

Roles of TTSS Effectors in Pathogenicity and Resistance

Although the mechanisms are not clear, TTSS effector proteins are predicted to (i) stimulate an increase in the pH and nutrient content of the plant apoplast, making the apoplastic fluids more hospitable for bacterial multiplication (2,3), (ii) activate the host defense responses through recognition by a corresponding host R protein (avirulence function; 12,49), (iii) inhibit the activation of host defense responses that are signaled by other TTSS avirulence effectors (44,65,67), or (iv) inhibit basal plant resistance mechanisms (38,80). Most of the over 40 known bacterial TTSS effectors were originally identified by their avirulence function in gene-for-gene interactions (12,52), and as a consequence, most research has focused on understanding how these proteins interact with plant proteins to activate defense responses (58). Such plant defense responses are characterized by many cellular and molecular events, including the formation of active oxygen species, defense gene induction, and in many cases, a rapid, localized cell death called the hypersensitive response (HR). Activation of the HR, which is a genetically controlled and regulated process similar to programmed cell death (35), is frequently used in studies to indicate activation of defense responses by effectors.

Since some TTSS effector proteins cause both disease and resistance phenotypes, studies into their roles in resistance have also identified possible plant virulence targets for the effector proteins. Interestingly, these plant virulence targets may or may not be the plant R gene product. Following, we summarize some of the current and simplest interpretations of how effectors function in disease and resistance.

Modification of plant interactors.

Some TTSS effectors modify plant target proteins inside the plant cell to stimulate either the activation of defense (Fig. 2A and B) or the induction of disease (Fig. 2C). Interactions involving TTSS effectors from *Pseudomonas syringae* and their target protein from *Arabidopsis thaliana* provide excellent examples of these possibilities. *P. syringae* TTSS effectors AvrPphB and AvrRpt2 are cysteine proteases whose proteolytic activity is essential for elicitation of the HR (4,71, Notes 1 and 2). HR elicitation by AvrRpt2 in *Arabidopsis* plants containing the corresponding resistance gene product, RPS2, requires cleavage of a plant membrane-bound target protein called RIN4. RIN4 is complexed with the RPS2 resistance protein, and cleavage of RIN4 by AvrRpt2 protease results in the release of RIN4 from the complex (protein degradation in Fig. 2A). Change in the overall structure of the complex is thought to be detected by RPS2, and thus the RPS2-mediated resistance response is signaled. Interestingly, two

Fig. 2. Some type III secretion system (TTSS) effectors modify a host target protein inside the plant cell promoting disease (C) or triggering resistance (A,B). The modification, which may be a conformational change, proteolytic cleavage, or phosphorylation of the target protein, is detected by the bound resistance gene product R (A). The effector also may interact directly with the R protein to trigger resistance (B). The conserved signal transduction factors are proteins present in both monocots and dicots that are essential for signaling the resistance response (41).

other bacterial effectors from *P. syringae*, AvrRpm1 and AvrB, which are not proteases, also use RIN4 as a plant target, but they signal resistance through another R protein, RPM1 (56), and by a different protein modification mechanism. AvrB and AvrRpm1 TTSS effectors induce phosphorylation of RIN4. The phosphorylation of RIN4 is detected by RPM1, and this activates the RPM1-mediated resistance response (protein modification in Fig. 2A).

The above studies show that the TTSS effector modification of a plant target protein is required for avirulence function. The requirement for modification by proteolytic cleavage or phosphorylation in the pathogenicity functions has not been determined, but it is speculated for AvrRpt2, again through interaction with the target RIN4 (4). Mackey et al. (55) suggest that the “normal” function of RIN4 may be to activate basal defense in plants. Cleavage

of RIN4 by effector AvrRpt2 protease in the absence of any R proteins is predicted to suppress these basal defense responses and, therefore, result in enhanced susceptibility. Indeed, in the absence of RPS2 (*rps2* plants), cleavage of RIN4 by AvrRpt2 leads to more pathogen growth in the tissues (55,56). Thus, RIN4 is the virulence target of AvrRpt2, and its proposed function in the plant cell is to regulate a basal level of defense. RIN4 may also function

Fig. 3. Model showing speculated pathways leading to resistance involving AvrBs3/PthA effectors. Some effector proteins in this family are transported to the plant cell nucleus where they may activate transcription of disease and/or defense response genes. It is not known where recognition of the AvrBs3/PthA protein by plant resistance (R) proteins occurs, i.e., in cytoplasm or nucleus. SS, secretion signal; LZ, imperfect leucine zipper; NLSs, nuclear localization signals; AAD, acidic activation domain; TTSS, type III secretion system; N, nucleus; C, cytosol.

in plant development because inactivation of RIN4 resulted in a dwarf phenotype in *Arabidopsis* (56).

RIN4 is likely a critical intermediary protein in plant–pathogen interactions in *Arabidopsis* because several TTSS effector proteins, including AvrRpt2, AvrRpm1, and AvrB, all interact with and modify this plant protein. It is particularly intriguing that pathogenic bacteria have evolved effectors with no sequence relationships that all target RIN4, and that plants have evolved different R proteins to monitor the condition of this target protein.

Other effectors, such as AvrBst (60) and AvrXo1 (B. Y. Zhao, E. Ardales, S. Hulbert, and J. Leach, *unpublished*) from *Xanthomonas* species, harbor sequences consistent with protease catalytic domains; however, whether or not they actually function as proteases has not been determined. Identification of plant virulence and avirulence targets for these and other effectors is an important step in addressing how general TTSS effector modification of host target proteins is in plant disease and defense.

Possible transcriptional activation of plant defense or disease response genes. The AvrBs3/PthA family is a group of highly conserved TTSS effectors prevalent in the genus *Xanthomonas* (30). The functions of these effectors in the host plant have been deduced from their unique protein structure (Fig. 3 and Table 2), which is composed of (i) a central region of multiple, nearly identical repeated sequences of 34 amino acid residues, (ii) three nuclear localization signals (NLSs) in the carboxyl terminus, and (iii) an acidic activation domain (AAD) also in the carboxyl terminus. Based on the last two structures, the NLSs and AAD, these effectors are predicted to function in the plant nucleus (for details see references on Table 2). Indeed, several family members were demonstrated to locate to the plant nucleus (78,81,86) and, in one case, to bind to DNA in vitro (85), which is consistent with a role in gene activation. Mutagenesis of both NLSs and AAD structures abolishes defense responses and reduces the ability of the pathogen to cause disease in various plant–pathogen interactions. One family member, AvrBs3 from *X. campestris* pv. *vesicatoria*, activates host genes and causes a characteristic hypertrophy in pepper; these activities require the repeats, NLSs and AAD (57). Whether these or other AvrBs3/PthA-like effectors directly activate plant defense genes remains unknown.

It is also unclear where, when, and how recognition of the AvrBs3/PthA effectors by the corresponding R proteins takes place. That is, it is not yet known if recognition for resistance takes place in the cytosol or nucleus, or if it is dependent or independent of nuclear localization. At least for one member of the family, AvrBs4 from *X. campestris* pv. *vesicatoria*, HR-mediated

resistance in tomato plants with the corresponding *Bs4* resistance gene is independent of nuclear localization (6). Progress in elucidating the function of the complex AvrBs3/PthA-like effectors will be expedited as more host interactors are identified.

Inhibition of defense response induction. Bacterial TTSS effectors can function to prevent host recognition and/or block activation of host defense responses (65,67,80). For example, the TTSS effector protein VirPphA from *P. syringae* pv. *phaseolicola* is proposed to interfere with signal transduction leading to the HR (44; pathogen effector 2 in Fig. 4). Delivery of the VirPphA from the bacteria to bean allows the pathogen to avoid HR-based resistance and to colonize the plants, even if the same pathogen also delivers a separate effector (pathogen effector 1 in Fig. 4) that is recognized by a host resistance gene product.

Interference with HR signaling is a plausible function for a virulence effector. In fact, this is the case for AvrPtoB, an effector protein from *P. syringae* pv. *tomato* that shares low sequence similarity to AvrPto. AvrPtoB inhibits HR-related programmed cell death in *Nicotiana benthamiana* (the signaling components of the tomato Pto-mediated resistance are conserved in *N. benthamiana*), thereby allowing the pathogen to colonize and cause disease (1). Interestingly, AvrPtoB is similar to VirPphA (57% identity at the protein level) and is widely distributed among several genera of phytopathogenic bacteria, including *Xanthomonas*, *Erwinia*, and *Pseudomonas*. This suggests the presence of a common structural feature for a virulence effector in plant-pathogenic bacteria and has great implications in plant disease. How AvrPtoB interferes with the HR is not known, but its interference occurs downstream of R protein recognition.

Recent work suggests that bacterial effectors encoded by *avrPto* and genes of the *avrBs3/pthA* family (*aplI* from *X. axono-*

podis pv. *citri* [46] and *avrXa10* from *X. oryzae* pv. *oryzae* [42]) suppress plant defense responses (T. Fujikawa, H. Ishihara, and S. Tsuyumu, *unpublished*). For these studies, a strain of the saprophytic bacteria *P. fluorescens* expressing the TTSS and an effector from *P. syringae* pv. *syringae* was used; this strain elicits a nonspecific HR in tobacco. When this strain was used to deliver AplI, AvrXa10, or AvrPto effector proteins to tobacco cells, the HR and other defense responses were suppressed. These bacterial effector proteins may be functioning in ways analogous to fungal suppressors of defense responses such as the one isolated from spore germination fluid of the pea fungal pathogen *Mycosphaerella pinodes* (72). This fungal suppressor retards defense responses in pea induced by a fungal nonspecific glycoprotein elicitor, but it functions as an elicitor of defense responses in cowpea (a nonhost plant). The fungal suppressor specifically inhibited NTPase (nucleoside triphosphatase) in pea cell walls, but not in cowpea walls. Interestingly, the bacterial Avr effectors delivered by *P. fluorescens* also specifically inhibit the NTPase in tobacco cell walls (T. Fujikawa, H. Ishihara, and S. Tsuyumu, *unpublished*).

Another mechanism by which TTSS effectors may suppress defense responses was recently described for *P. syringae* pv. *tomato*–*A. thaliana* interactions (38). The *P. syringae* pv. *tomato* strain DC3000 with a functional TTSS suppresses expression of a large set of *Arabidopsis* genes that encode putatively secreted cell wall and defense proteins such as extensins, hydroxyproline-rich proteins, germin-like proteins, lipid transfer proteins, and phosphatases. This suppression is caused by AvrPto that is translocated by the TTSS because AvrPto-expressing transgenic lines of *Arabidopsis* exhibited down regulation of the same cell wall and defense protein genes. Interestingly, extensins and hydroxyproline-rich proteins are components of defense response cell wall appositions

Table 2. Known functions of various domains found in AvrBs3/PthA family members

Domain	Function	Reference
Secretion signal, SS	Signal required for protein secretion through the type III secretion system	(78)
Repeat region	Essential for avirulence and pathogenicity. Biochemical function unknown	(40,57,85)
Imperfect leucine zipper, LZ	Biochemical function unknown. LZ motifs are involved in protein-protein and protein-DNA interactions	(30)
Nuclear localization signals, NLSs	Essential for avirulence and pathogenicity for most gene family members. Directs protein to plant nucleus. Second NLS of AvrBs3 binds to importin α	(6,32,77,78,81,85, 86,90)
Acidic activation domain, AAD	Essential for avirulence and pathogenicity. Activates transcription of a reporter gene in plant transient assays and in yeast cells, and activates host gene expression leading to pathogenic phenotype	(57,77,85,90)

called papilla that are initiated by bacterial pathogens lacking a functional TTSS as well as by saprophytes (8,13). These results suggest that TTSS effectors are suppressors of cell wall-based plant defense (Fig. 4).

In the absence of a functional TTSS, the cell wall-based defense is induced. Mutants of *P. syringae* pv. *tomato* DC3000 bacteria that are deficient in the TTSS induce significantly more callose deposition in *Arabidopsis* compared with wild type DC3000 (38). Callose is a major component of papilla. Again, this response can be suppressed by AvrPto, because AvrPto-expressing transgenic *Arabidopsis* plants showed low levels of callose deposition.

The fact that AvrPto suppresses both a cell wall-based defense (38) and an HR-based defense described above (T. Fujikawa, H. Ishihara, and S. Tsuyumu, *unpublished*) suggests that this TTSS effector uses different mechanisms of suppression. This raises the questions of how an effector could evolve with two different mechanisms of suppression or whether both of the mechanisms are the same or interconnected. In addition, while AvrPto suppresses the cell wall-based defense response as described above, this suppression does not likely require interaction with the R protein Pto, a serine/threonine protein kinase (17,70). Avirulence function, on the other hand, does require physical interaction with Pto in the plant cytoplasm (69,79). This demonstrates that, at least in some cases, the plant interactors for disease may be different than those for activation of defense.

Lessons Learned through Analysis of TTSS Effectors

The contributions of effector genes to pathogen avirulence and pathogenicity were determined by using gene-specific knockout mutants in the effector genes or by comparing naturally occurring variants of the genes isolated from field populations (31,52). Several important points relevant to the dual roles of effector genes can be derived from these studies. First, as discussed above, the molecular basis for how TTSS effector genes contribute to disease or resistance may vary, but there are commonalities that are emerging. For example, modification of a host target protein and detection of that modification by an R protein have been found for more than one TTSS effector/host interaction. Suppression of host defense responses by TTSS effectors has been described for bacterial interactions with bean, *Arabidopsis*, tomato, and tobacco (1,38).

Second, effector genes may contribute to different attributes of pathogenicity. In some cases, the effectors control intercellular multiplication (5,74,75), whereas in other cases, the effectors control the ability of the bacteria to exit the intercellular spaces to the leaf surface (87). Such an

increase in the release of bacteria to the leaf surface could have a major impact on the epidemic potential of the pathogen.

Third, not all effectors contribute measurably to pathogenicity, and those that do may have quantifiably different contributions. Mutations in *avrXa10* from *X. oryzae* pv. *oryzae* (5) and *avrBs3* from *X. campestris* pv. *vesicatoria* (81) did not cause a measurable loss in pathogen aggressiveness or bacterial multiplication in planta. On the other hand, the relative contributions of other *avrBs3/PthA* effectors to pathogenicity varied considerably (5,87). Inactivation of some individual members of the *avrBs3* family in *X. campestris* pv. *malvacearum* and *X. oryzae* pv. *oryzae* reduces disease symptom expression in cotton and rice, respectively. In both cases, the relative contribution by individual gene members varied. For example, the *X. oryzae* pv. *oryzae* with a mutation in the *avrXa7* gene caused much shorter lesions than the strain with mutations in other *avrBs3/pthA* family members (5).

Fourth, redundancy of function may make it difficult to measure a pathogenicity function for a given gene (22). In some

xanthomonads, there are many copies of members of the *avrBs3/pthA* gene family, and some of these contribute additively and redundantly to pathogenicity (87). In other cases, although the genes are redundant, the functions of those genes are not redundant, i.e., the pathogenicity function of the effector gene may be gene specific. In *X. oryzae* pv. *oryzae* mutants in *avrXa7*, the pathogenicity function could only be complemented by the *avrXa7* gene; the *avrXa7* mutant was not complemented by other highly related gene family members that also have a pathogenicity function such as *avrXa5* or *aB4.5* (5).

Fifth, some of the structural requirements for pathogenicity and avirulence functions are distinct. In a few cases, site-directed mutagenesis has allowed the separation of the pathogenicity and avirulence functions of effector genes. Strains of *P. syringae* containing AvrPto with single amino acid changes exhibited a loss of avirulence function, but not of pathogenicity function (70). The avirulence and pathogenicity functions of the AvrBs2 effector from *X. campestris* pv. *vesicatoria* have also been uncoupled (34).

Fig. 4. Type III secretion system (TTSS) effectors can inhibit the basal plant resistance (right) or the resistance (R) protein mediated resistance (left). The exact mechanisms are not known and there is likely more than one mode of inhibition. HR = hypersensitive response.

Sixth, to date, predicting the importance of effectors in pathogenicity and fitness from sequence structures has not been fruitful, mainly because few show similarities to known genes in databases. In a few cases, where effectors are related across pathovars, species, and even genera, and their roles in pathogenicity have been confirmed, their importance to pathogenic fitness has been speculated (48). For example, three related effectors, *avrE* from *P. syringae* pv. *tomato*, *dspE* from *Erwinia amylovora* (10,11), and *wtsE* from *Pantoea stewartii* subsp. *stewartii* (29), are critical for pathogenicity to each of the respective host plants, suggesting they may be important to pathogen fitness. As more commonalities in structure and function among effectors are observed and tested, it may be possible to predict the impact of loss of effector function on pathogen fitness.

Finally, the role of a particular effector gene in pathogenicity may vary in different bacterial genetic backgrounds. Inactivation of effector gene *avrPto* in *P. syringae* pv. *tomato* strain T1 affects pathogenicity, but in strain DC3000, inactivation of the *avrPto* gene has no effect (70). On the other hand, the *pthA* gene, which is required for virulence of *X. axonopodis* pv. *citri* to citrus plants, has different pathogenicity and avirulence functions when transferred to other xanthomonads (75).

Population Level Analyses of Effectors

Throughout the history of the use of single dominant R genes to control plant disease, we have faced “boom and bust” cycles, wherein the deployment of a new resistance gene controls disease for a limited time (boom), but then the pathogen population adapts to and overcomes the resistance (bust). The “bust” part of the cycle usually results from changes in the avirulence effector in the pathogen population, that is, mutations or loss of the bacterial effector genes that lead to loss of avirulence function and therefore breakdown in resistance. Many studies now have documented naturally occurring changes in effector genes that result in breakdown of resistance; these mutations can result from insertions, deletions, rearrangements, and loss of plasmids carrying the effector genes that function in avirulence (25,34,48,50,76,83). Since effector genes function in pathogenicity, loss of effector gene function also can result in reduced pathogenicity or aggressiveness (31,52,84). These mutations in effector genes that lead to reduced pathogenicity have been predicted to negatively impact pathogen fitness in field populations (52).

With increasing information on effector gene sequences and function, empirical tests of the prediction that mutations in

effector genes negatively impact pathogen fitness in field populations are possible. Vera Cruz et al. (83) established field plots to determine if the fitness cost associated with effector gene mutations was measurable in interactions between the bacterial blight pathogen, *X. oryzae* pv. *oryzae*, and rice. Previous laboratory studies had shown that *avrXa7*, the effector gene corresponding to the bacterial blight resistance gene *Xa7*, contributed to pathogen aggressiveness, whereas *avrXa10*, the effector gene corresponding to resistance gene *Xa10*, did not measurably contribute to aggressiveness (5; *avrXa7/avrXa10* double mutant in Fig. 5). Based on these findings, Vera Cruz and co-workers predicted that *avrXa7* but not *avrXa10* would be important to pathogen fitness in the field.

The field experiments took place over 3 years (six cropping seasons) at two sites endemic for bacterial blight in the Philippines. *X. oryzae* pv. *oryzae* populations on rice lines near-isogenic for the bacterial blight resistance genes *Xa7* and *Xa10* and the recurrent parent line IR24 were monitored. Neither *Xa7* or *Xa10* had been deployed in the Philippines prior to these experiments. Over the 3 years of the experiment, very little disease was observed on the rice line with *Xa7*, whereas severe disease was observed on the line with *Xa10* in the first year (83; Fig. 6). Clearly, loss of *avrXa10* function was not imposing a significant fitness cost on the field population.

X. oryzae pv. *oryzae* strains virulent to *Xa7* were detected during the course of the study (83). Based on the amount of disease they caused on plants with *Xa7*, these strains were divided into two groups, those that had completely lost *avrXa7* avirulence function and those that had partially lost avirulence function. Strains that had completely lost *avrXa7* avirulence function (virulent to rice with *Xa7*) were reduced in overall pathogenicity and did not increase, nor did they persist in the population. Sequence and complementation analyses confirmed that mutations in the *avrXa7* gene resulted in the loss of both avirulence and pathogenicity functions of the effector protein. These results confirmed that the mutation in *avrXa7* had imposed a significant cost on the overall fitness of the pathogen in the field.

The second group of strains isolated from the fields, i.e., those that show partial loss of *avrXa7* avirulence function and little loss of aggressiveness to susceptible rice, are intriguing because they persisted and increased in the field population during the study, yet did not cause an epidemic on *Xa7* rice (83). Significantly, continued samplings from one experimental site through 2002 show that this group of strains still persists, and yet still does not cause epidemic on *Xa7* (K. Linholm, J. E. Leach, and C. Vera Cruz, *unpublished*). The strains in this group cause a range in

Fig. 5. Contributions of individual *Xanthomonas oryzae* pv. *oryzae* *avrBs3/pthA* effector genes to pathogenicity in rice are different. Lesion lengths on susceptible rice caused by several *X. oryzae* pv. *oryzae* strains with the indicated *avrBs3/pthA* gene family member mutated. Mutations are in the background of wild-type strain PXO86. All double mutant strains harbor an insertion that inactivates the *avrXa7* gene (*avrXa7*⁻) and a second insertion that inactivates the other family member. Figure adapted from Bai et al. (5).

lesion lengths on susceptible rice (no *Xa7* gene), suggesting the possibility of many different mutations in the *avrXa7* gene that impact avirulence and pathogenicity differently. Indeed, sequence analysis of the *avrXa7* alleles from these mutants and reconstruction of some of the mutations in laboratory strains revealed a number of different changes in the *avrXa7* allele, including point mutations and rearrangements (G. Ponciano, K. Linholm, J. Bai, C. Vera Cruz, and J. Leach, *unpublished*). These changes could explain the wide variation in pathogenicity exhibited by strains harboring the *avrXa7* variants. Given that these strains persist and are increasing in the population, and that they are not as severely compromised in pathogenicity or fitness functions as the mutants with complete loss of avirulence function, it is intriguing that they have not yet caused an epidemic on *Xa7*.

From a population perspective, the detection of field variants that harbor effector alleles with changes that impact avirulence but not pathogenicity or fitness functions is of most concern. Alleles from field-derived mutants of another avirulence effector, *avrBs2* from *X. campestris* pv. *vesicatoria*, were characterized to understand adaptation to the pepper resistance gene *Bs2*. In 20 characterized alleles, several variations were detected, including deletions, insertions, and point mutations (34). To test the effects of the mutations on avirulence and aggressiveness, isogenic strains of *X. campestris* pv. *vesicatoria* that contained chromosomal versions of several mutant alleles were generated. In some cases, the mutations resulted in the loss of *avrBs2*-induced resistance on *Bs2* pepper plants and abolition of *avrBs2*-mediated enhancement of fitness on susceptible plants. In other cases, the mutations led to minimal reduction of virulence function but either minimal or intermediate levels of avirulence function. Thus, the effector *avrBs2* is evolving to decrease detection by the *Bs2* gene while at the same time maintaining its virulence function. It will be interesting to determine if there is any other cost to fitness unrelated to pathogenicity that is imposed by these mutations in *avrBs2*, for example, a reduction in ability to survive outside the plant.

The *avrBs2/Bs2* study described above emphasizes the importance of knowing if fitness and avirulence functions can be separated by mutations to different parts of the effector structure and the frequency at which this can occur. This is particularly critical when trying to relate the genetics of pathogen fitness to R gene durability, that is, why the boom cycle lasts longer for some R genes than for others (27,82). In the field study described by Vera Cruz and coworkers (83) that empirically tested the linkage between R gene durability and effector fitness, the resistance gene (*Xa7*) that targeted a pathogen effector important

for fitness (*avrXa7*) was not overcome by *X. oryzae* pv. *oryzae* variants. On the other hand, the resistance gene *Xa10*, which targeted a pathogen effector with no evidence of fitness contribution, was readily overcome. The authors suggested that *Xa7* is more durable than *Xa10* because the *avrXa7* gene is more important for pathogen fitness than the *avrXa10* gene. Now that many effector genes from many plant pathogens have been sequenced and characterized, the generality of the linkage of pathogen fitness to R gene durability can be tested.

Summary and Future Perspectives

Our understanding of the structure, location, and function of many bacterial pathogen effector genes and proteins and the nature of their interactions with plant virulence target and resistance proteins has progressed tremendously since the first avirulence gene was cloned in 1984 (73). With these advances, we have gained many insights into both how plants detect and respond to bacterial pathogens, and how those pathogens cause disease on plants. The models that have developed from these studies have provided insights into new directed strategies for enhancing resistance not just to one pathogen, but to multiple pathogens. For example, knowing that RIN4 (or its homologs in other species) is the virulence target of several effector proteins and that its structural integrity is monitored by different R proteins suggests that this protein might be an ideal candidate for modification, i.e., to avoid negative interactions with the effectors.

Understanding the relative contribution of various effectors to fitness is also useful information for predicting how durable a given resistance gene might be, or for designing plant genotypes that contain optimally effective combinations of resistance genes. Kousik and Ritchie (51), for exam-

ple, used knowledge of the *X. campestris* pv. *vesicatoria* effector genes and their corresponding resistance genes to develop pepper lines containing combinations of defeated resistance genes with residual effects. Guided studies such as these are an important research avenue for determining the practical implications of the cost of fitness concept.

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Notes Added in Press

Since this article was written, the following two articles of great relevance have been published.

1. Axtell, M. J., Chisholm, S. T., Dahlbeck, D., and Staskawicz, B. 2003. Genetic and molecular evidence that the *Pseudomonas syringae* type III effector protein AvrRpt2 is a cysteine protease. *Mol. Microbiol.* 49:1537-1546.
2. Shao, F., Golstein, C., Ade, J., Stoutemyer, M., Dixon, J. E., and Innes, R. W. 2003. Cleavage of *Arabidopsis* PBS1 by a bacterial type III effector. *Science* 301:1230-1233.

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Fig. 6. Rice field in the Philippines showing resistant rice carrying the *Xa7* bacterial blight resistance gene (cultivar IRBB7) and diseased rice lacking the *Xa7* gene (plot in right foreground, cultivar IR24, no *Xa7* or *Xa10*; plot in right background, cultivar IRBB10 with *Xa10*). Photo courtesy of the International Rice Research Institute.

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Dr. Tsuyumu is a professor in the Laboratory of Plant Pathology, Faculty of Agriculture, Shizuoka University, Japan. He received his B.S. from Shizuoka University. His M.A. and Ph.D. are in microbiology from U.C. Davis and the University of Hawaii, respectively. After a postdoctoral position in H. E. Umbarger's lab in the Department of Biological Sciences at Purdue University, he joined the faculty of Shizuoka University, where he started the work on the regulatory mechanism of pectinases in soft-rot *Erwinia* spp. and the interactions between the citrus canker pathogen, *Xanthomonas axonopodis* pv. *citri*, and host and nonhost plants.

Hiromichi (Hiro) Ishihara

Mr. Ishihara is a graduate student working to earn his Ph.D. in Dr. Tsuyumu's lab in the Laboratory of Plant Pathology, Faculty of Agriculture, Shizuoka University, Japan. He received his B.S. degree from Shizuoka University. His research involves determining critical features of the *avrBs3/pthA* effector gene family members that govern function in avirulence and virulence to citrus, rice, and other host plants. He also studies a gene isolated from *Xanthomonas axonopodis* pv. *citri* that contributes to in planta growth. Mr. Ishihara joined Dr. Leach's laboratory for 3 months in 2001 to initiate the collaboration between the labs.

Jan Leach

Dr. Leach is a professor at Kansas State University and an adjunct scientist at the International Rice Research Institute in the Philippines. She received her B.S. and M.S. degrees in microbiology from the University of Nebraska-Lincoln and her Ph.D. from the University of Wisconsin-Madison. After a postdoctoral position at East Malling Research Station, Kent, England, Dr. Leach joined the faculty at KSU where she began her studies of the interactions between the bacterial blight pathogen, *Xanthomonas oryzae* pv. *oryzae*, and rice.

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