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Our long term aim over our many years of generous DOE-BES funding was to understand mechanisms by which the pathogen virulence factors (called 'type III effectors') AvrRpm1 and AvrB activate the plant NLR immune receptor RPM1. In general effectors are delivered from the infecting bacteria into host cells by the type III pilus, where they manipulate host machinery to help pathogens overcome host defense. Delivery of effectors to increase virulence is a general feature of all classes of plant pathogens, from fungi to insects to oomcyetes and bacteria. Hence, understanding the overall diversity of effectors, their myriad delivery systems and their effectors on host cell biology, is of central importance in plant immunology.

In principle, pathogen effectors could interact with targets in the plant cell after delivery, contributing to virulence. We did find evidence for this in both the specific cases we studied and in general. Also in principle, the effectors could either directly activate the host receptor that recognizes their presence, in a ligand receptor model. However, we found that the activation of the RPM1 receptor was in fact driven indirectly, by the action of the effectors on their host target for virulence, a protein called RIN4. Our DOE-BES funded work, along with parallel funding from NSF to study the RPM1 receptor, helped drive a paradigm shift in how we understand the plant immune system, summarized in two very influential reviews (Dangl and Jones, 2001; Jones and Dangl, 2006).

We demonstrated that several Type III effectors, including the AvrRpm1, AvrB, and AvrPphB proteins from *P. syringae*, are targeted to the host plasma membrane and that efficient membrane association enhances their function as either an Avr protein or, in the case of AvrRpm1, a virulence factor. Efficient localization of all three Avr proteins requires consensus myristoylation sites and at least AvrRpm1 and AvrB proteins can be myristoylated inside the host cell. These prokaryotic Type III effectors thus utilize a eukaryote-specific post-translational modification to access the subcellular compartment where they function. These results are very exciting as they predict that the role of these three Avr proteins, and several other *P. syringae* Type III effectors which also have consensus acylation sequences, in virulence of bacterial pathogens is carried out at the inside face of the host plasma membrane (Nimchuk et al., 2000). We also identified another NLR receptor that responds weakly to AvrB (Eitas et al., 2008).

The isolation of RIN4 via its interaction with AvrB was the first step in an ongoing process to understand how effectors manipulate target biochemistry to provide a hospitable environment for the pathogen, and how the host immune system has evolved to be activated by these manipulations. RIN4, like AvrB and AvrRPm1, is acylated into the host plasma membrane (Kim et al., 2005a) where it interacts with RPM1 in plants expressing the *RPM1* gene (Mackey et al., 2002). In addition to AvrB and AvrRpm1 targeting RIN4, we and others showed that a third type III effector called AvrRpt2 does as well (Mackey et al., 2003). AvrRpt2 is a cysteine protease that cleaves RIN4 in two homologous positions, thus activating a second NLR receptor, RPS2, also at the plasma membrane (Mackey et al., 2003). At rest, RIN4 interacts with different pools of RPM1 and RPS2. Because AvrRpt2 eliminates RIN4 by cleavage, *rin4* null mutants are lethal as RPS2 is ectopically activated (Belkhadir et al., 2004a). The real function of RIN4 is illuminated in the absence of RPM1 or RPS2; RIN4 negatively regulates plant defense against pathogen pattern molecules like flagellin (Kim et al., 2005b).

In collaboration with my colleague John Sondek, co-crystalized a fragment of RIN4, containing the AvrRpt2 cleavage site and the domain that interacts with AvrB, and we used this structure as a guide to define AvrB residues required for RIN4 interaction and its activity during RPM1 activation (Desveaux et al., 2007). The presence of AvrB and AvrRpm1 inside the plant cell lead to post-translational modifications on RIN4 (Mackey et al., 2002). AvrB, acting with a host kinase called RIPK, leads to accumulation of extra phosphorylation on several sites of RIN4 and we have shown that one of these pThr166, is necessary and sufficient to activate RPM1 (Chung et al., 2011) at the plasma membrane (Gao et al., 2011). However, when microbes are sensed, for example, via recognition of their flagellin by

the FLS2 cell surface receptor, other kinases act on different RIN4 residues to de-repress host defense responses. In this case, accumulation of RIN4 pSer141 is both necessary and sufficient (Chung et al., 2014). These findings allowed us to build an evolutionarily consistent model wherein RIN4 normally suppresses host defense. At rest, phosphorylation of RIN4 on Thr166 and Ser141 are at some equilibrium that maintains the defense system in an off state. Upon recognition of microbial patterns like flagellin, phosphorylation on RIN4 Ser141 de-represses the host defense system, to the detriment of the microbe. If that microbe delivers effectors like AvrB, then the effector enhances RIPK activity on RIN4 Thr166. We showed that excess pThr166 suppresses the accumulation of pSer141, proving that AvrB locks RIN4 into an off state to suppress defense and contribute to pathogen virulence (Chung et al., 2014).

We also established that AvrRpm1 interacts with RIN4 at the general positions, but with slightly different precise amino acid requirements (Chung et al., 2011). AvrRpm1 is very difficult to purify and our work has been hampered by this. However, structural modeling allowed us to suggest that AvrRpm1 encodes a poly-ADP-ribosylation protein and residues required for this catalytic function are required for its ability to activate RPM1 and to contribute to virulence (Cherkis et al., 2012).

AvrRpm1 is carried on a transmissible plasmid of over 40kb that we sequenced (Rohmer et al., 2003). This exercise led us to discover that diverse evolutionary mechanisms shape type III effector evolution (Rohmer et al., 2004) and to also consider, using the then newly emerging high throughput DNA sequencing technologies (Jeck et al., 2007; Reinhardt et al., 2009), the entire effector repertoire of a particular bacterial species (Baltrus et al., 2011). Using RNSseq, we were able to completely define the type III regulon (specifically defined as that set of genes controlled by HrpL), leading to the definition of new virulence factors and evolutionary mechanisms by which bacterial genes are recruited into, and lost from, this critical virulence regulon (Mucyn et al., 2014). Our sequencing exercise opened the floodgates for further sequencing of *P. syringae* isolates from around the world, and the DOE-JGI IMG database now has upwards of 70 different pathovars, most submitted by us.

That RIN4 is targeted by multiple type III effectors also suggested that knowing the entirety of the type III effector collection from a broad phylogenetic representation of a single bacterial species, *Pseudomonas syringae*, might help us to 'collapse' the huge diversity of effectors, from all pathogen types, down onto a limited number of shared host targets. These host targets, in turn, would define the sets of machinery that are generically required for defense, deduced because evolution had seen fit to target them repeatedly with pathogen effectors. We combined forces with several labs to do this experiment as part of a large interactome project. We used pathogen effectors from bacterial and from an oomycete and a fungal pathogen separated by 2 billion years of independent evolution. Amazingly, these sequence unrelated effector suites did collapse down onto a set of host proteins, most of which have mutant phenotypes consistent with a role in defense (Mukhtar et al., 2011; Wessling et al., 2014). Our work on this project has spurred other examples and the concept that effectors from diverse pathogens evolve to target a core set of host machines is now well established in the field.

Our P. syringae genome sequences also propelled new biology in our DOE-BES project. Again in collaboration with the Sondek lab, we solved structures for a chaperone-effector pair called HopF2. The structure allowed us to define key conserved residues on HopF2 required for both virulence function and the ability to trigger a host NLR protein (Singer et al., 2004). This finding is consistent with the idea that manipulation of a host target for effector virulence function is essential for its recognition in plants that are genetically programmed to do so via the presence of an NLR genes. Using the same logic, we were able to define key residues required for virulence and receptor recognition in the AvrPphE family (Nimchuk et al., 2007). We identified a novel effector, crystallized it and used it to define a new, and novel, TIR only NLR receptor (unpublished). We also have identified a novel effector that blocks the Yang cycle required to cycle methionine into ethylene. These studies will be the last published citing support from the DOE-BES funding.

Of course, given the high profile of our publications, we have been asked to provide many reviews. Each has its important points. Our reviews in the framework of the DOE-BES project are best summarized as three sets. In one set, we summarized models for NLR activation and we reviewed data consistent with the idea that NLR receptors acted in pairs and that these could be homologous or heterologous (Belkhadir et al., 2004b; Eitas and Dangl, 2010; Nimchuk et al., 2003; Nimchuk et al., 2001). In the second set, we were able to summarize the knowledge at the time about type III effectors from a variety of plant pathogenic bacteria (Chang et al., 2004; Desveaux et al., 2006; Grant et al., 2006; Mole et al., 2007). In the third set, we were asked to summarize how a strong community focus on Arabidopsis has led to overall conceptual breakthroughs in plant immunology over time and how that might be deployed to facilitate crop improvement (Dangl et al., 2013; Nishimura and Dangl, 2010).

In sum, our DOE-BES funding has made a very large contribution to the overall understanding of how the plant immune system is wired. We are extremely grateful for such long term, continuous support from DOE-BES.

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