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Effector Trafficking: RXLR-dEER as Extra Gear for Delivery into Plant Cells

When driving a car with automatic transmission, one hardly notices that extra gears give more power to the car. But in a car with manual transmission, one is constantly aware that even one gear shift helps to reach your goal much more efficiently. For Phytophthora pathogens, a domain characterized by the amino acid motifs RXLR and dEER seems to function as a special gear. In this issue of The Plant Cell, Dou et al. (2008b) report that an RXLR-dEER domain embodies a complete machinery that the pathogen needs to deliver effectors into host cells. This is in contrast with the bacterial type III secretion system, which requires a multitude of proteins to accomplish this task (see figure panels A and B).

Many microorganisms are successful plant pathogens because they have the ability to manipulate the host and suppress plant defenses and hence can invade and colonize plant cells without any hindrance. To manipulate plant innate immunity, pathogens secrete effector proteins that negatively affect the well-being of the plant. In the arms race, plants have evolved resistance (R) proteins that recognize effectors. This often leads to cell death thus arresting pathogen growth. Since resistance against hemibiotrophic Phytophthora species, such as P. infestans and P. sojae, is governed by cytoplasmic R proteins containing nucleotide binding site and leucine-rich repeat domains, it was anticipated that the cognate effectors should also reside in the cytoplasm of host cells. There is now ample evidence that several oomycete effectors are recognized intercellularly; they become avirulence proteins in the presence of their respective R proteins.

To address the question of how oomycete effectors are delivered into plant cells, Dou et al. (2008b) describe a set of elegant experiments investigating the role of the conserved motifs RXLR and dEER that were discovered in 2004 during a Phytophthora genome annotation jamboree (Govers and Gijzen, 2006). At that time, the first few oomycete avirulence (Avr) genes were isolated by positional cloning, and a genome mining and alignment expedition resulted in the discovery of these conserved motifs in all oomycete Avr proteins and Avr homologs. The motifs are located in the N terminus adjacent to the signal peptide in a domain christened RXLR-dEER (see figure panel C). As yet, no conserved motifs have been identified in Avr proteins from fungal biotrophic pathogens (Catanzariti et al., 2007), and the presence of a conserved motif in oomycete Avr proteins came as a true surprise. As it happened, shortly after the 2004 jamboree, it was reported that many effector proteins of the malaria parasite Plasmodium falciparum share the conserved motif RXLX^{V/I}D. This motif, with a striking resemblance to RXLR, plays a role in translocating effectors across membranes that surround vacuoles harboring malaria parasites in red blood cells. Delivery into the cytoplasm of host cells enables these Plasmodium effectors to carry out virulence and host remodeling functions (Templeton and Deitsch, 2005). This led to the hypothesis that oomycete RXLR effectors are also targeted into host cells.

The RXLR-dEER effector gene investigated by Dou et al. (2008b) is P. sojae Avr1b-1, the counterpart of the soybean R gene Rps1b in a gene-for-gene interaction and the first oomycete Avr gene that was cloned (Shan et al., 2004). To prove Avr function, Shan et al. (2004) infiltrated Avr1b protein into the apoplast and observed Rps1b-specific defense responses. This suggested extracellular recognition of the Avr1b protein, an issue that was controversial in light of the intracellular nature of R proteins. Subsequently, other oomycete Avr genes were identified, and transient in planta coexpression assays with their cognate R genes pointed toward intracellular recognition (Kamoun, 2006). These assays also revealed that the recognition domains of RXLR-dEER effectors are located in the C terminus and not the N terminus that harbors the RXLR and dEER motifs. Earlier this year Dou et al. (2008a), provided evidence that Avr1b is in fact recognized intracellularly and defined certain domains and amino acid residues in the C terminus of Avr1b that are involved in the avirulence function or suppression of cell death. Cobombardment of Rps1b soybean leaves with leaderless Avr1b-1 constructs driven by the 35S CaMV promoter and a GUS construct resulted in a significant reduction of GUS-positive spots. To facilitate comparison of test and control bombardments, the authors modified a standard Bio-Rad Gene Gun with a special device: a double barrel to shoot two different DNA samples side by side into a leaf in the same shot. This innovative device enables transient biologic expression to be internally controlled and quantified and represents a valuable addition to the toolbox of researchers studying effectors and plant-microbe interactions in general. In their study, Dou et al. (2008b) used the modified Gene Gun extensively to test Avr1b-1 constructs with mutations in the RXLR and dEER motifs.

So what then is the function of the RXLR-dEER domain? Dou et al. (2008b) first showed that the RXLR and dEER motifs are essential for avirulence function of Avr1b. They transformed a P. sojae strain that is virulent on Rps1b plants with Avr1b-1, with and without mutations in RXLR and dEER. Transgenic strains complemented with wild-type Avr1b-1 could no longer infect Rps1b plants and were avirulent. By contrast, the phenotype of strains complemented with Avr1b-1 constructs having mutated motifs did not change, thus demonstrating that dEER and RXLR are pivotal. These results are similar to those reported by Whisson et al. (2007), who found gain of avirulence on R3a-potato in P. infestans.
Transgenic strains complemented with wild-type Avr3a but not with Avr3a mutated in the RXLR and dEER motifs. Unlike Avr3a, Avr1b has two adjacent RXLR motifs, 1 and 2, seven amino acids apart. Only mutations in RXLR2, and not RXLR1, abolished avirulence. As described below, Dou et al. (2008b) exploited this feature to demonstrate the importance of sequences flanking the motif. Since dEER and RXLR2 are not required for triggering cell death when Avr1b-1 is bombarded into Rps1b-leaves, a function in effector delivery seemed apparent.

The next step was to investigate what happens when Avr1b, produced in planta upon bombardment with an Avr1b-1 construct, has a secretory leader. Whereas secreted Avr1b triggered Rps1b-mediated cell death with the same efficiency as Avr1b lacking the secretory leader, secreted Avr1b with mutated RXLR2 and dEER motifs triggered no response. This suggested that the motifs mediate reentry of Avr1b into the cell and, more strikingly, that the entry does not require the presence of the pathogen. To assess stability of Avr1b and mutated Avr1b, with and without secretory leader, GFP fusions were made and bombarded in onion bulb epidermal cells. Accumulation in the cytoplasm or the apoplast could be distinguished, and especially after plasmolysis there was a clear difference between secreted and nonsecreted Avr1b and between secreted wild-type and mutated Avr1b. These experiments confirmed a role for RXLR2 and dEER in reentry of Avr1b. Autonomous uptake in plant cells was also demonstrated with Avr1b-GFP fusion proteins synthesized in Escherichia coli. Soaking root tips of soybean seedlings for 12 h in a solution containing partly purified fusion protein resulted in penetration of Avr1b-GFP protein into the roots tips up to 10 cell layers deep, including uptake into cells. Also in this assay, mutations in the RXLR or dEER motifs abolished uptake. In retrospect, this could be an explanation for the controversial findings of Shan et al. (2004) that infiltrations of Avr1b into the apoplast elicited Rps1b-specific cell death.

Avr1b-1 is one of the hundreds of RXLR-dEER genes known. The size of this gene family in each of the sequenced Phytophthora genomes is astonishing. Jiang et al. (2008) reported 396 RXLR-dEER genes in P. sojae and 374 in P. ramorum. With the same mining strategy of reiterated BLAST searches and Hidden Markov Models (HMM), ~560 family members were found in P. infestans (R.H.Y. Jiang, personal communication). Despite the fact that other mining strategies and algorithms resulted in different numbers (Whisson et al., 2007; Win et al., 2007; Jiang et al., 2008), there is no doubt that the family is extremely large and diverse. The extensive sequence diversity in the C-terminal domains is reminiscent of rapid birth and death evolution and is consistent with a role in interactions with host plants. As yet, dispute about the best mining strategy continues, but it is clear that a simple RXLR string search is not sufficient. As shown by Dou et al., mutations in RXLR1 do not compromise the avirulence function of Avr1b, and this is in line with the low HMM score of RXLR1 compared with RXLR2. This HMM was created using RXLR flanking regions (10 amino acid residues to the left and right) and could clearly separate sequences with a random RXLR motif (HMM < 5) from sequences in a curated set of high-quality
candidate RXLR effectors (Jiang et al., 2008) and in known Avr proteins (P. infestans Avr3a and Hyaloperonospora parasitica ATR1 and ATR13). Altogether, these results implied that sequences flanking RXLR are important to the activity of an RXLR motif.

Dou et al. (2008b) next conducted experiments to show that RXLR-dEER domains in proteins other than Avr1b can functionally replace the RXLR-dEER domain in Avr1b. Fusion of two RXLR-dEER proteins (HMM > 5) with the C-terminal domain of Avr1b resulted in hybrids that rendered transgenic P. sojae strains avirulent on Rps1b plants. Similarly, by replacing the RXLR-dEER domain in Avr1b with host cell targeting domains of three Plasmodium proteins, the cell death–inducing activity of secreted Avr1b in biolistic assays was retained. Mutant analysis in Plasmodium revealed that the RXLR domain of the RXLR effectors of plant pathogens and Plasmodium are functionally interchangeable, one could wonder whether the uptake machinery is common to plants and mammals and, if so, what the intrinsic function is. Can every RXLR-dEER effector exploit this uptake machinery and always in an autonomous manner? Why does the host maintain such a system? Are there conserved host cell targeting motifs in fungal effectors that are yet to be discovered, and do they use the same host machinery? These are just a few of the many questions that remain to be answered before we fully understand the effector trafficking process and the power of the extra gear of these devastating pathogens.

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