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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; pages 1930–1947)** report that an RXLR-dEER domain embodies the 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$^{E}/_{Q}$. 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 *Rps*1b 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 *Rps*1b-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 *Rps*1b 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 biolistic 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 *Rps*1b plants with *Avr1b*-1, with and without mutations in RXLR and dEER. Transgenic strains complemented with wild-type *Avr1b*-1 could no longer infect *Rps*1b 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*

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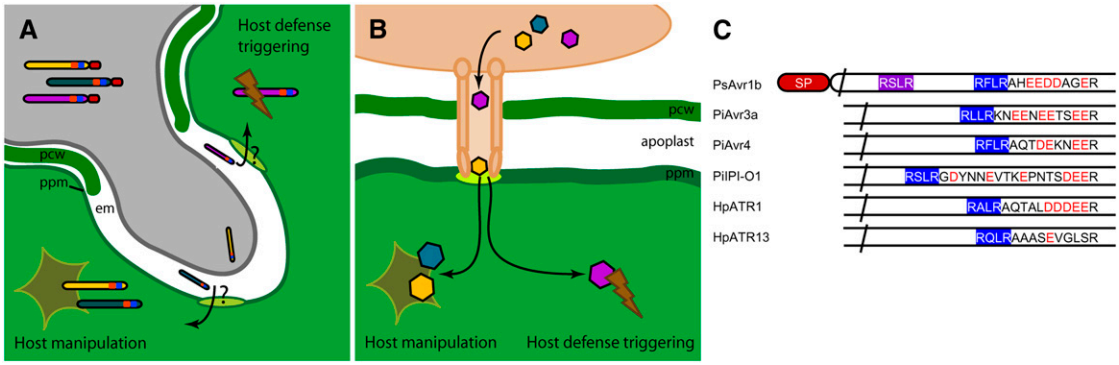

Effector Trafficking and the RXLR-dEER Domain.

Pathogens deliver effector proteins into the cytoplasm to suppress host defenses and to manipulate the host. When recognized by R proteins, defense responses are triggered.

(A) Effector proteins from *Phytophthora* are secreted from haustoria into the extrahaustorial matrix (em) and cross the plant plasma membrane (ppm). This trafficking is mediated by the RXLR-dEER domain located in the N terminus of effector proteins. In the model proposed by Dou et al. (2008b), RXLR-dEER proteins exploit a targeted host machinery (?) for uptake and do not require other proteins from the pathogen for translocation.

(B) By contrast, the type III secretion system of bacteria requires a multitude of proteins from the pathogen to form a molecular syringe that penetrates the plant cell wall (pcw) and plasma membrane, through which effectors are injected into host cells.

(C) The N-terminal part of Avr1b contains a signal peptide (SP) and the RXLR-dEER domain with two adjacent RXLR motifs (RXLR1 in purple and RXLR2 in blue) and a dEER motif. As shown by Dou et al. (2008b), RXLR1 is not essential for avirulence function of Avr1b. Alignment of RXLR-dEER domains of avirulence proteins from *P. sojae* (Ps), *P. infestans* (Pi), and the downy mildew *H. parasitica* (Hp) (Shan et al., 2004; Kamoun, 2006; van Poppel et al., 2008) shows that the dEER motif is less conserved than the RXLR motif but is characterized by a stretch of mainly acidic amino acids (red).

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 *Rps*1b-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 *Rps*1b-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 *Rps*1b-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

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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 *Rps*1b 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 $RXLX^{E}/_{Q}$ motif by itself is not sufficient to translocate proteins across membranes into red blood cells but requires flanking regions enriched in acidic and hydrophilic residues. Since Dou et al. (2008b) did not perform mutant analysis of the flanking domains, there is as yet no direct evidence that flanking regions of Avr1b are required for autonomous uptake. Dou et al. (2008b) further investigated whether a polyarginine (Arg_9) and a protein transduction domain in an HIV-1 protein, both of which are capable of autonomously carrying proteins across membranes in animal systems, can functionally replace RXLR2. Biolistics showed they can but, as yet, it is not clear how to interpret these findings in relation to the mechanisms of effector delivery into plant cells. Macropinocytosis, a specialized form of endocytosis, is one option, but Dou et al. (2008b) favor the hypothesis of receptor-mediated uptake (see figure panel A). This would require a targeted machinery of host origin that is exploited by pathogens. A precedent for this is a membrane-spanning lectin receptor kinase in *Arabidopsis* that binds an RXLR-dEER effector from *P. infestans* (Gouget et al., 2006).

Oomycetes and *Plasmodium* belong to the same eukaryotic supergroup, the Chromalveolates (Govers and Gijzen, 2006). The similarity between the RXLR motif in oomycetes and $RXLX^{E}/_{Q}$ in *Plasmodium* could point to a common evolutionary origin of the domains carrying these motifs. But what about the host uptake machinery? How widespread is this machinery and how ancient? Considering the fact that the host cell targeting domains in *Phytophthora* 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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