Showdown at the RXLR motif: Serious differences of opinion in how effector proteins from filamentous eukaryotic pathogens enter plant cells

Jeffrey G. Ellis[1] and Peter N. Dodds

Commonwealth Scientific and Industrial Research Organization, Division of Plant Industry, Canberra, Australia

Plant pathology has entered the era where it seems possible to understand the basis of pathogenicity in molecular terms. The key has been the identification of the role of pathogen effectors, proteins secreted at infection sites, some of which enter host cells to dampen host immunity and modify metabolism to pathogens' advantage. Knowledge of how effectors function and how their contributions to plant disease can be blocked promises ways of controlling crop diseases (1).

An Oomycete Effector Translocation Signal

Fungal and oomycete pathogens (filamentous eukaryotic microbes) that are the major pathogens of rice, corn, wheat, soybeans, and potatoes, the big 5 crops feeding the world, secrete effectors that enter plant cells to reach their site of action. How do effectors cross the host membrane, and what is their function inside of host cells? Studies with the effectors Avirulence protein 3a (AVR3a) from *Phytophthora infestans* and Avirulence protein 1b (AVR1b) from *P. sojae*, pathogens of potato and soybean, respectively, have provided important insights. Both effectors carry an uptake signal motif with consensus sequence arginine, any amino acid, leucine, arginine (RXLR) that is common to a large repertoire of oomycete effectors and located near each protein's N terminus, which directs effector uptake by plants (2, 3). However, the identification of this signal did not reveal the mechanism of effector uptake by host cells.

RXLR and Phosphatidyl Inositol Phosphate Binding

In the work by Kale et al. (4), data were presented indicating that the RXLR domain mediates binding of AVR1b to phosphatidyl inositol phosphates (PIPs). The experimental evidence for this hypothesis was generated through immunological detection of specific interactions of the purified *P. sojae* effector and various forms of PIP (differing by the position or number of phosphate groups on the inositol ring) immobilized on nitrocellulose membranes or exposed on the surface of artificial vesicles. Specifically, full-length AVR1b, which penetrated plant cells, bound PI3P and PI4P, whereas AVR1b with the RXLR motif (in this effector, RFLR) mutated to glutamine phenylalanine leucine arginine (QFLR) or alanine (AAAA) failed to penetrate plant cells and bind to either PIP. The N-terminal region of AVR1b (containing the RXLR motif) still bound PI4P but interestingly, not PI3P, and this binding was also dependent on the RXLR motif.

The sum of these data and data with other effectors was used to postulate that the RXLR motif mediates effector entry into host cells through the uptake motif binding to PIPs exposed on the external surface of the plant plasma membrane, which stimulates endocytosis of effectors into plant cells (4). Additionally, a role for PIP binding in effector translocation was supported by inhibition of effector uptake by plant cells by exogenous PIPs and PIP binding proteins and inositol phosphate head groups of PI3P and PI4P.

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Showdown at the RXLR

Now, a paper (1) in PNAS challenges the RXLR × PIP interaction = uptake story. Yaeno et al. (1), working with AVR3a from *P. infestans* and AVR1b from *P. sojae*, confirm that both effectors interact with PIPs. However, contradicting the work by Kale et al. (4), the work by Yaeno et al. (1) finds that mutations in the RXLR (AAAA substitution for AVR3a and AVR1b and QFLR for AVR1b) do not disrupt interaction with PIPs in filter-binding assays. Furthermore, deletion analysis shows that the PIP binding of AVR3a is mediated by a C-terminal fragment lacking the RXLR domain. Conversely, an N-terminal fragment of AVR3a containing the RXLR domain fails to interact with PIPs. Yaeno et al. (1) also produce an NMR structure of an amino acid sequence-related effector AVR3a4 (but not Avr3a and AVR1b because of solubility and protein-folding problems) and show by structural modeling that AVR3a4, AVR3a, and AVR1b are structurally related proteins comprised of four similarly placed alpha-helices. Each effector contains sequence-related surface patches of positively charged amino acids at the equivalent location in each structure, which Yaeno et al. (1) postulate could bind to negatively charged PIPs. Yaeno et al. (1) show that mutations in the positive patch of AVR1b and AVR3a diminish or abolish their PIP interactions. Unfortunately, AVR3a4, with its determined solution structure, does not bind to PIPs, possibly because of partial disruption of the positive patch by nearby negative charges. This finding precluded NMR studies to confirm and map AVR-PIP binding in solution. Nevertheless, the results and conclusions of this work contradict the data in the work by Kale et al. (4), which found a PIP–RXLR interaction.

Given that Yaeno et al. (1) find no PIP binding to the RXLR, what function does PIP binding to the positive patch of Avr3a have? Avr3a has four biological activities detected so far: (*i*) secretion from *P. infestans* determined by the N-terminal secretion signal, (*ii*) host cell penetration controlled by RXLR, (*iii*) induction of host defense responses in plants expressing the immune receptor R3a, and (*iv*) in the absence of R3a, interaction with a host E3 ubiquitin ligase CMPG1, causing suppression of host cell death induced by certain pathogen protein toxins and pathogen associated molecular patterns (PAMPs) (5, 6). The PIP binding domain of Avr3a is not required for secretion from the *P. infestans* (*i*) (2). When transiently expressed in host cells, mutants of AVR3a that do not bind PIPs do not abolish R3a activation and conversely, an allelic version of AVR3a that does not activate R3a does bind PIPs. Therefore, PIP binding is not

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See companion article on page 14682.

[1]To whom correspondence should be addressed. E-mail: Jeff.Ellis@csiro.au.

involved in recognition by the host R3a immune receptor (*iii*). However, a mutant of AVR3a that does not bind PIP does not suppress the host cell death response mediated by the AVR3a-CMPG1 interaction (*iv*), and therefore, Yaeno et al. (1) propose that binding of AVR3a to PIPs is associated with the intracellular virulence enhancing activity of the effector, possibly through internal membrane association because of effector–PIP interaction. Given this focus on an intracellular function for PIP binding, the work by Yaeno et al. (1) importantly omits to test the requirement for PIP-positive patch interaction for effector uptake by host cells (*ii*).

Rapid Resolution Now Needed

Given the current mutually exclusive nature of the data and conclusions in these two papers, where does this standoff leave the field of plant pathogen effector biology? The answer is in a bit of a mess. There is now an urgent need for this situation to be cleared up by independent repetition of the experiments using exactly the same mutants and deletions of both AVR1b and AVR3a. Kale et al. (4) did not report PIP binding assays using AVR1b deleted for its N-terminal region carrying RXLR. There is also a need to develop an NMR-based solution assay to confirm and map the AVR-PIP binding using an AVR protein that binds PIP in the filter-binding assay. Equally important, there is a need to establish whether there is a requirement for the PIP binding to the positive patch for effector uptake by plant cells. As pointed out by the work by Kale et al. (4) an understanding of how to block uptake and function of effectors, the basis of plant diseases, has the potential to provide new therapeutic agents for disease control to assist meeting food security targets. Given the current significance of this goal, the confusion needs rapid resolution. The potential translational application of effector biology underlines the critical requirement to get the basic science right.

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