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The RXLR motif of oomycete effectors is not a sufficient element for binding to phosphatidylinositol monophosphates

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The translocation of effector proteins into the host plant cells is essential for pathogens to suppress plant immune responses. The oomycete pathogen *Phytophthora infestans* secretes AVR3a, a crucial virulence effector protein with an N-terminal RXLR motif that is required for this translocation. It has been reported that the RXLR motif of *P. sojae* Avr1b, which is a close homolog of AVR3a, is required for binding to phosphatidylinositol monophosphates (PIPs). However, in our previous report, AVR3a as well as Avr1b bind to PIPs not via RXLR but via lysine residues forming a positively-charged area in the effector domain. In this report, we examined whether other RXLR effectors whose structures have been determined bind to PIPs. Both *P. capsici* AVR3a11 and *Hyaloperonospora arabidopsidis* ATR1 have an RXLR motif in their N-terminal regions but did not bind to any PIPs. These results suggest that the RXLR motif is not sufficient for PIP binding.

Filamentous plant pathogens, including oomycete and fungi, secrete a number of effector proteins that accumulate in apoplastic spaces or enter host plant cells to modulate host immune responses.[1,2] AVR3a, an effector protein secreted from the oomycete pathogen *Phytophthora infestans* causing potato late blight disease, has the characteristic RXLR motif sequence of amino acids Arg-X-Leu-Arg (where X is any amino acid) at the N-terminus and an effector domain harboring virulence functions at the C-terminal end. AVR3a is translocated into the host cells in an RXLR-motif dependent manner.[3,4]

In our previous work, we determined the protein structure of *P. capsici* AVR3a4, which is a close homolog of AVR3a, by NMR analysis.[5] The NMR-derived model structure of AVR3a showed that the effector domain comprises four α-helices, but the N-terminal region including the RXLR motif is disordered (**Fig. 1**). Kale et al.[6] have reported that the RXLR motif of Avr1b, which is a close homolog of AVR3a in *P. sojae*, binds to phosphatidylinositol monophosphate (PIPs) lipids on the surface of host cells and hypothesized that this binding is required for pathogen-independent entry of the protein into host cells. On the contrary, we found that AVR3a as well as Avr1b bound to PIPs not via the RXLR motif, but via lysine residues forming a positively-charged area in the effector domain.[5] In agreement with our findings, it was recently shown that the PIP-binding abilities of AVR3a are mediated by its effector domain, not its RXLR motif.[7] However, it was reported that the MiSSP7 effector protein secreted from *Laccaria bicolor*, a mutualistic ectomycorrhizal symbiont of poplar, can enter host plant cells via an RXLR-like motif, RALG, and that the motif binds to PIPs.[8] To resolve these discrepancies, it is necessary to perform further studies on whether other RXLR effectors also bind to PIPs.

In addition to AVR3a4, the protein structures of AVR3a11 from *P. capsici* as well as ATR1 from *Hyaloperonospora arabidopsidis,* an oomycete downy mildew pathogen, were determined.[9,10] We therefore investigated whether these RXLR effectors bind to PIPs. The GST fusions of these proteins were used for a lipid overlay assay as described in Yaeno et al.[5] As shown in **Figure 2**, AVR3a11 and ATR1 does not bind to PIPs, even though they harbor the RXLR motif at the N-terminus, suggesting that the RXLR motif is insufficient for PIP binding. These RXLR effectors also have the WY motif in the effector domain as a conserved structural fold.[11] Thus, the WY motif is unlikely to be involved in PIP binding.

Recently, consistent in principle with our findings,[5] Sun et al.[12] showed that the *P. sojae* RXLR effector Avh5 bound to PIPs predominantly via the lysine residues of the C-terminal effector domain. The mutations in the RXLR motif of Avh5 did not have much effect on PIP binding. This is inconsistent with the finding by Kale et al.[6] showing that Avh5 binds to PIPs via the RXLR motif and the reason for this discrepancy is unclear.

The PIP-binding ability of the effector domain in AVR3a may be essential for protein stability inside the host cells.[5] Similarly, PIP binding confers thermal stability and a protective effect against trypsin proteolysis to Avh5. As the results were obtained from NMR analysis and circular dichroism spectra, the tested

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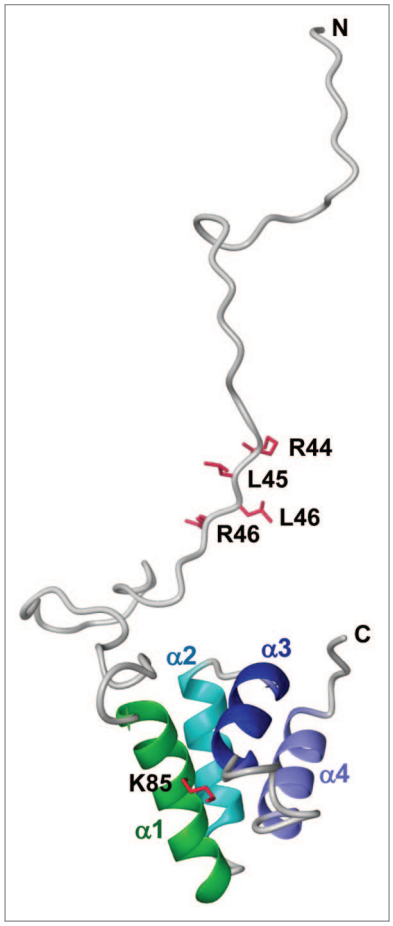

Figure 1. The NMR-derived model structure of AVR3a with the RXLR motif. The residues for the RXLR motif (R44, L45, L46 and R47) and PIP binding (K85) are mapped on the ribbon diagram as red sticks.

Avh5 protein is likely to be properly structured.[12] In contrast, it was reported that PIP binding was observed in only denatured AVR3a proteins in vitro and was physiologically irrelevant.[7] Thus the physiological roles of PIP binding in the effector domains remain to be elucidated.

A major point of contention in studies on effector translocation is whether or not the PIP-binding abilities of effectors are involved in host cell entry. AvrM, an effector from *Melampsora lini,* the flax rust fungus, enters host plant cells but has no obvious motif in the region required for entry.[13] Gan et al.[14] found that although AvrM bound to PIPs, the binding was independent of the region required for host cell entry. SpHtp1, an effector from the fish pathogenic oomycete *Saprolegnia parasitica*, enters fish cells in an RXLR-like motif dependent manner. However, this process is not mediated by PIP binding.[15] The effectors of the human malaria parasite *Plasmodium falciparum* which are delivered into host cells also have an RXLR-like motif RxLxE/D/Q required for translocation across the parasitiphorous vacuolar membrane into the host erythrocyte cytoplasm.[16,17] Interestingly, the RxLxE/D/Q motif binds to PI3P in parasite endoplasmic reticulum (ER) membranes in the process of export to the erythrocyte.[18] However, in fact, the motif is cleaved by a protease in the parasite ER before export, and furthermore, the cell surfaces of host erythrocytes do not have detectable levels of PI3P.[6,19,20] Thus, the entry of *Plasmodium* effectors cannot be explained by PIP-binding of their RXLR-like motif to host PIPs. Even if the RXLR motif itself has a PIP-binding ability similar to the RxLxE/D/Q motif, the mechanism of cell entry appears to differ between RXLR effectors and *Plasmodium* effectors. This is because unlike *Plasmodium* effectors, RXLR effectors can enter host cells without the requirement of pathogen-encoded machinery.[4,21,22] Clearly, many questions remain to be resolved to elucidate the mechanisms underlying host cell entry of effector proteins, including the relationship between PIP binding and cell entry.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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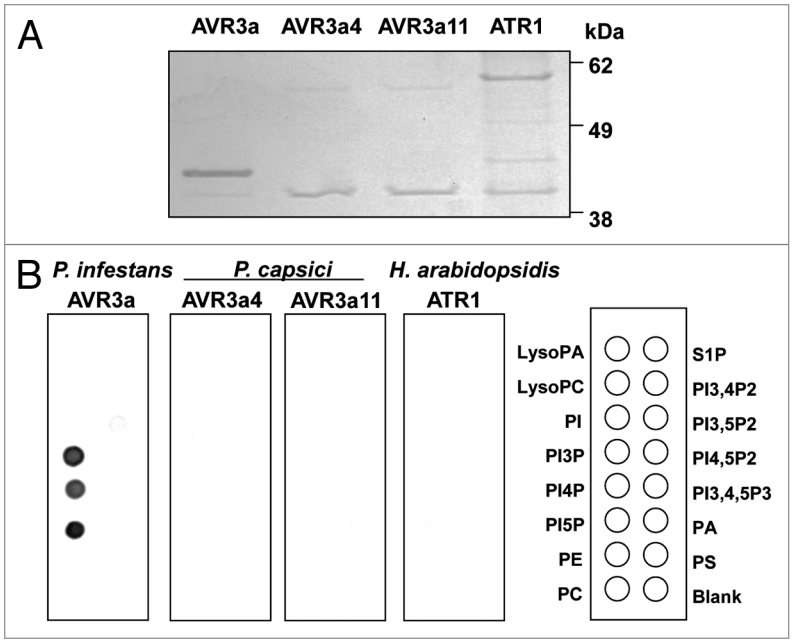

Figure 2. Lipid overlay assay of oomycete RXLR effectors, AVR3a, AVR3a4, AVR3a11 and ATR1. (**A**) *Escherichia coli* strain BL21-AI was transformed with pDEST24 constructs for AVR3a (Asp23-Tyr147), AVR3a4 (Asn22-Tyr122), AVR3a11 (Asn22-Val132) or ATR1-Emwa1 (Ser22-Glu324). Protein expression and purification were performed as described in Yaeno et al.[5] The purified C-terminal GST fusion proteins were checked by SDS-PAGE stained with InstantBlue (Expedeon) and equal amounts of proteins were used for the lipid overlay assay. (**B**) Nitrocellulose membranes spotted with 100 pmol of various lipids (PIP Strips; Echelon Biosciences) were blocked in 1% nonfat milk in PBS for 1 h and then incubated with 1 μg/mL C-terminal GST fusions of *P. infestans* AVR3a, *P. capsici* AVR3a4, *P. capsici* AVR3a11 and *H. arabidopsidis* ATR1 overnight at 4°C. After washing with PBS-T, the bound proteins were detected using anti-GST-HRP antibodies (GE Healthcare) diluted to 1:2,000. PA, phosphatidic acid; PC, phosphatidyl-choline; PE, phosphatidyl-ethanolamine; PI, phosphatidylinositol; PI3P, PI-3-phosphate; PI4P, PI-4-phosphate; PI5P, PI-5-phosphate; PI3,4P2, PI-3,4-biphosphate; PI3,5P2, PI-3,5-biphosphate; PI4,5P2, PI-4,5-biphosphate; PI3,4,5P3, PI-3,4,5-triphosphate; PS, phosphatidylserine; S1P, sphingosine-1-phosphate.

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