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

Takashi Yaeno* and Ken Shirasu

Plant Science Center; RIKEN; Tsurumi, Yokohama, Kanagawa, Japan

Keywords: oomycete, effector, RXLR motif, phosphatidylinositol phosphate, host cell entry

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 arabidopsis 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. 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. In our previous work, we determined the protein structure of P. capsici AVR3a4, which is a close homolog of AVR3a, by NMR analysis. 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. 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. 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. However, it was reported that the MiSSP7 effector protein secreted from Laccaria bicolor, a mutualistic ectomycorrhizal symbiont of popular, can enter host plant cells via an RXLR-like motif, RALG, and that the motif binds to PIPs. 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 and Hyaloperonospora arabidopsis ATR1 were determined. 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. 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. Thus, the WY motif is unlikely to be involved in PIP binding.

Recently, consistent in principle with our findings, Sun et al. 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 Yaeno et al. 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. 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
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.\(^7\) Gan et al.\(^8\) 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.\(^9\) The effectors of the human malaria parasite *Plasmodium falciparum* which are delivered into host cells also have an RXLR-like motif RlxLxD/Q required for translocation across the parasitophorous vacuolar membrane into the host erythrocyte cytoplasm.\(^10,11\) Interestingly, the RlxLxD/Q motif binds to PI3P in parasitophorous reticulum (ER) membranes in the process of export to the erythrocyte.\(^12\) 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.\(^6,13,14\) 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 RlxLxD/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.\(^4,15\) 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.

Acknowledgments

We thank Pamela Gan for critical reading of the manuscript; and Kaori Takizawa and Yoko Nagai for technical supports. This work was supported by JSPS KAKENHI Grant Numbers 24228008 (to K.S.) and 24780046 (to T.Y.).

References

1. Kamoun S. A catalogue of the effector secretome of plant pathogenic oomycetes. Annu Rev Phytopathol 2006; 44:41-60; PMID:16448329; http://dx.doi.org/10.1146/annurev.phyto.44.070505.134336.

2. Oliva R, Win J, Raffaele S, Bouteemy L, Borkari TO, Chaparro-Garcia A, et al. Recent developments in effector biology of filamentous plant pathogens. Cell Microbiol 2010; 12:705-15; PMID:20374248; http://dx.doi.org/10.1111/j.1462-5822.2010.01471.x.

3. Birch PRJ, Boevink PC, Gilroy EM, Hein I, Pritchard L, Whisson SC. Oomycete RXLR effectors: delivery, functional redundancy and durable disease resistance. Curr Opin. Plant Biol 2008; 11:373-9; PMID:18511334; http://dx.doi.org/10.1016/j.pbi.2008.04.005.

4. Whisson SC, Boevink PC, Moleleki L, Avrova AO, Morales JG, Gilroy EM, et al. A translocation signal for delivery of oomycete effector proteins into host plant cells. Nature 2007; 450:115-8; PMID:17914356; http://dx.doi.org/10.1038/nature06203.

5. Yano T, Li H, Chaparro-Garcia A, Schornack S, Koshiba S, Watanabe S, et al. Phosphatidylinositol monophosphate-binding interface in the oomycete RXLR effector Avr3a is required for its stability in host cells to modulate plant immunity. Proc Natl Acad Sci USA 2011; 108:14682-7; PMID:21821794; http://dx.doi.org/10.1073/pnas.1013270108.

6. Kale SD, Gu B, Capelluto DGS, Doh D, Feldman E, Rumsie A, et al. External lipid PI3P mediates entry of eukaryotic pathogen effectors into plant and animal host cells. Cell 2010; 142:284-95; PMID:20655469; http://dx.doi.org/10.1016/j.cell.2010.06.008.

7. Wawra S, Agacan M, Boddey JA, Davidson I, Gachon CMM, Zanda M, et al. A virulence protein 3a (Avr3a) from the potato pathogen *Phytophthora infestans* forms homodimers through its predicted translocation region and does not specifically bind phospholipids. *J Biol Chem* 2012; 287:38101-9; PMID:22977236; http://dx.doi.org/10.1074/jbc.M112.395129.

8. Plett JM, Kempainen M, Kale SD, Kohler A, Legué V, Brun A, et al. A secreted effector protein of *Laccaria bicolor* is required for symbiosis development. *Curr Biol* 2011; 21:1197-203; PMID:21757352; http://dx.doi.org/10.1016/j.cub.2011.05.033.

9. Bouteemy LS, King SRF, Win J, Hughes RK, Clarke TA, Blummenstein TMA, et al. Structures of *Phytophthora* RXLR effector proteins: a conserved but adaptable fold underpins functional diversity. *J Biol Chem* 2011; 286:35834-42; PMID:21813644; http://dx.doi.org/10.1074/jbc.M111.262503.

10. Chou S, Krasiljev KV, Holton JM, Steinhrenner AD, Albet T, Staskawicz BJ. *Psilotrema arachidopсидis* ATR1 effector is a repeat protein with distributed recognition surfaces. *Proc Natl Acad Sci USA* 2011; 108:13323-8; PMID:21788488; http://dx.doi.org/10.1073/pnas.1107911108.

11. Win J, Krasiljev KV, Kamoun S, Shirazu K, Staskawicz BJ, Banfield MJ. Sequence divergent RXLR effectors share a structural fold conserved across plant pathogenic oomycete species. *PLoS Pathog* 2012; 8:e1002400; PMID:22253591; http://dx.doi.org/10.1371/journal.ppat.1002400.
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-Emw1 (Ser22-Glu324). Protein expression and purification were performed as described in Yaeno et al. 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 fusion proteins. The bound proteins were detected using anti-GST antibodies (GE Healthcare) diluted to 1:2,000. PA, phosphatidic acid; PC, phosphatidyl-choline; PE, phosphatidyl-ethanolamine; PI, phosphatidylinositol; P13P, PI-3-phosphate; P14P, PI-4-phosphate; P15P, PI-5-phosphate; P13,4P2, PI-3,4-biphosphate; P13,5P2, PI-3,5-biphosphate; P14,5P2, PI-4,5-biphosphate; P13,4,5P3, PI-3,4,5-triphosphate; PS, phosphatidylserine; S1P, sphingosine-1-phosphate.

12. Sun F, Kale SD, Azurmendi HF, Li D, Tyler BM, Capelluto DG. Structural basis for interactions of the *Phytophthora sojae* RxLR effector Avr5 with phosphatidylinositol 3-phosphate and for host cell entry. Mol Plant Microbe Interact 2012; In press; PMID:23075041; http://dx.doi.org/10.1094/MPMI-07-12-0184-R.

13. Rafiq M, Gan PHP, Ravensdale M, Lawrence GJ, Ellis JG, Jones DA, et al. Internalization of flax rust avirulence proteins into flax and tobacco cells can occur in the absence of the pathogen. Plant Cell 2010; 22:2017-32; PMID:20525849; http://dx.doi.org/10.1105/tpc.109.072983.

14. Gan PHP, Rafiq M, Ellis JG, Jones DA, Hardham AR, Dodd PT. Lipid binding activities of flax rust AVR and AVR-L67 effectors. Plant Signal Behav 2010; 5:1272-5; PMID:20855950; http://dx.doi.org/10.4161/psb.5.10.13013.

15. Wawra S, Bain J, Durward E, de Bruijn I, Minor KL, Matena A, et al. Host-targeting protein 1 (SpHtp1) from the oomycete *Saprolegnia parasitica* translocates specifically into fish cells in a tyrosine-O-sulphate-dependent manner. Proc Natl Acad Sci USA 2012; 109:2096-101; PMID:22308362; http://dx.doi.org/10.1073/pnas.1113775109.

16. Hiller NL, Bhattacharjee S, van Ooij C, Liolsos K, Harrison T, Lopez-Estraño C, et al. A host-targeting signal in virulence proteins reveals a secretome in malarial infection. Science 2004; 306:1934-7; PMID:15591203; http://dx.doi.org/10.1126/science.1102737.

17. Marty M, Good RT, Rug M, Knuepfer E, Cowman AF. Targeting malaria virulence and remodeling proteins to the host erythrocyte. Science 2004; 306:1934-7; PMID:15591203; http://dx.doi.org/10.1126/science.1102452.

18. Bhattacharjee S, Stahelin RV, Speicher KD, Speicher DW, Haldar K. Endoplasmic reticulum PI(3)P lipid binding targets malaria proteins to the host cell. Cell 2012; 148:201-12; PMID:22265412; http://dx.doi.org/10.1016/j.cell.2011.10.051.

19. Boddey JA, Hodder AN, Günther S, Gilson PR, Patsiouras H, Kapp EA, et al. An aspartyl protease directs malaria effector proteins to the host cell. Nature 2010; 463:2017-32; PMID:20130644; http://dx.doi.org/10.1105/tpc.107.056093.

20. Russo I, Babbitt S, Muralidharan V, Burler T, Okman A, Goldberg DE. Plasmepsin V licenses *Plasmodium* proteins for export into the host erythrocyte. Nature 2010; 463:632-6; PMID:20130644; http://dx.doi.org/10.1038/nature08726.

21. de Koning-Ward TF, Gilson PR, Boddey JA, Rug M, Smith BJ, Papenfuss AT, et al. A newly discovered protein export machine in malaria parasites. Nature 2009; 459:945-9; PMID:19536257; http://dx.doi.org/10.1038/nature08104.

22. Dou D, Kale SD, Wang X, Jiang, RHY, Bruce NA, Arredondo FD, et al. RXLR-mediated entry of *Phytophthora sojae* effector Avr1b into soybean cells does not require pathogen-encoded machinery. Plant Cell 2008; 20:1930-47; PMID:18621946; http://dx.doi.org/10.1105/tpc.107.056093.