Structural Basis of Host Autophagy-related Protein 8 (ATG8) Binding by the Irish Potato Famine Pathogen Effector Protein PexRD54*§**

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Filamentous plant pathogens deliver effector proteins to host cells to promote infection. The Phytophthora infestans RXLR-type effector PexRD54 binds potato ATG8 via its ATG8 family-interacting motif (AIM) and perturbs host-selective autophagy. However, the structural basis of this interaction remains unknown. Here, we define the crystal structure of PexRD54, which includes a modular architecture, including five tandem repeat domains, with the AIM sequence presented at the disordered C terminus. To determine the interface between PexRD54 and ATG8, we solved the crystal structure of potato ATG8CL in complex with a peptide comprising the effector’s AIM sequence, and we established a model of the full-length PexRD54-ATG8CL complex using small angle x-ray scattering. Structure-informed deletion of the PexRD54 tandem domains reveals retention of ATG8CL binding in vitro and in planta. This study offers new insights into structure/function relationships of oomycete RXLR effectors and how these proteins engage with host cell targets to promote disease.

During selective autophagy, specific cellular constituents can be targeted to autophagic pathways for subcellular trafficking or degradation (1–3). The autophagy toolkit includes around 40 ATG (autophagy-related) proteins. Together, they help initiate, regulate, and form the constituents of autophagic pathways. The role of selective autophagy in the response to pathogen challenge in animal cells is increasingly being appreciated and includes direct elimination of microorganisms and control of immunity-related signaling (4, 5). In turn, microorganisms have developed mechanisms to perturb host-selective autophagy to either shut it down and promote infection (4, 5) or activate it and re-direct nutrients to the parasite (6). There is also evidence that membrane formation and trafficking, as controlled by ATG proteins, are exploited by numerous viruses (7). To date, the role of host-selective autophagy in host-microbe interactions has mostly been studied in mammals. The role of host-selective autophagy in plant-microbe interactions, and how it is manipulated by plant pathogen, remains poorly understood.

ATG8 is a ubiquitin-like protein that performs multiple functions in autophagy. It is cycled, via conjugation and deconjugation reactions, to the membrane lipid phosphatidylethanolamine, and this localization is important for autophagy. ATG8 functions in autophagy. It is cycled, via conjugation and deconjugation reactions, to the membrane lipid phosphatidylethanolamine, and this localization is important for autophagy. ATG8 also serves as an adaptor to interact with proteins containing an ATG8-interacting motif (AIM).³ AIM-containing proteins can serve as receptors for cargo destined for autophagosomes. The core AIM sequence is defined as LXXΦΦΦ, where Φ is an aromatic amino acid (Trp, Tyr, or Phe); X is any residue, and Ψ is an aliphatic amino acid (Leu, Ile, and Val) (10–12). Frequently, residues just to the N terminus of the LXXΦΦΦ motif are acidic in nature. Structural studies have elucidated how the AIM sequence binds ATG8, with key features including the Ω and Ψ residues binding within hydrophobic pockets, and the motif adopting a β-strand structure that extends the β-sheet of ATG8 (1, 13–15). It is generally thought that AIMS adopt a disordered or flexible conformation in the absence of a binding partner (11, 16). Mechanisms for pathogens to perturb host-selective autophagy include delivery of factors that interfere with recruitment of endogenous AIM-containing proteins to ATG8 or that re-direct additional cellular components to autophagosomes.

Filamentous plant pathogens cause devastating diseases of crops that are of both historical significance (17) and relevant to global agriculture today (18). Phytophthora infestans, the Irish potato famine pathogen, facilitates disease on its hosts by delivering effector proteins that modulate host cell processes to the

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‡ This article was selected as a Paper of the Week.

§ This article contains supplemental Video.

The atomic coordinates and structure factors (codes SL75 and SL83) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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³ The abbreviations used are: AIM, ATG8-family interacting motif; SPR, surface plasmon resonance; NTA, nitritoliacetic acid; RFP, red fluorescent protein; ITC, isothermal titration calorimetry; SAXS, small angle x-ray scattering; NSD, normalized spatial discrepancy.
Structure/Function of PexRD54

benefit of the parasite (19), a strategy used by many biotrophic plant pathogens (20–22). Many putative P. infestans effectors contain a conserved N-terminal RXLR (Arg-Xaa-Leu-Arg) motif for host translocation (23). Furthermore, about half of these effectors are predicted to adopt the conserved WY domain fold in their C-terminal regions, which encodes their biochemical activity (24–26). Although recent studies have begun to elucidate the virulence-associated targets and functions of P. infestans RXLR effectors (27–34), these have yet to be identified for the vast majority of these proteins.

Recently, a P. infestans RXLR effector, PexRD54, which contains an AIM sequence Trp-Glu-Ile-Val “WEIV” positioned at the C terminus (residues 378–381), was identified (35). It was shown that PexRD54 specifically interacts with a member of the ATG8 family of proteins from potato, ATG8CL, in vitro and in planta. In plant cells, PexRD54 activates selective autophagy by increasing the number of ATG8CL-containing autophagosomes and stabilizing ATG8CL. Furthermore, PexRD54 was shown to antagonize the function of the host autophagy cargo receptor Joka2 by competing for binding with ATG8CL. As Joka2 contributed toward immunity against P. infestans, which was counteracted by PexRD54, it was concluded that this effector acts as an inhibitor of Joka2 function.

To better understand how PexRD54 interacts with potato ATG8CL to perturb host-selective autophagy, we have investigated the structural basis of effector–host target interaction. We determined the crystal structures of PexRD54 and ATG8CL in complex with the C-terminal AIM peptide of this effector. We also obtained a structure of the PexRD54-ATG8CL complex by docking the crystal structures into an envelope derived from solution scattering data. Site-directed mutagenesis of the PexRD54 C-terminal AIM region, and ATG8CL binding to a PexRD54 AIM-based peptide array, mapped the key residues that define the PexRD54-ATG8CL interface. Finally, we used structure-informed deletions to show that the WY domains of PexRD54 are dispensable for ATG8CL binding suggesting an alternative function for these domains. Together, these data provide a mechanistic understanding of how translocated effectors engage with their host targets and offer new methods for engineering control of plant diseases.

Results

PexRD54 Forms a Stable Complex with ATG8CL in Vitro—To investigate complex formation between PexRD54 and ATG8CL, we expressed both proteins separately in Escherichia coli and purified them to homogeneity (Fig. 1A). To determine whether the two proteins form a stable complex in solution, we mixed them in an equimolar ratio prior to injection on a Superdex S75 10/300 analytical gel filtration column and compared the resulting elution volume to the elution volumes of the individual proteins. As shown in Fig. 1A, PexRD54 elutes at 10.9 ml and ATG8CL at 13.1 ml when these proteins are run independently. After mixing, a new peak at an earlier elution volume (10.2 ml) is apparent, and SDS-PAGE analysis shows this peak contains both proteins. This shift in the elution peak is indicative of complex formation and that this complex is stable over the time course of the experiment. Based on a calibration curve, elution volumes from this column of 10.9, 13.1, and 10.2 ml correspond to ~44, ~18, and ~58 kDa. All these represent overestimates of the predicted molecular masses of the proteins on their own or in complex (PexRD54 ~34 kDa, ATG8CL ~15 kDa, and PexRD54-ATG8CL complex ~49 kDa) but indicate monomeric forms of each state exist in solution.

Next, we determined whether the PexRD54-ATG8CL complex could be formed on purification following co-expression in E. coli. We cloned PexRD54 and ATG8CL into different expression vectors, with only the ATG8CL containing a His6 tag (see under “Experimental Procedures”). Following expression and preparative tandem immobilized metal affinity chromatography/gel filtration chromatography of the clarified cell lysate, a
single peak was obtained at an elution volume consistent with a complex between PexRD54 and ATG8CL (Fig. 1B). Using this technique, we determined that the affinities of complex formation between PexRD54 and ATG8CL is likely formed in cells and can be purified from cell culture directly.

Finally, we used surface plasmon resonance (SPR) to investigate the affinities of complex formation between PexRD54 and ATG8CL (Fig. 1C). Using this technique, we determined that PexRD54 binds to ATG8CL with a $K_d$ of 388 ± 47 nM. The AIM motif disrupting PexRD54-Trp378-AEIA-381 variant (where the Trp and Val of the “WEIV” AIM motif are replaced by alanine) did not bind to ATG8CL using SPR, consistent with previous results (35). The overall fold of the PexRD54-Trp378-AEIA-381 variant was equivalent to wild-type protein as assessed by circular dichroism (CD) spectroscopy (Fig. 2).

**PexRD54 Is a Tandem Repeat WY Domain Effector with a Disordered C-terminal AIM**—To discover the molecular architecture of PexRD54, we determined the crystal structure of the effector domain of this protein (residues Val92 to Val381) at 2.90 Å resolution. Although PexRD54 could be crystallized alone, the crystal that gave rise to the best X-ray dataset was obtained from a sample including both PexRD54 and ATG8CL after co-expression in *E. coli* (see under “Experimental Procedures”). Although SDS-polyacrylamide gel analysis of dissolved crystals showed that both proteins were present in these crystals, no electron density for ATG8CL was observed. The structure of PexRD54 was solved using single wavelength anomalous diffraction, and the final model was refined to final $R_{work}$ and $R_{free}$ values of 23.1 and 25.6%, respectively (Table 1). Inspection of the packing of PexRD54 revealed that ATG8CL could be accommodated in the crystal, within a region of unaccounted for space near the C terminus of the effector. The structure of PexRD54 includes 16 α-helices (Fig. 3A and supplemental video 1). Five N-terminal residues (92–96), the residues in two loops (248–250 and 331–334), and 11 C-terminal residues (371–381), which include the AIM motif, were not included in the final model due to poor electron density in these regions.

Previous bioinformatics analysis predicted the presence of multiple DY domains in PexRD54 (24). Our structural analysis revealed that PexRD54 includes five tandem WY domains that pack to form an elongated molecule (Fig. 3A). This is a conformation not yet observed for RXLR effectors with multiple WY domains. The WY domain is a conserved structural unit consisting of three α-helices and two characteristic hydrophobic amino acids, frequently W (Trp) and Y (Tyr), which contribute to a stable hydrophobic core (24, 25). Structural superposition of the archetypal WY domain of the *Phytophthora capsici* RXLR-WY effector AVR3a11 on each of the WY domains of PexRD54 is shown in Fig. 3B, with root mean square deviations derived from each superposition given in Table 2. As more structures are determined, it is increasingly clear that WY domains can tolerate variations at the Trp and Tyr positions, while maintaining the hydrophobic core and overall fold. This is in addition to the remarkable overall structural conservation among WY domains despite a lack of pairwise sequence identity, which is as low as 13% between PexRD54 and AVR3a11 (Table 2).

**Host Protein ATG8CL Binds the PexRD54 AIM Sequence via Two Hydrophobic Pockets**—In the PexRD54 structure, we did not observe the last 10 amino acids that contain the AIM motif, or the ATG8CL protein itself, in the electron density. Therefore, to visualize the interaction between PexRD54 and ATG8CL, we determined the crystal structure of ATG8CL in complex with a PexRD54 C-terminal pentapeptide. This pentapeptide includes the AIM motif, with residues Asp–377–Trp–378–Glu–379–Ile–380–Val–381. To produce crystals of ATG8CL + pentapeptide, we used an ATG8CL construct lacking four N-terminal residues and five C-terminal residues.

The structure of the complex was solved by molecular replacement and refined to 1.90 Å with final $R_{work}$ and $R_{free}$ values of 17.6 and 19.9%, respectively (Table 1). Positive difference electron density within the likely AIM binding region of ATG8CL indicated the presence of bound pentapeptide. The final model contains two molecules of ATG8CL + pentapeptide in the asymmetric unit. The electron density maps for both complexes were of equivalent quality, and subsequent analysis focuses on one representative monomer.

The structure of ATG8CL contains two domains, an N-terminal helical domain (α1 and α2) and a C-terminal domain that adopts a β-grasp (ubiquitin-like) fold of four β-strands (β1–β4) flanked by two helices (α3 and α4) (Fig. 4A). ATG8CL adopts a very similar structure to that observed for ATG8s from other organisms. For example, ATG8CL overlays on the structures of GATE-16 (Protein Data Bank code 1EO6, 60% sequence identity with ATG8CL) and GABARAP (Protein Data Bank code 4XC2, 57% sequence identity with ATG8CL) with a root mean square deviation of 0.8 and 0.9 Å, respectively, for 115 α-carbons.

In the complex, the pentapeptide adopts an extended conformation forming a parallel β-sheet with β2 of ATG8CL. The peptide binds within a narrow channel at the surface of ATG8CL via hydrophobic and hydrogen bond interactions (Fig. 4A). The side chain of PexRD54 Trp–378 is contained within a hydrophobic pocket formed at the interface between the β-grasp and N-terminal helical domains of ATG8CL, whereas the side chain of PexRD54 Val–381 binds a distinct hydrophobic pocket between β2 and an adjacent helix on the
C-terminal domain of ATG8CL (Fig. 4A). In addition to hydrophobic interactions, the indole nitrogen of Trp-378 forms a hydrogen bond with the side chain of ATG8CL Glu-18 (Fig. 4A). The side chain of PexRD54 Glu-379 makes hydrogen bonds and ionic interactions with the side chains of ATG8CL Lys-47 and ATG8CL Arg-68 (Fig. 4A). Another prominent ionic interaction is formed between the side chain of PexRD54 Asp-377, Trp-378, Glu-379, Ile-380, and Val-381. Each of these proteins was expressed and purified as described for wild type. We then used analytical gel filtration to qualitatively assay whether these variants support complex formation with ATG8CL. As predicted, we did not observe interaction of PexRD54 W378A with ATG8CL (Fig. 7). For each of the other mutations, we still observed an interaction with ATG8CL (Fig. 7). For each of the other mutations, we observed interaction with ATG8CL, including PexRD54 V381A.

To build on the structural studies above, we used two complementary biochemical approaches to investigate the role of individual residues in the AIM region of PexRD54 in binding to ATG8CL. First, we used alanine-scanning mutagenesis to substitute Ala at six positions in the PexRD54 AIM region, Pro-373, Asp-377, Trp-378, Glu-379, Ile-380, and Val-381. Each of these proteins was expressed and purified as described for wild type. We then used analytical gel filtration to qualitatively assay whether these variants support complex formation with ATG8CL. As predicted, we did not observe interaction of PexRD54 W378A with ATG8CL (Fig. 7). For each of the other mutations, we still observed an interaction with ATG8CL, including PexRD54 V381A. Second, we designed a nitrocellulose-anchored pentapeptide array of 200 variant AIM peptides, based on the final 10 amino acids of PexRD54, where each amino acid was changed to all other possible amino acids. The peptides were anchored at the N terminus to best mimic the presentation of the PexRD54 AIM region to ATG8CL. We visualized

### Table 1: PexRD54/ATG8CL x-ray data collection and refinement statistics

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<th>Native</th>
<th>Iodide</th>
<th>ATG8CL native</th>
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<td>86.09–1.90 (1.90–1.95)</td>
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<td>100 (86.4)</td>
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<td><strong>Refinement and model statistics</strong></td>
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<td>Resolution (Å)</td>
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<td>86.09–1.90 (1.95–1.90)</td>
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<td>Rwork, Rfree (%)</td>
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<td>17.6/19.9 (24.2/25.3)</td>
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<td>MolProbity Score</td>
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<td>1.14 (100th percentile)</td>
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* The highest resolution shell is shown in parentheses.
* Data are as calculated by MolProbity.
ATG8CL binding to the peptide array using an ATG8CL fusion with glutathione S-transferase (GST) and a His tag (see “Experimental Procedures”), followed by incubation with an anti-GST-HRP antibody (Amersham Biosciences) and detection of chemiluminescence (Fig. 4B). The results of the peptide array clearly highlight the importance of the hydrophobic residues 378 and 381 of the PexRD54 AIM motif (Trp and Val) in binding ATG8CL. For position 378, the strongest binding was seen for Trp and Phe, with limited binding of Tyr and the aliphatic amino acids. Position 381

### Table 2

<table>
<thead>
<tr>
<th>r.m.s.d.</th>
<th>Residue range</th>
<th>WY amino acids</th>
<th>Sequence identity to AVR3a11</th>
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<td>WY-2</td>
<td>2.35/32 Asn-151–Gly-198</td>
<td>LM</td>
<td>18</td>
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<tr>
<td>WY-3</td>
<td>2.89/39 Asn-199–Asn-247</td>
<td>WY</td>
<td>16</td>
</tr>
<tr>
<td>WY-4</td>
<td>2.80/41 Phe-251–Ser-299</td>
<td>FL</td>
<td>14</td>
</tr>
<tr>
<td>WY-5</td>
<td>1.73/41 Ser-302–Ile-354</td>
<td>WY</td>
<td>20</td>
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ATG8CL binding to the peptide array using an ATG8CL fusion with glutathione S-transferase (GST) and a His tag (see “Experimental Procedures”), followed by incubation with an anti-GST-HRP antibody (Amersham Biosciences) and detection of chemiluminescence (Fig. 4B). The results of the peptide array clearly highlight the importance of the hydrophobic residues 378 and 381 of the PexRD54 AIM motif (Trp and Val) in binding ATG8CL. For position 378, the strongest binding was seen for Trp and Phe, with limited binding of Tyr and the aliphatic amino acids. Position 381
favors the bulky aliphatic amino acids, with limited binding also observed by bulky hydrophobic residues. Interestingly, with the exception of Pro at position 379, any amino acid can be accommodated at positions 379 and 380, and binding is still observed. Furthermore, any amino acid can be accommodated at positions 372–377 without a significant reduction in binding, suggesting that these residues may only act as a linker between the WY domain region of PexRD54 and the C-terminal AIM motif.

**WY Domains of PexRD54 Are Dispensable for the Interaction with ATG8CL in Vitro and in Planta**—Although the AIM region of PexRD54 appears necessary and sufficient for the interaction with ATG8CL, we explored whether the WY domains of PexRD54, which include 96% of the protein expressed here, impact the binding of the effector to ATG8CL. For this, we produced two structure-informed deletions of PexRD54, removing either the first three WY domains (but leaving the C-terminal helix of WY-3, which forms an N-terminal extension of WY-4), generating PexRD54 deletions retain the ability to bind ATG8CL in planta by co-immunoprecipitation (co-IP) from Nicotiana benthamiana leaves transiently expressing these proteins following delivery of the genes by infiltration with Agrobacterium tumefaciens (agroinfiltration). In these assays both RFP-PexRD54 deletion mutants still interacted with ATG8CL (Fig. 8C).

![Diagram](image)

**FIGURE 4.** Crystal structure of ATG8CL bound to the PexRD54(377–381)-peptide and specificity of peptide binding. A, schematic representation of ATG8CL/PexRD54(377–381)-peptide complex highlighting key interactions. ATG8CL is shown in magenta schematic representation with the molecular surface that contacts the PexRD54(377–381)-peptide shown in orange. The PexRD54(377–381)-peptide is shown as sticks with yellow carbon atoms. The electron density omit map of the peptide ligand (Foobs −Fcals map) is shown in blue mesh and contoured at 2 σ. Electrostatic interactions are indicated with black dashed lines. B, results of the peptide array analyzing the effect of single amino acid substitutions(top) at all positions of 10-mer peptide of PexRD54 (Lys-372–Val-381, side). GST-tagged ATG8CL was visualized using an anti-GST-HRP antibody.

![Graph](image)

**FIGURE 5.** Analysis of SAXS data. A, P(r) distribution curves used for ab initio modeling. Left, PexRD54; right, PexRD54-ATG8CL complex. Dmax was set at 92 nm (PexRD54) and 120 nm (PexRD54/ATG8CL complex). Data were cropped at 0.35 Å⁻¹ for analysis. B, left, fit of the theoretical scattering curve of PexRD54 from CRYSOL (red) to the PexRD54 scattering data (black). Right, fit of the theoretical scattering curve of the PexRD54-ATG8CL complex from CORAL (red) to the PexRD54-ATG8CL scattering data (black).
RFP-PexRD54 and the AIM motif disrupting variant RFP-PexRD54-378-AEIA-381 were used as controls.

Discussion

Understanding the mechanistic basis of translocated effector protein function in support of pathogen infection and colonization is a major focus of research in plant-microbe interactions. Such studies reveal how manipulation of host cell processes by pathogen-derived molecules can promote virulence and also identify plant systems, such as autophagy, whose importance in disease or general host cell physiology may be underappreciated. In a few cases, the structural basis for bacterial plant pathogen effector interaction with a host protein or peptide has been described (37–40). However, such studies of filamentous plant pathogen effectors are lacking. The *P. infestans* RXLR-type effector PexRD54 (PITG_09316) perturbs host-selective autophagy for the benefit of the pathogen via interaction with ATG8CL (35). Here, we focused on the biochemical and structural basis of PexRD54’s interaction with ATG8CL to understand how the pathogen co-opts autophagic pathways.

Structural conservation in RXLR-type effectors from the oomycetes, in the absence of confidently assignable sequence similarity, has previously been established (24, 25). Although each of the five structurally conserved three-helical bundle (WY domain) repeats in PexRD54 adopts the same overall fold, they pack together to form a unique structure different from that of the two WY domain repeats of *Hyaloperonospora arabidopsidis* (41). Detailed analysis of the PexRD54 structure suggests trajectories for the evolution of WY domain proteins through gain or loss of functional units presented on the N or C terminus of the core three-helical bundle. First, the minimal three helix WY domain fold seen in
PexRD54 is found in *P. infestans* effector PexRD2 (24), but in other RXLR-type effectors of known structure an N-terminal helix is present resulting in a four-helical bundle. Interestingly, in PexRD54, the C-terminal helices of WY-1, WY-3, and WY-4 are positioned such that they also serve as N-terminal helical extensions to WY-2, WY-4, and WY-5 to build four-helical bundles as observed in AVR3a4 (42), AVR3a11, and ATR1. Second, in ATR1 the tandem repeats of the four helix bundle are separated by a fifth “linker” helix. When the first WY domain of ATR1 is overlaid on WY-5 of PexRD54, the fifth linker helix is positioned on the final helix of PexRD54 (*brown* in Fig. 3A). In both protein structures, this helix then serves to present the proximal regions, either a second WY domain as seen in ATR1 or the AIM region as seen in PexRD54. Finally, PexRD54-WY-3 does not have an N-terminal helix and does not form a four helical bundle. This correlates with a significant kink in the PexRD54 structure between WY-2 and WY-3. Each of these observations serves to highlight the plasticity of the WY-fold and how it can be utilized to deliver new template structures with the potential for functional diversification. It is interesting to note that conserved structure in the absence of confidently assignable sequence similarity is emerging as a recurring theme for filamentous plant pathogen effectors (43, 44).

Little is known about how plant autophagic pathways are controlled and manipulated by pathogens. The structure of ATG8CL bound to the PexRD54 ATG8 peptide revealed the fundamental mechanisms of AIM recognition by plant ATG8s are similar to those seen in other organisms. The two critical hydrophobic residues of the ΩXXΨ motif, Trp and Val in PexRD54, are bound in two hydrophobic pockets on the surface of ATG8CL (Fig. 4A). Furthermore, our mutagenesis and peptide-binding studies confirm the important roles for these residues in the interaction. The identity of the residues to the N terminus of the AIM, which in other systems comprise acidic residues (11), do not seem to be important in this case. Previously, it was shown that the binding of PexRD54 to another ATG8 family member, ATG8IL, was weaker *in planta* and *in vitro*. These two proteins share 50% sequence identity. Interestingly, three amino acids are changed between ATG8CL and ATG8IL at the ATG8CL/PexRD54 AIM peptide interface: I33V, L56M, and V164I. ATG8CL Ile-33 is located at the base of the pocket that binds PexRD54 Trp-378, whereas ATG8CL Leu-56 and ATG8CL Val-64 are both located in the second hydrophobic pocket that faces PexRD54 Val-381. The interactions between ATG8s and AIM peptides are dominated by hydrophobic interactions, and the subtle changes delivered by these mutations may be responsible for the weaker binding affinity of ATG8IL over ATG8CL, although this remains to be tested *in vitro* and will be the subject of future work.

The previous study (35) and the work described here reveal the importance of the interaction between PexRD54 and ATG8CL, as mediated by the effector’s C-terminal AIM region. This region includes only ~3% of the amino acids downstream of the RXLR-deER motif, but deletion of WY domains 1–4 does not significantly affect ATG8CL binding *in vitro* or *in planta*. This raises the following question. How do the five WY domains contribute to PexRD54 function? This effector has been shown to stimulate host autophagosome formation, and it was hypothesized that the pathogen exploits this for its own benefit in either promoting nutrient recycling or counteracting defense. Future work will address how the PexRD54 WY domains may contribute to autophagosome formation and/or act as a receptor to localize specific cellular cargo to autophagic pathways.

**Experimental Procedures**

**Gene Cloning**

All constructs were verified by DNA sequencing.

PexRD54—For protein expression in *E. coli*, DNA encoding PexRD54 residues Val-92 to Val-381 was amplified from RFP-PexRD54 (35) and cloned into pOPINA or pOPINS3C (45) by In-Fusion cloning (Clontech). The resultant vectors expressed PexRD54 protein without a fusion tag (pOPINA) or with the N-terminal His<sub>6</sub>-SUMO tag (pOPINS3C), respectively. DNA encoding PexRD54 residues Arg-219 to Val-381 was amplified from pOPINA-PexRD54 and cloned into pOPINS3C. DNA encoding PexRD54 residues Ser-299 to Val-381 was amplified from pOPINA-PexRD54 (and cloned into pOPINS3C) or from pOPINS3C-PexRD54 (and cloned into pOPINA). Single point mutants within the AIM region of PexRD54 were encoded...
within primers that were then used to amplify the full-length construct from pOPINS3C-PexRD54 followed by ligation into pOPINS3C. For protein expression in planta, DNA encoding PexRD54 residues Arg-219 to Val-381 or Ser-299 to Val-381 were amplified from RFP-PexRD54 and cloned into pENTR (Thermo-Fisher, UK). The expression constructs RFP-PexRD54/H9004218 and RFP-PexRD54/H9004298 were generated by Gateway LR reaction (Invitrogen) using the destination vector pH7WGR2 (N-terminal RFP fusion).

ATG8CL—For protein expression in E. coli, DNA encoding Met-1 to Phe-119 of ATG8CL was amplified from pOPINF-ATG8CL (35) and cloned into pOPINE, producing ATG8CL with a non-cleavable C-terminal His6 tag. DNA encoding Ser-5 to Asn-114 of ATG8CL was amplified from pOPINF-ATG8CL and cloned into pOPINF, expressing ATG8CL with a cleavable N-terminal His6 tag (called ATG8CL* hereafter). For probing the peptide array, DNA encoding ATG8CL residues Met-1 to Phe-119 was amplified from pOPINE-ATG8CL and cloned into pOG3182 (Oxford Genetics). DNA encoding the ATG8CL-GST fusion was amplified from ATG8CL-pOG3182 and cloned into pOPINE. The resultant pOPINE-ATG8CL-GST vector expressed ATG8CL protein with a non-cleavable C-terminal GST-His6 tag. For protein expression in planta, GFP-EV and GFP-ATG8CL constructs were described previously (35).

Heterologous Protein Production and Purification

Purified proteins were concentrated and stored in 20 mM HEPES buffer, pH 7.5, containing 150 mM NaCl, except where stated.

PexRD54 and Its Variants—For analytical gel filtration and ITC, all PexRD54 proteins were produced using E. coli BL21-arabinose-inducible cells and purified as described previously (35). For SPR, the same purification protocol was followed, with the exception of the final gel filtration step, which used 20 mM HEPES, pH 7.5, 500 mM NaCl.

ATG8CL—ATG8CL, expressed from pOPINF, was produced in E. coli BL21(DE3) and purified as described previously (35). When produced from pOPINE, a single Ni2+/NTA cap-ture step followed by gel filtration produced soluble protein. The same strategy was used for purifying pOPINE-ATG8CL-GST-His. For SPR, ATG8CL was purified using 20 mM HEPES, pH 7.5, 500 mM NaCl in the gel filtration step. For crystallization, pOPINF-ATG8CL* was expressed and purified as for pOPINF-ATG8CL, except auto-induction media were used to culture the E. coli.

PexRD54-ATG8CL Complex—For crystallization and SAXS analysis of the complex, pOPINA-PexRD54 and pOPIN-
ATG8CL were co-transformed and expressed in BL21(DE3). Purification used the same protocol as for ATG8CL produced from pOPINE.

**Protein-Protein Interaction Studies**

**Analytical Gel Filtration**—Analytical gel filtration chromatography was performed at 4 °C using a Superdex 75 10/300 column (GE Healthcare) pre-equilibrated in 20 mM HEPES, pH 7.5, 150 mM NaCl. 100 µl of sample was injected at a flow rate of 0.8 ml/min, and 0.5-ml fractions were collected for analysis. To study complex formation, proteins were mixed and incubated on ice for at least 1 h prior to loading.

**Surface Plasmon Resonance**—SPR experiments were performed at 18 °C using a BIAcore T200 system (GE Healthcare) and an NTA sensor chip (GE Healthcare). Protein samples were prepared in 20 mM HEPES, pH 7.5, 500 mM NaCl, and all the measurements were recorded in the same buffer at a flow rate of 30 µl/min. A single cycle kinetics approach was used to study the interaction between PexRD54 and ATG8CL. The NTA chip was activated by injecting 10 µl of 0.5 mM NiCl₂ over flow cell 2, which was also used to immobilize His-tagged ATG8CL to a response level of 85 ± 2. Increasing concentrations of PexRD54 (20, 200, 600, 1000, and 2000 nM) were injected over flow cell 1 and 2 for 90 s. After the final injection, the dissociation was recorded for 300 s. Two startup cycles were run where the chip was activated and ATG8CL immobilized in the same manner, but buffer only was injected instead of PexRD54. This was subtracted to account for any dissociation of ATG8CL from the sensor chip. The sensor chip was regenerated by injecting 10 µl of 350 mM EDTA. The data were analyzed using BIAcore T200 BIAevaluation software (GE Healthcare) and then plotted with Microsoft Excel.

**Isothermal Titration Calorimetry**—Calorimetry experiments were recorded at 15 °C in 20 mM HEPES, pH 7.5, 150 mM NaCl, using an iTC200 instrument (MicroCal Inc.). The calorimetric cell was filled with 80 µM PexRD54 truncation (PexRD54D218 or PexRD54D208) and titrated with 0.8 mM ATG8CL from the syringe. A single injection of 0.5 µl of ATG8CL was followed by 19 injections of 2 µl each. Injections were made at 120-s intervals with a stirring speed of 750 rpm. The raw titration data were integrated and fitted to a one-site binding model using the MicroCal Origin software.

**In Plant Co-immunoprecipitation**—3–4-week-old *N. benthamiana* plants were used for transient expression experiments. T-DNA expression vectors encoding PexRD54 constructs, ATG8CL constructs, or empty vector were transformed into the *A. tumefaciens* GV3101 strain. Transformed agrobacteria were diluted in 5 mM MES, 10 mM MgCl₂, pH 5.6, and mixed in 1:1 ratio to a final A₆₀₀ of 0.2 prior to leaf infiltration.

*N. benthamiana* leaves transiently expressing proteins were harvested 2 days post-infiltration. Protein extraction, immunoprecipitation, and Western blotting analyses were performed as described previously (35). For blots shown in Fig. 8, mouse monoclonal single step GFP-HRP antibody (Santa Cruz Biotechnology) was used for GFP immunoblot experiments. For RFP blots, polyclonal RFP antibody (Invitrogen) was used as primary antibody and anti-rat HRP antibody (Sigma, UK) was used as secondary antibody.

**Crystallization, Data Collection, and Structure Solution**

PexRD54 (in the Presence of ATG8CL)—For crystallization, the PexRD54-ATG8CL complex produced by co-expression was concentrated to 10 mg/ml in 20 mM HEPES, 150 mM NaCl, pH 7.5. Crystallization experiments used 4-µl hanging drops with a 2:1 protein/precipitant ratio. For data collection, crystals were grown in 18% PEG 10K, 0.1 M sodium acetate, pH 5.0, 0.18 M tri-ammonium citrate and transferred to a cryoprotectant solution consisting of 22% PEG 10K, 0.1 M sodium acetate, pH 5.0, 0.18 M tri-ammonium citrate and 10% ethylene glycol. To enable structure solution, crystals were soaked for ~45 s in well solution supplemented with 500 mM potassium iodide and then cryoprotected as above.

Native and single wavelength anomalous diffraction x-ray data sets were collected at the Diamond Light Source, United Kingdom, beamline I02. The datasets were processed using the Xia2 pipeline (46), see Table 1. The structure was solved using the single wavelength anomalous diffraction approach with the data collected from the crystal soaked in potassium iodide solution. Iodide sites were identified with Phenix (47). These positions were used to estimate initial phases using Phaser EP from the CCP4 suite (48), followed by density improvement with PARROT (49). An initial model was built using Buccaneer (50) followed by manual rebuilding and refinement using COOT (51) and REFMAC5 (52). Next, molecular replacement with Phaser, followed by the Phenix AutoBuild wizard, was used to produce an initial model of PexRD54 using the native x-ray data. The final model was produced through iterative rounds of refinement using REFMAC5 and manual rebuilding with COOT. Structure validation used the tools provided in COOT and MOLPROBITY (53).

ATG8CL—ATG8CL* mixed with a 3-fold molar excess of pentapeptide (Asp-Trp-Glu-Ile-Val) was incubated at 4 °C for 24 h and concentrated to 80 mg/ml in 20 mM HEPES, 150 mM NaCl, pH 7.5. Crystallization experiments used 2-µl sitting drops with a 1:1 protein/precipitant ratio. Crystals were produced in 0.2 M ammonium sulfate, 0.1 M Tris buffer, pH 8.0, and 36% PEG3350 and transferred to the precipitant solution with the addition of 10% ethylene glycol as a cryoprotectant. X-ray diffraction data were collected at the Diamond Light Source, UK, beamline I04, and the data were processed as above (Table 1). The structure was solved by molecular replacement using Phaser, as implemented in Phenix. The molecular replacement search model was generated by submitting the complete sequence of ATG8CL to the Phyre web server (54). Based on the solution, an initial model was produced using the AutoBuild wizard in Phenix. At this stage, clear electron density was apparent for the Asp-Trp-Glu-Ile-Val pentapeptide in both molecules of ATG8CL*. The final model was completed and validated as described for PexRD54. Data collection and refinement statistics for PexRD54 and ATG8CL are given in Table 1.

**SAXS Measurements, Data Processing, and Analysis**

SAXS data were collected at the ESRF beamline BM29 (Grenoble, France (55, 56)) and at the Diamond Light Source, UK, beamline B21. For BM29, measurements were made at an energy of 12.5 keV, camera length of 2.81 m, and q range...
Circular Dichroism Spectroscopy

CD spectroscopy experiments were performed using a Chirascan-Plus CD spectrophotometer (Applied Photophysics). Purified proteins in 20 mM HEPES, pH 7.5, 150 mM NaCl at a concentration of at least 10 mg/ml were diluted to 0.2 mg/ml in 20 mM di-potassium phosphate, pH 7.2. CD measurements were carried out in a quartz glass cell with a 0.5-mm path length. To obtain overall CD spectra, wavelength scans between 190 and 260 nm were collected at 15 °C using a 2.0-nm bandwidth, 0.5-nm step size, and time per point of 1 s. The data were collected over four accumulations and averaged. The raw data in millidegree units were corrected for background and converted to mean residue molar ellipticity.

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Structural Basis of Host Autophagy-related Protein 8 (ATG8) Binding by the Irish Potato Famine Pathogen Effector Protein PexRD54
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