Avirulence Protein 3a (AVR3a) from the Potato Pathogen Phytomonothora infestans Forms Homodimers through Its Predicted Translocation Region and Does Not Specifically Bind Phospholipids

The mechanism of translocation of RxLR effectors from plant pathogenic oomycetes into the cytoplasm of their host is currently the object of intense research activity and debate. Here, we report the biochemical and thermodynamic characterization of the Phytophthora infestans effector AVR3a in vitro. We show that the amino acids surrounding the RxLR leader mediate homodimerization of the protein. Dimerization was considerably attenuated by a localized mutation within the RxLR motif that was previously described to prevent translocation of the protein into host. Importantly, we confirm that the reported phospholipid-binding properties of AVR3a are mediated by its C-terminal effector domain, not its RxLR leader. However, we show that the observed phospholipid interaction is attributable to a weak association with denatured protein molecules and is therefore most likely physiologically irrelevant.

The oomycetes (or water molds) encompass many devastating pathogenic species (1, 2). Among them, Phytophthora infestans causes potato late blight, a disease of concern for global food security. Like all successful pathogens, oomycetes are able to evade the defense reactions of their hosts. One of their common strategies is to manipulate their host’s immune system by secreting effectors that interfere with intracellular components of basal or inducible immunity (e.g. Refs. 3–5).

Recently, it was shown that several plant pathogenic oomycetes contain genes that encode putative secreted proteins that all contain a conserved tetrameric amino acid sequence motif: RxLR. This Arg-Xaa-Leu-Arg motif is found within four amino acids following the predicted cleavage sites of canonical signal peptides and is often followed by a Glu-Glu-Arg (EER) motif (6–8).

To date, the best characterized RxLR effector is AVR3a from P. infestans (see Scheme 1A for a schematic summary of the current published literature). AVR3a was originally identified as the cognate avirulence protein recognized by the cytosolic potato R3a resistance protein (9). AVR3a is a small secreted protein that is delivered into host cells by the pathogen via an unknown mechanism dependent on the N-terminal RxLR leader (8). In planta coexpression of R3a and different AVR3a truncation constructs showed that amino acids 73–147 were sufficient to trigger the R3a-mediated hypersensitive response, but the response was reduced compared with AVR3a lacking only the signal peptide (residues 1–21) or the signal peptide and the RxLR leader (amino acids 1–59). Furthermore, deletion of the last 16 residues abolished any recognition by R3a (10). Two isoforms of AVR3a have been identified in P. infestans isolates that differ by only three amino acids. Two of these changes, at
positions 80 and 103, are present in the mature proteins lacking the signal peptide. AVR3a(C19)K80I103 is referred to here as AVR3aKI, and AVR3a(S19)K80M103 is referred to as AVR3aEM. Whereas AVR3aKI is quite efficiently recognized by R3a, AVR3aEM is weakly recognized. Thus, strains expressing only the AVR3aEM isoform evade recognition by plants carrying the R3a resistance gene and do not induce a defensive hypersensitive response (9, 10).

AVR3a modulates the plant immune response by stabilizing the host ubiquitin E3 ligase CMPG1. By doing so, it suppresses a plant defense response against another P. infestans secreted protein, INF1 (5). Crucial for this biological function of AVR3a is the presence of a freely accessible C-terminal aromatic residue (Tyr-147). However, modification or deletion of Tyr-147 from the AVR3aKI C terminus did not interfere with R3a recognition (5). An explanation for this observation was suggested by the recently published structural model of AVR3a. This model was based on the NMR structure of the Phytophthora capsici homolog, AVR3a4 and shows that the C terminus is flexible and angled away from the four-bundle helical protein core. However, no defined structural information could be obtained from the RxLR leader of P. capsici AVR3a4 to potentially shed light on the requirements for translocation, despite this region being included in the recombinant protein under study (4).

In 2010, Kale et al. (11) reported that RxLR and RxLR-like proteins are translocated into eukaryotic cells via an RxLR-dependent, pathogen-independent mechanism. In addition, the authors claimed that RxLR or RxLR-like motifs mediate translocation through binding to phosphoinositol 3-phosphate molecules exposed at the cell surface. However, this view has been recently challenged by two independent studies (4, 12). Using recombinant AVR3a from P. infestans, Yaeno et al. (4) showed that the effector domain, rather than the RxLR leader, binds to phospholipids (PLPs).3 Using in planta expression of AVR3a effector domain point mutants (i.e. K85E) that did not show any PLP binding in vitro, they reported that this effect correlates with the ability of AVR3a to stabilize the host ubiquitin ligase CMPG1 (5).

Ellis and Dodds (13) highlighted the urgent need to test the requirement of PIP binding for effector uptake by host cells. Indeed, despite good structural models and extensive information about the biological effects of various mutations within the AVR3a protein effector domain, negligible data are available describing the influence of the RxLR leader on protein stability and dynamics. Here, we evaluated the effect that the RxLR leader exerts on the dynamics of the protein using recombinant protein constructs, and we describe an in-depth analysis of the lipid-binding properties of AVR3a. In addition, we analyzed the effect of a mutation within the AVR3a RxLR motif previously shown to prevent protein translocation into host cells, and we found that it impaired homodimerization of this effector.

3 The abbreviations used are: PLP, phospholipid; GdnHCl, guanidine hydrochloride; AUC, analytical ultracentrifugation; ITC, isothermal titration calorimetry; PI3P, phosphoinositol 3-phosphate; Ins-1,3-P2, inositol 1,3-bisphosphate.
AVR3aKI protein fragments were studied (Scheme 1B). The AVR3a fragment comprising amino acids 22–147 lacked only the signal peptide that is expected to be missing in the mature protein. For the AVR3a effector domain construct, residues 60–147 were chosen because this sequence has the same R3a recognition potential in planta as residues 22–147 (10). The RxLR leader construct comprised only amino acids 22–59. This exact sequence was previously shown to be important for the delivery of AVR3a by *P. infestans* into host cells (8) and does not affect the in planta recognition by R3a (10). Furthermore, a protein was produced in *Escherichia coli* in which the AVR3a RxLR motif was mutated, AVR3a(22–59)-His6-KMIK. This mutant was found to impair the delivery of the full-length protein from the pathogen into host cells (8). All AVR3a fragments were fused to the His6 tag of pET21b at the C terminus and were characterized in detail prior to use (supplemental Fig. S1 and Note 1).

**N-terminal RxLR Leader of AVR3a Mediates Dimerization of the Protein in Vitro**—Size exclusion chromatograms revealed a significant difference in the chromatogram of AVR3a(60–147)-His6 compared with that of AVR3a(22–147)-His6 (Fig. 1A). The apparent molecular mass calculated for AVR3a(22–147)-His6 (see supplemental Fig. S2 for additional information) showed that this construct corresponded to that of a dimeric protein, whereas AVR3a(60–147)-His6, in which the RxLR leader was deleted, behaved like a monomer. Under the same conditions, the AVR3a RxLR leader construct, AVR3a(22–59)-His6, showed three peaks corresponding to a tetramer, dimer, and monomer, with the dimer as the dominant species (Fig. 1B and supplemental Fig. S2A).

To confirm these findings at lower, biologically more relevant concentrations, all constructs that showed dimerization were evaluated by AUC. AUC monitors the sedimentation of macromolecules in solution, which allows the characterization of protein oligomers. Each protein sample was analyzed at different concentrations, and the data were combined to calculate the respective dissociation constants (*K*D). Consistent with the gel filtration results, AVR3a(22–147)-His6 was identified as a homodimer. The *K*D determined for the AVR3a(22–147)-His6 dimerization was 10.83 ± 1.8 μM, which nearly matched the *K*D obtained for the RxLR-only construct, AVR3a(22–59)-His6 (9.8 ± 1.45 μM) (Fig. 1C and supplemental Appendix 1). For both constructs, the dimer concentrations were inversely proportional to the protein concentration under these conditions. The *K*D dimerization constant of AVR3a(22–147)-His6 also coincided with the concentration that resulted in a half-maximal drop in the melting temperature (*T*m) that was observed when the thermostability of the protein was characterized in a concentration-dependent manner (supplemental Fig. S3). A *T*m difference of ~12 °C was observed when the heat denaturation of low concentration solutions was compared with that of high concentration solutions. The concentration obtained that cor-

---

**SCHEME 1.** A, schematic summary of the currently published literature on AVR3a. Dotted arrows highlight the conclusions of the cited references, and the solid arrows indicate the suggested additions/modifications based on the data presented here. B, schematic representations of the AVR3a constructs investigated in this work.
AVR3a Dimerizes and Does Not Specifically Bind Phospholipids

responded to $T_{\text{m}}(\text{C})$ was $\sim 15 \, \mu M$. This shows that AVR3a(22–147)-His$_6$ is slightly more (thermo)stable at low concentrations. In contrast to the gel filtration runs, no tetrameric species for AVR3a(22–59)-His$_6$ were observed with AUC. Overall, these observations show that, in vitro, AVR3a is an oligomeric protein, principally a homodimer, and that dimerization is facilitated by the RxLR leader region.

To evaluate the effect that the C-terminal His$_6$ tag fusions might have on the dimerization constants of AVR3a, amino acids 22–147 were C-terminally fused with monomeric red fluorescent protein, a protein roughly double the size of AVR3a, and analyzed by AUC. A fluorescent reporter protein was chosen because these are commonly used to study protein localization and/or trafficking. This bulky fusion construct, AVR3a(22–59)-His6 were observed with AUC. Overall, these observations show that, in vitro, AVR3a is an oligomeric protein, principally a homodimer, and that dimerization is facilitated by the RxLR leader region.

Mutations of RxLR Amino Acids RLLR to KKKM Leads to Structural Changes in the Leader and Inhibits Dimerization of AVR3a—The homologous replacement of the RLLR sequence in AVR3a to KKKM was shown to abolish AVR3a delivery by the pathogen to potato host cells (8). CD spectroscopic comparison of the AVR3a(22–59)-His$_6$, WT RxLR leader peptide and the AVR3a(22–59)-His$_6$-KKKM mutant peptide revealed that this mutation decreased the content of random coil structure at all protein concentrations tested (37, 74, and 111 $\mu M$) (Fig. 1C and supplemental Appendix 1). Therefore, we expect that the C-terminal His$_6$ tag only marginally decreases the dimerization of AVR3a.

The gel filtration chromatogram of AVR3a(22–59)-His$_6$-KKKM indicated that this polypeptide adopted a dimer form at high concentrations (652 $\mu M$) (Fig. 1B and supplemental Fig. S2A). However, subsequent analysis by AUC revealed only sedimentation coefficients consistent with a monomeric polypeptide at all protein concentrations tested (37, 74, and 111 $\mu M$) (Fig. 1C and supplemental Appendix 1). A control experiment showed that this discrepancy could not be ascribed to an ionic strength effect of the 500 mM NaCl used in the size exclusion chromatography buffer (Fig. 1C, last two rows, and supplemental Appendix 1). In the presence of 500 mM NaCl and at all tested concentrations (37, 74, and 111 $\mu M$), only monomeric species were detected for AVR3a(22–59)-His$_6$-KKKM. Therefore, we attribute the discrepancy for AVR3a(22–59)-His$_6$-KKKM to the difference in the protein concentrations utilized by AUC and gel exclusion. Due to the low extinction coefficient of the RxLR leader constructs at 280 nm (2980 M$^{-1}$ cm$^{-1}$), high protein concentrations (600 – 800 $\mu M$) were required to obtain good quality gel filtration chromatograms.

We conclude that the AVR3a(22–59)-His$_6$-KKKM homodimer forms only at concentrations that exceed those utilized for AUC (111 $\mu M$). Therefore, this mutant construct is most likely a monomer at biologically relevant concentrations.

RxLR Leader of AVR3a Facilitates Intermolecular Dimerization with the Predicted $\alpha$-Helix 3 and/or $\alpha$-Helix 4—To further analyze the dimerization of AVR3a, cross-linking experiments were performed using formaldehyde. At ambient temperature, formaldehyde forms predominantly immonium cations that are reactive toward nucleophiles, such as the N terminus of a protein and the lateral chains of cysteine, lysine, histidine, and tyrosine residues (14). Formaldehyde is ideal to detect specific protein-protein interactions due to (i) a short reaction time that minimizes nonspecific cross-linking, (ii) the formation of highly reactive intermediates that allow fixation of transient protein-protein interactions due to (i) a short reaction time that minimizes nonspecific cross-linking, (ii) the formation of highly reactive intermediates that allow fixation of transient interactions, and (iii) a short cross-linking distance (0.2–0.3 nm) (15). Cross-linking conditions were optimized for AVR3a(22–147)-His$_6$ at room temperature. However, we noted that formaldehyde treatment reduced the efficiency of His tag detection by Western blotting. Therefore, all Western blots were slightly overexposed to visualize effects better. The example shown in Fig. 2 is representative for all experiments performed.

The different AVR3a constructs were cross-linked at concentrations of 20 $\mu M$ alone and in combination with one another. Subsequently, the samples were analyzed by SDS-PAGE/Western blotting and compared with the untreated controls. Cross-linking of AVR3a(22–59)-His$_6$ (RxLR leader only) did not yield detectable amounts of covalently linked dimers (Fig. 2, lanes 1 and 2). Therefore, none of the lysines, tyrosines, and histidines (cysteine not present), highlighted in the sequence alignment in supplemental Fig. S4, are within 0.2–0.3
AVR3a Dimerizes and Does Not Specifically Bind Phospholipids

|            | MW   | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 |
|------------|------|----|----|----|----|----|----|----|----|----|----|----|----|
| AVR3a<sub>22–59</sub>-His<sub>6</sub> | 6.8kDa |   |   |   |   |   |   |   |   |   |   |   |   |
| AVR3a<sub>22–147</sub>-His<sub>6</sub> | 16kDa |   |   |   | x |   |   |   | x |   | x |   |   |
| AVR3a<sub>22–59</sub>-His<sub>6</sub> (KMIK) | 6.7kDa |   |   |   |   | x |   | x |   |   |   |   |   |
| AVR3a<sub>60–147</sub>-His<sub>6</sub> | 12kDa |   |   |   | x |   | x | x | x |   | x | x |   |
| Formaldehyde | ..... | x |   | x | x | x | x | x | x | x | x | x |   |

**FIGURE 2. Detection of AVR3a dimer complexes after formaldehyde cross-linking.** Cross-linking was carried out using 2% formaldehyde with the indicated proteins at a concentration of 20 μM in 50 mM sodium phosphate buffer (pH 7.2) for 5 min. Compared with untreated controls, no additional bands for the dimeric polypeptide AVR3a(22–59)-His<sub>6</sub> (lanes 1 and 2) were detected after formaldehyde treatment, indicating that no Lys, Tyr, or His is a 2–3 Å distance to one another in the dimer complex. The same was found for AVR3a(22–59)-His<sub>6</sub>-KMIK, which was exclusively a monomer at a concentration of 20 μM (lanes 6 and 7). Formaldehyde treatment of AVR3a(22–147)-His<sub>6</sub> led to the appearance of a new band (full ovals) on the SDS-polyacrylamide gel, consistent with the dimeric size of the protein (lanes 3 and 4). When cross-linking of AVR3a(22–147)-His<sub>6</sub> was carried out in the presence of the AVR3a RxLR leader construct (AVR3a(22–59)-His<sub>6</sub>), a new protein band appeared (lane 5, arrowhead), in addition to the AVR3a(22–147)-His<sub>6</sub> dimer complex. The size corresponded to a complex of both proteins, which could not be detected when AVR3a(22–59)-His<sub>6</sub> was substituted with AVR3a(22–59)-His<sub>6</sub>-KMIK (lane 8). The AVR3a(60–147)-His<sub>6</sub> monomeric protein did not cross-link to itself with formaldehyde (lanes 9 and 10). Surprisingly, AVR3a(60–147)-His<sub>6</sub> could be cross-linked to AVR3a(22–147)-His<sub>6</sub> (lane 11, dotted oval) but not to AVR3a(22–59)-His<sub>6</sub> (lane 12). See supplemental Appendix 3 for further information.

To further characterize the AVR3a dimerization, samples of the monomer and dimer bands of AVR3a(22–147)-His<sub>6</sub> were cut from the gel, digested with trypsin, separated by liquid chromatography, and subsequently analyzed by MALDI-TOF mass spectrometry (supplemental Appendix 2). The chromatograms were very similar for both the dimer and monomer samples, with the exception of one distinct peak that appeared only in the dimer sample. MALDI-TOF lift experiments revealed peptides covering AVR3a amino acids 59–79 (APNFPNALSLNEEMFNVAALTK); these amino acids are located C-terminal after the EER motif, including the beginning of the predicted helix α1, amino acids 110–120 (VTLDQIDTFLK), nearly the complete predicted α-helix 3, amino acids 131–147 (YNQIYNSYMMHLGLTGY), and the predicted α-helix 4 (AVR3a helix prediction according to Ref. 4). In addition, a partial proteolytic digest of AVR3a(22–147)-His<sub>6</sub> was performed to identify the possible starting point of the AVR3a effector domain (supplemental Fig. S5). This experiment showed a proteinase K-resistant C-terminal domain starting at amino acid 68. Therefore, we conclude that AVR3a residues 59–68 are located close to the effector domain of the partner molecule in the tertiary dimer complex.

**Lipid-binding Property of the AVR3a Effector Domain Results from a Denatured Protein Fraction**—Since PLP binding of oomycete RxLR effectors is currently controversial (13), we investigated the lipid-binding ability of AVR3a using lipid spot membranes. We first confirmed the observations made by Yaeno et al. (4) that indeed the effector domain and not the RxLR leader of AVR3a binds membrane-attached PLPs (Fig. 3A). As already underlined by these authors, this result is in contrast with a previous report claiming that the interaction of oomycete RxLR proteins with PLPs is attributed to the RxLR leader (motif) (10). To investigate the possible physiological significance of PLP binding, experiments were performed utilizing denatured
AVR3a Dimerizes and Does Not Specifically Bind Phospholipids

**A**

AVR3a\textsubscript{22–147}(His\textsubscript{6}). The urea transition of AVR3a(22–147)-His\textsubscript{6} was measured by following the loss of secondary structure with increased urea concentrations using CD spectroscopy. The urea EC\textsubscript{50} is 4.7 M (Fig. 3B), and the protein is completely unfolded at concentrations above 6 M. Surprisingly, when the PLP binding assay was performed in the presence of 8 M urea, the protein was able to bind to PLPs on lipid spot membranes. No lipid binding could be detected for the R\textsubscript{x}LR leader construct. The membranes were incubated for 20 min with 20 \textmu M protein. The lipid recognized on membrane A was sulfatide; those on membrane B were phosphatidylinositol 3-, phosphatidylinositol 4-, and phosphatidylinositol 5-phosphate; and those on membrane C were phosphatidyglycerol, cardiolipin, and phosphatidylinositol 4-phosphate. B, urea- but not guanidine-denatured AVR3a(22–147)-His\textsubscript{6} binds to PLPs. The urea transition point was 4.68 M. To analyze the lipid-binding ability of unfolded AVR3a(22–147)-His\textsubscript{6}, the lipid binding assay was performed in the presence of 8 M urea and 6 M GdnHCl, respectively. No difference in the lipid binding of native and urea-denatured AVR3a(22–147)-His\textsubscript{6} could be found, whereas the presence of a 6 M concentration of the charged chemical denaturant GdnHCl completely abolished the lipid binding of AVR3a(22–147)-His\textsubscript{6}.

**B**

CD-spectroscopic determination of the AVR3a(22–147)(His\textsubscript{6}) urea transition.

**C**

Membrane A

- Sphingosine
- Sphingosine-1-phosphate (S1P)
- Phosphorylcholine
- Ceramide
- Sphingomyelin
- Lyso-phosphatidylcholine (LPC)
- Lyso-phosphatic acid (LPA)
- Myristolein

Membrane B

- Lyso-phosphatidic acid (LPA)
- Lyso-phosphocholine (LPC)
- Phosphatidylinositol 3-phosphate (PI(3P))
- Cholesterol
- Phosphatidylycholine (PC)

Membrane C

- Triglyceride
- Diacylglycerol (DG)
- Phosphatidylcholine (PC)
- Phosphatidylethanolamine (PE)
- Cardiolipin

The urea EC\textsubscript{50} is 4.7 M (Fig. 3B), and the protein is completely unfolded at concentrations above 6 M. Surprisingly, when the PLP binding assay was performed in the presence of 8 M urea,
we found that denatured AVR3a(22–147)-His₆ interacted with PLPs with the same specificity as the non-denatured protein solutions (Fig. 3B). In contrast, no PLP binding of AVR3a(22–147)-His₆ was detectable when the assay was carried out with a 6 M concentration of the charged chemical denaturant GdnHCl instead of urea (Fig. 3B). These observations demonstrate that PLP binding of AVR3a does not require an intact three-dimensional structure, that it is likely based on charge-charge interactions, and that the headgroups of the recognized PLPs are mainly responsible for this interaction. Thus, we characterized this binding by isothermal titration calorimetry (ITC) utilizing both native and urea-denatured AVR3a(22–147)-His₆ (268 μM) indicates a weak interaction between both molecules under these conditions (red and dark red traces). However, the thermograms show that the stoichiometry was greater than 1:8 (concentration range covered by the titration) because there is no obvious enthalpy change visible in the titration profile. In addition, the binding constant (Kₜₐₜ) must be >1.92 mM. The dialysis buffer for this type of experiment was 50 mM sodium phosphate (pH 7.2) containing 8 M urea. All titrations were carried out at 20 °C. B, lipid binding of 20 μM AVR3a(22–147)-His₆ to lipids on lipid spot membranes was suppressed by the presence of ADP-bound DnaK at a molar concentration (1 μM) that represents 5% of the AVR3a(22–147)-His₆ amount. C, lipid binding of 20 μM AVR3a(22–147)-His₆ in the presence of DnaK amounts corresponding to 3% (0.6 μM) and 1% (0.2 μM) of the AVR3a(22–147)-His₆ molar concentration. Lipid spot identity can be found in Fig. 3C.

Since charge-charge dominated protein-ligand interactions are normally characterized by endothermic ITC profiles (16), the actual amount of complex detected in the membrane assay had to be below the detection limit of ITC. This suggested that a small fraction of unfolded AVR3a(22–147)-His₆ molecules might bind to the PLPs. To test this hypothesis, ITC titrations utilizing Ins-1,3-P₂ and AVR3a(22–147)-His₆ were performed in the presence of 8 M urea. The thermograms show signals indicative of nonspecific interactions, and only a marginal change in enthalpy could be observed within the investigated concentration range (Fig. 4A). These experiments show that the binding stoichiometry must be above 1:8 (concentration range covered by the titra-
tions) and that the binding constant has to exceed the final ligand concentration of 1.92 mM. Therefore, it should be possible to block the observed lipid binding of AVR3a(22–147)-His$_6$ on spot membranes with substoichiometric amounts of a chaperone that specifically binds unfolded protein molecules. To test this, we utilized the E. coli chaperone DnaK. DnaK was purified in the absence of its co-chaperones DnaJ (the DnaK hydrolysis factor) and GrpE (the nucleotide exchange factor) in an ADP-bound state, also referred to as the high affinity state (17–21). In this form, DnaK binds proteins that expose hydrophobic surface patches. The ADP-DnaK-unfolded protein complexes usually have dissociation half-times of minutes to even hours (17). Thus, a nearly 1:1 binding of DnaK to misfolded AVR3a(22–147)-His$_6$ can be assumed during the 20-min incubation time of the performed lipid spot assay. No AVR3a(22–147)-His$_6$ could be immunodetected on the lipid spot membranes following co-incubation of 20 $\mu$M AVR3a(22–147)-His$_6$ and 1 $\mu$M DnaK, even after prolonged film exposure (Fig. 4, B and C). This demonstrates that <5% of the AVR3a molecules are able to bind to the PLPs and that either no or only a very slow transition toward a lipid-binding conformational state exists in AVR3a(22–147)-His$_6$ molecules.

To investigate the binding to PLPs presented in a more natural context, AVR3a(22–147)-His$_6$ was probed for its ability to bind PolyPIPosomes in different buffers (supplemental Fig. S10). Consistent with the lipid spot membrane experiments, AVR3a(22–147)-His$_6$ was found to bind PI3P-containing PolyPIPosomes when denatured with 8 M urea and in the absence of the stabilizing ions phosphate and/or sulfate. To evaluate the competition effect of phosphate ions on PI3P binding, we tested the p40 PX-binding domain in the presence and absence of these. 50 mM sodium phosphate buffer slightly inhibited the PI3P-p40 PX binding, but the interaction could still be clearly detected (supplemental Fig. S10, lower). These observations confirm that denatured AVR3a(22–147)-His$_6$ has an affinity for PI3P and that phosphate and/or sulfate ions are crucial for structural stabilization of this protein construct.

**DISCUSSION**

Protein self-association to form dimers is widely found in biology (22). We have shown here that recombinant AVR3a forms a homodimer in vitro (Figs. 1 and 2). Its dissociation constant is ~10 $\mu$M, a value often found in other homodimers (for examples, see Refs. 23–25). Dimerization of AVR3a is mainly (but not solely) facilitated by the RxLR leader because the recombinant protein comprising these residues exhibited the same $K_D$ as the full-length protein (Fig. 1 and supplemental Appendix 1). In addition, the dimerization constant of the AVR3a RxLR leader is independent of the solvent ionic strength (Fig. 1C and supplemental Appendix 1), suggesting that this complex formation is not driven by charge-charge interactions. Interestingly, the authors of a recent study tested whether AVR3a dimerizes in planta (26). For this purpose, the authors coexpressed two different N-terminally tagged (FLAG and GFP) AVR3a constructs in planta but were unable to detect any interaction between these two proteins in co-immunoprecipitation experiments. However, this finding does not necessarily mean that the dimer form of AVR3a is biologically irrelevant. It still could play a role during trafficking and secretion from the parasite or during translocation into the host cells. In addition, we studied solely C-terminally tagged recombinant constructs to investigate the role that the N-terminal RxLR leader plays for the protein because direct addition of a tag to a sequence of interest can alter the biological function of the respective polypeptide (see review, see Ref. 27). We found that a C-terminal extension to AVR3a of ~28 kDa still allowed dimerization but also slightly reduced the respective $K_D$ value. It is possible that N-terminal tags more strongly affect the structure and dynamics of the short and flexible AVR3a RxLR leader because we found that parts of the RxLR leader are in 2–3-Å contact with some amino acids of the effector domain (Fig. 2 and supplemental Appendix 2). We are aware that those studying the effects that AVR3a facilitates on the host prefer detectable modifications on the N terminus of the protein because a free C terminus is crucial for the suppression of the plant immune defense response against INF1 (4, 5, 10). Nevertheless, on the basis of our findings, we believe that the biological significance of AVR3a dimerization should be investigated with constructs containing small C-terminal tags.

In addition, we investigated a previously described mutation of the RxLR amino acids within the leader of AVR3a (residues 43–47, RRLR changed to KKMIK) (8). Interestingly, this mutation altered the secondary structure ensemble of the RxLR leader (supplemental Fig. S7A), and the respective polypeptide showed at least a 10-fold increased dimerization constant in vitro (Fig. 1, B and C, and supplemental Appendix 1). Furthermore, the mutation reduced the ability of the RxLR leader peptide to interact with full-length AVR3a (Fig. 2 and supplemental Fig. S7, B and C, and Note 2). Since the investigated mutant showed strongly altered characteristics compared with the WT, we believe that the biologically observable effects of RxLR motif mutations require in vitro characterization of the respective mutants to better understand the nature of the observed effects.

Several recent publications reported that some oomycete and fungal effectors interact with the headgroups of phosphoinositol monophosphates (4, 11, 12, 28). In addition, claims were made that, for oomycete RxLR proteins, RxLR or RxLR-like motifs in their respective N-terminal regions facilitate pathogen-independent translocation. Through binding to cell surface-exposed PI3P (11, 28). This view has been discussed (13) and challenged by two independent groups (4, 12). While studying the lipid binding of AVR3a, we found that the protein was indeed detectable by antibodies on certain matrix-associated PLPs. In addition, we could reproduce the findings by Yaeno et al. (4) that the C-terminal effector domain, not the RxLR leader of AVR3a, bound the PLPs. However, we found that this PLP-binding ability of AVR3a did not require an intact three-dimensional structure and that only a small subtraction of presumably misfolded molecules are responsible (Figs. 3 and 4 and supplemental Fig. S10). Furthermore, we were unable to detect any signs of a physical interaction between native AVR3a and the PLP headgroup Ins-1,3-P$_2$ or Ins-1,5-P$_2$, using ITC (Fig. 4A). Furthermore, AVR3a seems to only bind PI3P-containing PolyPIPosomes when denatured or not sufficiently stabilized (supplemental Fig. S10). Our data also suggest that either no or only a very slow equilibrium toward a lipid-binding conforma-
AVR3a Dimerizes and Does Not Specifically Bind Phospholipids

Affiliations—We thank Sharon Kelly for use of the CD spectrophotometer, Prof. Ian Booth for use of the ITC200 system, and Prof. Neil A. R. Gow for helpful suggestions and discussions.

REFERENCES

1. Phillips, A. J., Anderson, V. L., Robertson, E. J., Secombes, C. J., and van West, P. (2008) New insights into animal pathogenic oomycetes. Trends Microbiol. 16, 13–19
2. Gu, B., Kale, S. D., Wang, Q., Wang, D., Pan, Q., Cao, H., Meng, Y., Kang, Z., Tyler, B. M., and Shan, W. (2011) Rust secreted protein Ps87 is conserved in diverse fungal pathogens and contains an RXL-like motif sufficient for translocation into plant cells. PLoS ONE 6, e27217
3. Kale, S. D., and Tyler, B. M. (2011) Entry of oomycete and fungal effectors into plant and animal host cells. Cell. Microbiol. 13, 1839–1848
4. Yaeno, T., Li, H., Chaparro-Garcia, A., Schornack, S., Koshiba, S., Watanahe, S., Kigawa, T., Kamoun, S., and Shirasu, K. (2011) 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. U.S.A. 108, 14682–14687
5. Bos, J. I., Armstrong, M. R., Gilroy, E. M., Boevink, P. C., Hein, I., Taylor, R. M., Zhendong, T., Engelhardt, S., Vetukuri, R. R., Harrower, B., Dixelius, C., Bryan, G., Sadanandom, A., Whisson, S. C., Kamoun, S., and Birch, P. R. (2010) Phytophthora infestans effector AVR3a is essential for virulence and manipulates plant immunity by stabilizing host E3 ligase CMGPI. Proc. Natl. Acad. Sci. U.S.A. 107, 9909–9914
6. Birch, P. R., Rehmany, A. P., Pritchard, L., Kamoun, S., and Beynon, J. L. (2006) Trafficking arms: oomycete effectors enter host plant cells. Trends Microbiol. 14, 8–11
7. Rehmany, A. P., Gordon, A., Rose, L. E., Allen, R. L., Armstrong, M. R., Whisson, S. C., Kamoun, S., Tyler, B. M., Birch, P. R., and Beynon, J. L. (2005) Differential recognition of highly divergent downy mildew avirulence gene alleles by RPP1 resistance genes from two Arabidopsis lines. Plant Cell 17, 1839–1850
8. Whisson, S. C., Boevink, P. C., Moleleki, L., Avrova, A. O., Morales, J. G., Gilroy, E. M., Armstrong, M. R., Grouffaud, S., van West, P., Chapman, S., Hein, I., Toth, I. K., Pritchard, L., and Birch, P. R. (2007) A translocation signal for delivery of oomycete effector proteins into host plant cells. Nature 450, 115–118
9. Armstrong, M. R., Whisson, S. C., Pritchard, L., Bos, J. I., Venter, E., Avrova, A. O., Rehmany, A. P., Böhme, U., Brooks, K., Cherevach, I., Hamlin, N., White, B., Fraser, A., Lord, A., Quail, M. A., Churcher, C., Hall, N., Berriman, M., Huang, S., Kamoun, S., Beynon, J. L., and Birch, P. R. (2005) An ancestral oomycete oocyte locus contains late blight avirulence gene AVR3a, encoding a protein that is recognized in the host cytoplasm. Proc. Natl. Acad. Sci. U.S.A. 102, 7766–7771
10. Bos, J. I., Kanneganti, T. D., Young, C., Cakir, C., Huisema, E., Win, J., Armstrong, M. R., Birch, P. R., and Kamoun, S. (2006) The C-terminal half of Phytophthora infestans RXLR effector AVR3a is sufficient to trigger R3a-mediated hypersensitivity and suppress INF1-induced cell death in Nicotiana benthamiana. Plant J. 48, 165–176
11. Kale, S. D., Gu, B., Capelluto, D. G., Dou, D., Feldman, E., Rumore, A., Arredondo, F. D., Hanlon, R., Fudal, I., Rouxel, T., Lawrence, C. B., Shan, W., and Tyler, B. M. (2010) External lipid PI3P mediates entry of eukaryotic pathogen effectors into plant and animal host cells. Cell 142, 284–295
12. Gan, P. H., Rafiqi, M., Ellis, J. G., Jones, D. A., Hardham, A. R., and Dodds, P. N. (2010) Lipid binding activities of flax rust AvrM and AvrL567 effectors. Plant Signal. Behav. 5, 1272–1275
13. Ellis, J. G., and Dodds, P. N. (2011) Showdown at the RXLR motif: serious differences of opinion in how effector proteins from filamentous eukaryotic pathogens enter plant cells. Proc. Natl. Acad. Sci. U.S.A. 108, 14381–14382
14. Toews, J., Rogalski, J. C., Clark, T. J., and Kast, J. (2008) Mass spectrometric identification of formaldehyde-induced peptide modifications under in vivo protein cross-linking conditions. Anal. Chem. Acta 618, 168–183
15. Sutherland, B. W., Toews, J., and Kast, J. (2008) Utility of formaldehyde cross-linking and mass spectrometry in the study of protein-protein interactions. J. Mass Spectrom. 43, 699–715
16. Matulis, D., Rouzina, I., and Bloomfield, V. A. (2000) Thermodynamics of DNA binding and condensation: isothermal titration calorimetry and electrostatic mechanism. J. Mol. Biol. 296, 1053–1063
17. Packschies, L., Theyssen, H., Buchberger, A., Buku, A., Goody, R. S., and Reinstein, J. (1997) GrpE accelerates nucleotide exchange of the molecular chaperone DnaK with an associative displacement mechanism. Biochemistry 36, 3417–3422
18. Slepenkov, S. V., and Witt, S. N. (2002) The unfolding story of the Escherichia coli Hsp70 DnaK: is DnaK a holdase or an unfoldase? Mol. Microbiol. 45, 1197–1206
19. Russell, R. J., and McMacken, R. (1998) Kinetic characterization of the ATPase cycle of the DnaK molecular chaperone. Biochemistry 37, 596–607
20. Buku, A., and Horwich, A. L. (1998) The Hsp70 and Hsp60 chaperone machines. Cell 92, 351–366
21. Ellis, R. J., and Hartl, F. U. (1999) Principles of protein folding in the cellular environment. Curr. Opin. Struct. Biol. 9, 102–110
22. Marimanayagam, N. J., Sunde, M., and Matthews, J. M. (2004) The power of two: protein dimerization in biology. Trends Biochem. Sci. 29, 618–625
23. Nickerson, D. P., and Wong, L. L. (1997) The dimerization of Pseudomonas putida cytochrome P450cam: practical consequences and engineering of a monomeric enzyme. Protein Eng. 10, 1357–1361
24. Burrows, S. D., Doyle, M. L., Murphy, K. P., Franklin, S. G., White, J. R., Brooks, L., McNulty, D. E., Scott, M. O., Knutson, J. R., and Porter, D. (1994) Determination of the monomer-dimer equilibrium of interleukin-8 reveals it is a monomer at physiological concentrations. Biochemistry 33, 12741–12745
25. Phizicky, E. M., and Fields, S. (1995) Protein-protein interactions: methods for detection and analysis. Microbiol. Rev. 59, 94–123
26. Boutemy, L. S., King, S. R., Win, I., Hughes, R. K., Clarke, T. A., Blumenschein, T. M., Kamoun, S., and Banfield, M. J. (2011) Structures of Phytophthora RXLR effector proteins. A conserved but adaptable fold underpins functional diversity. J. Biol. Chem. 286, 35834–35842
27. Fidzinski, P., Wawra, M., Bartsch, I., Heinemann, U., and Behr, J. (2012) High-frequency stimulation of the temporomammal pathway induces input-specific long-term potentiation in subicular bursting cells. Brain Res. 1430, 1–7
28. Plett, J. M., Kemppainen, M., Kale, S. D., Kohler, A., Legué, V., Brun, A., Tyler, B. M., Pardo, A. G., and Martin, F. (2011) A secreted effector protein of Laccaria bicolor is required for symbiosis development. Curr. Biol. 21, 1197–1203
29. Wawra, S., Bain, J., Durward, E., de Bruijn, I., Minor, K. L., Matena, A., Löbach, L., Whisson, S. C., Bayer, P., Porter, A. J., Birch, P. R., Seconomes, C. J., and van West, P. (2012) Host-targeting protein 1 (SpHtp1) from the oomycete Saprolegnia parasitica translocates specifically into fish cells in a tyrosine-O-sulfate-dependent manner. Proc. Natl. Acad. Sci. U.S.A. 109, 2096–2101