The RxLR Motif of the Host Targeting Effector AVR3a of Phytophthora infestans Is Cleaved before Secretion

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INTRODUCTION

Many eukaryotic pathogens including plant pathogenic fungi and oomycetes use effector proteins to successfully infect their hosts. Effectors are secreted molecules that help both the invasion and the propagation of the pathogen by suppressing host defense responses as well as adapting host metabolism (Wawra et al., 2012a). The phylogenetically related malaria parasites (Plasmodium spp) and the plant pathogenic water molds (oomycetes) have numerous effector proteins, which are characterized by conserved N-terminal amino acid sequences (Hiller et al., 2004; Marti et al., 2004) that play a role in the host cell targeting process (Whisson et al., 2007; Boddey et al., 2009). In malaria parasites, the Plasmodium export element (PEXEL) consists of Arg-Xaa-Leu-Xaa-Glu/Asp/Gln (with Xaa being any amino acid) positioned close after the signal peptide cleavage site (Hiller et al., 2004; Marti et al., 2004). This sequence element is cleaved by endoplasmic reticulum-located proteases and the N terminus becomes acetylated (Ac-Xaa-Glu/Asp/Gln) (Chang et al., 2008; Boddey et al., 2009). In many eukaryotic pathogens including plant pathogenic fungi and oomycetes use effector proteins to successfully infect their hosts. These findings imply a role for the RxLR motif in the secretion of AVR3a by the pathogen, rather than a direct role in the host cell entry process itself.
followed by another second conserved motif Ser/Asp-Glu-Glu-Arg (s/dEER; reviewed in Wawra et al., 2012a). Effector secretion, and presumably entry into the host cell, happens at the haustorium, an infection-specific interface between pathogen and host that forms after the pathogen has colonized the host (Whisson et al., 2007; Rafiqi et al., 2010). Haustoria are always surrounded by a host plant-derived membrane (Lu et al., 2012). Evidence exists that RxLR effectors translocate after secretion from the extrahaustorial space into plant cells (Whisson et al., 2007; van Poppel et al., 2008; Lokossou et al., 2010; Chou et al., 2011; Gilroy et al., 2011; Saunders et al., 2012). RxLR and RxLR-like effectors may also be present in fungi (summarized in Kale and Tyler, 2011), and different models for the translocation process have been proposed, but the precise mechanism is unclear (Petre and Kamoun, 2014).

Some reports suggest that the RxLR sequences are directly involved in host cell entry. For the RxLR effector avirulence protein 3a (AVR3a) from the potato blight pathogen Phytophthora infestans, it was shown that the RxLR motif is required for host cell translocation based on a loss-of-function infection assay (Whisson et al., 2007). In addition, oomycete and fungal RxLR and RxLR-like effectors have been reported to translocate into host cells in the absence of the pathogen (Plett et al., 2011; Gu et al., 2011; summarized in Kale and Tyler, 2011). The autonomous translocation was proposed to occur after binding of the RxLR motif to lipids via phospholipid-mediated endocytosis (Kale et al., 2010). However, this was not observed uniformly for all RxLR effectors and excludes phospholipid binding as a general host entry mechanism (Gan et al., 2010; Yaeno et al., 2011; Wawra et al., 2012b). Furthermore, a recent study indicates that cell-based reentry assays do not support models of pathogen-independent translocation of effectors into plant cells and detected cytosolic amounts of proteins are presumably based on accumulation due to the overexpression of effector proteins (Petre et al., 2016; Na et al., 2013).

The oomycete RxLR motif might have a function similar to the PEXEL motif as a potential internal sorting signal. A fusion protein containing the effector core domain of AVR3a from P. infestans coupled to the PEXEL sequence of HRP2 (histidine rich protein II) from Plasmodium falciparum is delivered into the plant by P. infestans (Grouffaud et al., 2008). The equivalent experiment in Plasmodium, the delivery of HRP2 with the RxLR leader of AVR3a, was also reported to be successful (Bhattacharjee et al., 2006), although this observation was not reproducible in a recent study (Boddey et al., 2016). Nevertheless, RxLR motifs reside within flexible protein regions (Yaeno et al., 2011; Wawra et al., 2012b; Sun et al., 2013) and could be easily accessed by endopeptidases and modified by acetytransferases. In addition, P. infestans also possesses a putative protease similar to the protease responsible for the PEXEL cleavage in Plasmodium (Boddey et al., 2010; Kay et al., 2011).

Here, we investigated the biochemical integrity of the native RxLR effector AVR3a secreted into culture filtrate of axenically grown P. infestans. In combination with the structural analysis of recombinant AVR3a by NMR spectroscopy, the obtained data support a model comprising the processing of the effector RxLR sequence before secretion.

**RESULTS**

To investigate the potential processing of the RxLR motif of RxLR effector proteins from P. infestans, ideally the proteins should be obtained from the interhaustorial space of infected plant material. However, as the quantities of effector produced during infection are negligible it is not feasible with the current available technology to isolate and purify suitable amounts of secreted effectors for biophysical studies. Therefore, an RxLR effector protein from culture filtrate was purified. The only validated effector candidate that is secreted in reasonable quantities into the culture filtrate of P. infestans is the RxLR effector AVR3a (Torto et al., 2003). Since tagging and/or modification of the sequence can impair the biophysical properties of the effector (Wawra et al., 2012b), we focused on native AVR3a secreted during axenic growth of in vitro-cultivated P. infestans. The enrichment of the naturally occurring variant AVR3aEM [AVR3a(S19)E80M103] from the supernatant of an axenic culture was optimized and native AVR3a was purified (Figure 1A). The corresponding bands on SDS-PAGE were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS), and AVR3aEM (gi|289470504) was identified through the detection of several peptides with significant thresholds using two different mass spectrometers (Figure 1B). Interestingly, no peptides were detected N-terminally of the RxLR motif, but a “KNEENETSEER” peptide starting immediately after the RxLR motif was identified (Figure 1C; ion score, 54; e-value, 3.8e-01). The weak MS/MS fragmentation of the “KNEENETSEER” peptide is likely caused by the acidic character (6 × Glu), which results in elution very early in the LC run and insufficient ionization for mass spectrometry. A control spectrum of a 10-fold amount of recombinant AVR3a22-147 protein containing the residues N-terminal to the RxLR motif resulted in a similar weak spectrum for the same peptide (ion score, 21; e-value, 1.8e-01). The mass spectrometric data also support the prediction of the N-terminal acetylation of native AVR3a (e-value, 1.7e+02), which would be possible only if the RxLR is cleaved before secretion of AVR3a, rendering the N terminus of the corresponding lysine accessible to acetyltransferases, which are exclusively intracellular.

To further test for the potential cleavage of the RxLR motif in AVR3a, native AVR3a was separated by reverse-phase (RP) chromatography and the main constituent of the sample was subsequently analyzed by MALDI-TOF to determine its absolute molecular mass (Figure 1D). Several peaks were observed, but most interestingly in the mass range of 11.5 to 13 kD, peaks showed a Gaussian distribution with a separation of 11,621 D was confirmed after internal calibration with cytochrome
**Figure 1.** Purification of Native Processed AVR3a from *P. infestans* Culture Filtrate and MALDI-TOF Analysis.

(A) SDS-PAGE of native AVR3a purified from axenic growth culture of *P. infestans*. All bands on the gel were identified by LC-MS/MS as AVR3aEM, which seems to form SDS-resistant multimers (asterisks).

(B) Amino acid sequence of AVR3aEM from *P. infestans* isolate 88069. Peptides are highlighted according to their detection by LC-MS/MS (green), nanoRSLC-HPLC (orange), or both methods (blue). The RxLR motif is underlined, and the cleavage site is highlighted by an asterisk.
The occurrence of a single peak indicates that there was complete deglycosylation of the up to nine hexose units in native AVR3a and thereby excluded the presence of multiple cleaved polypeptides. The closest mass match of AVR3a to the measured 11,621 D is a polypeptide including amino acids 48 to 147 (11,577 D), especially considering that processing from the C terminus is unlikely due to the physiological importance of Tyr147 of AVR3a (Bos et al., 2010).

However, the observed +44 D difference between the theoretical mass and the experimentally determined mass of AVR3aEM is too large to be considered an error. Indeed, a polypeptide mass difference of +44 D would correspond exactly to an acetyl modification (with an additional H+) of AVR3aEM, which is in perfect agreement with the data obtained from the initial LC-MS/MS analysis (Figure 1B). To validate the LC-MS/MS data that indicated an N-acetylation at amino acid 48 (RLLR*K), Edman degradation was performed. Edman degradation results in chemical cleavage of the N-terminal peptide bond of a polypeptide chain only when the amino group is sterically accessible and not modified in any way (Edman, 1950). To reduce potential side reactions, Edman degradation was performed with deglycosylated AVR3a and, indeed, no significant amino acid sequence could be detected despite employing a sufficient quantity of AVR3a (data not shown). Consequently, the experimentally obtained mass difference of +44 D between the measured and the theoretical expected mass for AVR3aEM was +44 D, and the N-terminal blockage observed by Edman sequencing indicates that native AVR3a secreted by an axenic Phytophthora capsici culture into the medium is cleaved at R47 after the RxLR motif and probably is N-acetylated at Lys-48, the first amino acid downstream of the RxLR.

Since Kay et al. (2011) described a phylogenetic homology between the PEXEL-cleaving plasmin V from P. falciparum and PIAP12 of P. infestans, we overexpressed the protease domain of PIAP12 (79–432 amino acids) in Escherichia coli and purified the soluble fraction (Figure 2A, left). We incubated the PIAP12-containing fractions with 5 μg AVR3aEM for 1 h at 37°C. However, no differences in the band patterns were observed compared with the control, even after reduction of the pH (Figure 2A, right). Since impurities might disturb the activity of the protease, we performed another purification of the elution fractions under denaturing conditions. After refolding, PIAP12 remained unable to process recombinant AVR3a (Figure 2B). Hence, we cloned the other 10 aspartic protease domains of P. infestans to avoid any precipitation and inactivation of the proteases during the purification procedure, we cotransformed the catalytic domain of each protease with AVR3aEM into E. coli. We reasoned that the simultaneous expression of both proteins should increase our chances of detecting any potential proteolytic activity. Although each of the aspartic protease domains could be detected in the soluble fraction without significant degradation, no additional bands for AVR3aEM could be observed, indicating a lack of cleavage (Figure 2C).

Another interesting biochemical feature of native AVR3aEM seems to be the formation of stable multimers that can be observed after SDS-PAGE (asterisks in Figure 1A). To explore this multimer formation further, 15N-labeled AVR3aEM was overexpressed (Figure 3A), the backbone atoms assigned, and the relaxation times measured by NMR spectroscopy. From the relaxation times, we estimated the rotation correlation time (τr), which correlates to the size of the protein (Figure 3B). The τr/T2 constant ratio was 30.5 ± 2.7 ms and equal to a molecular mass of 24 kD, confirming the dimerization of recombinant AVR3aEM. By contrast, AVR3aEM was shown to behave like a monomer (Wawra et al., 2012b), which is in line with our relaxation data (τr/T2 = 6.9 ± 0.3 ms) and an estimated molecular mass of 11 kD.

For a detailed understanding of the dimerization interface, several 2D-, 3D-, and 4D-NMR spectra of AVR3aEM were measured, and the backbone as well as side chain atoms assigned because the monomeric state is more suitable for NMR studies. The NMR solution structure of AVR3aEM adopted the typical 4 α-helix bundle in the core domain with a flexible, disordered N terminus until Glu-70 (Figure 3C, Table 1) that was also observed for the Phytophthora capsici homolog AVR3a4 (Yaeno et al., 2011). The alignment of the AVR3aEM structure with the structures of AVR3a4 and AVR3a11 from P. capsici showed high similarity, with RMSD values of 3.1 and 3.3 Å, respectively (Figure 3D). Furthermore, the electrostatic potential of the molecular surface of AVR3aEM was calculated with PyMol using the Poisson Boltzmann Equation (Baker et al., 2001). As already speculated from homology models of AVR3aEM, a positively charged patch on the surface of the core domain was formed by Lys-85, Lys-86, Lys-89, Arg-124, Lys-125, and Lys-130 (Figure 3E). The formation of a positively charged patch seems to be restricted to AVR3a-like effectors since no patches could be found in effector proteins like PexRD2 or ATR1 although their folds are highly similar (data not shown; Win et al., 2012).

Based on our above results, we concluded that (1) AVR3aEM is a monomer, while AVR3aEM forms a homodimer, and (2) residues 48 to 59 (KNEENETSEEER) are essential for the multimerization of native as well as recombinant AVR3a as already reported.
postulated by Wawra et al. (2012b). To evaluate the influence of the positively charged poly-E linker on the multimerization of AVR3a, the charge of the N terminus (K48-R59) was changed by mutating all six glutamates to glutamines. In line with the hypothesis of an electrostatic-mediated dimerization, the mutant was monomeric according to the time constant ratio of 10.1 ± 0.6 ms, which indicated a mass of 13 kD similar to that of AVR3aEM (Figure 3B). The tendency for the multimerization of wild-type AVR3a48-147 in contrast to the monomeric state of the poly-E/Q mutant was also observed by size-exclusion chromatography (SEC) (Figure 3F).

**DISCUSSION**

This study shows that native AVR3aEM is cleaved and probably N-acetylated downstream of the RxLR motif by *P. infestans* before secretion into culture filtrate and that it forms stable multimers. N-acetylation is mediated by N-terminal acetyltransferases. Since N-terminal acetyltransferases are exclusively intracellular enzymes, N-acetylation of proteins after secretion is not possible (Starheim et al., 2012). Hence, the intracellular cleavage of AVR3a is a prerequisite for the potential acetylation of the N-terminal Lys-48 and cleavage by proteases of *P. infestans* coexpressed into the culture filtrate can be excluded.

The cleavage site directly after the RxLR sequence and the high conservation among other effector proteins suggest that the RxLR motif might play a crucial role in the intracellular processing before secretion. Notably, the RxLR effector Avh241 from *Phytophthora sojae* contains a myristoylation motif (GAAAK) after its RxLR motif (Yuet et al., 2012). In general, myristoylation sites are located at the N terminus of a protein. Based on our model invoking RxLR processing, this myristoylation modification site in Avh241 would no longer lie within the sequence but instead would be present at the very N terminus after removal of the RxLR motif (RWLR).

We have not directly shown that processed AVR3a is able to translocate into plant cells; thus, we cannot completely rule out that an alternative pathway might be involved during infection. However, the model whereby the RxLR motif is involved in secretion rather than working as an uptake motif is strongly supported by the fact that an RxLR deletion construct of AVR3a still showed translocation activity (Kemen et al., 2011). Based on our study, mutations or deletions of RxLR leaders could result not only in mis cleavage and concomitant missorting, but also possibly protein destabilization. Hence, loss-of-function phenotypes can even occur when the effector translocation process is not impaired. Accordingly, the loss-of-recognition phenotype of *P. infestans*-delivered AVR3a mutants (Whisson et al., 2007) or *P. sojae*-delivered AVR1b mutants (Dou et al., 2008) could be caused by defects of effector sorting and/or stabilization. Furthermore, results of studies in which chimeric proteins containing the effector domain of AVR3a and N-terminal sequences of other effectors, especially in model systems without the natural host-pathogen interaction, should be revisited (Anderson et al., 2012; Stassen et al., 2013). Negative results might be due to the lack of the correct processing of the N terminus and concomitant secretion and ultimately not caused by the loss of their translocation abilities into the host.

In general, the current models for pathogen-independent host cell uptake and endocytosis of effector proteins via RxLR binding to plant cell surface phospholipids should probably be revisited (Kale et al., 2010; Ellis and Dodds, 2011; Wawra et al., 2012b). Our study also strengthens the model whereby the lipid binding of effector proteins is mainly mediated by a positively charged lysine patch on the surface of the core domains rather than the RxLR motif in the N terminus (Sun et al., 2013; Yaeno and Shirasu, 2013; Lu et al., 2013). Since the lipid binding and the multimerization are mediated by the same patch, masking of the lysine patch by the N terminus of AVR3a might also have a protective function to avoid unspecific interactions during the translocation process.
The NMR solution structure of AVR3a60-147 reflects mainly the results obtained from an AVR3a4-based homology model (Yaeno et al., 2011). AVR3a comprises a three-helix WY domain with an N-terminal extension that forms a typical four-helix bundle (Win et al., 2012). The rigid core domain contains the amino acids Glu-70 to Tyr-147, while the N terminus is flexible and disordered and not incorporated into the core structure that is an essential prerequisite for the cleavage by a protease. Notably, the N terminus (Lys48-Arg59) of AVR3a48-147 is also involved in the dimerization, as demonstrated by earlier cross-linking studies (Wawra et al., 2012b). Our results suggest that an initially weak multimerization of the AVR3a core domain is highly stabilized by the electrostatic interaction between the negatively charged N terminus containing the poly-E stretch and the positively charged patch at the surface of the core domain (Figure 3). The poly-E-mediated multimerization of AVR3a likely does not influence the lipid binding.
properties of AVR3a because mutation of the RxLR motif of the effector AVR1d (RxLR/EEEE) does not have an effect on lipid binding (Na et al., 2013). Thus, as mentioned above, the N terminus serves to mask the positively charged patch of the core domain to avoid unspecific interaction during secretion and translocation until the point of membrane-mediated uptake into the host cell. Mutlimerization of another effector protein, PexRD2, is essential for its function in planta (King et al., 2014). Hence, the dimerization of AVR3a might be essential for its interaction with potato R3a since the different R3a recognition sites (K/E80 and I/M103), which are important for the hypersensitive response in planta, do not interfere with the multimerization (Bos et al., 2006).

The processing of native AVR3a secreted from P. infestans is indeed remarkably similar to the cleavage observed for the PEXEL motif of effectors from P. falciparum (Chang et al., 2008; Boddey et al., 2009; Russo et al., 2010) (Figure 4) and also for the TEXEL motif of effectors from T. gondii (Coffey et al., 2015; Curt-Varesano et al., 2016). However, the RxLR motif is not recognized and cleaved by the ER-located plasmepsin V protease of P. falciparum (Boddey et al., 2016). Furthermore, despite the phylogenetic relationship between plasmepsin V from P. falciparum and PiAP12 (Kay et al., 2011), we could not detect any proteolytic activity against recombinant AVR3a22-147 for PiAP12 nor for any of the other 10 aspartic protease domains (Figure 2C). An analogous system might exist in P. infestans with a similar mode of action as in P. falciparum but having evolved from a different origin. In contrast to the PEXEL motif, which is partly retained after processing (Ac-X-E/D/Q) (Chang et al., 2008), the RxLR sequence of AVR3aEM is cleaved off completely. The cleaved PEXEL motif is involved in the binding of the respective effector to the PTEX red blood cell import translocon (Boddey et al., 2009; Russo et al., 2010). It is intriguing to speculate what role might be played by the retained s/dEER sequence that is conserved in many RxLR effectors. Although the protein export and host cell translocation mechanisms between the pathogens are not interchangeable, probably due to their

Table 1. Statistics for the Solution Structure Calculation of AVR3a60-147

<table>
<thead>
<tr>
<th>Constraint Type</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total NOEs</td>
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</tr>
<tr>
<td>Intraresidual NOEs</td>
<td>269</td>
</tr>
<tr>
<td>Sequential NOEs (i to i + 1)</td>
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<tr>
<td>Medium-range NOEs (i to i + 2,3,4)</td>
<td>311</td>
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<tr>
<td>Long-range NOEs</td>
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<tr>
<td>Dihedral angle restraints (φ + ψ)</td>
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<tr>
<td>RMSD to the mean structure (Å)</td>
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<tr>
<td>RMSD for backbone atoms (T80-G144)</td>
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<tr>
<td>Average target function</td>
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<td>Average of upper distance (Å)</td>
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<td>Average of maximum torsion angle (°)</td>
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<tr>
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<tr>
<td>Residues in the disallowed regions (%)</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Data are derived from a set of the 10 lowest-energy structures calculated by CYANA 2.1 using automated NOE (nuclear Overhauser effect) assignment without a homology model. RMSD, root mean square deviation.

Figure 4. Comparison of the Processing of the PEXEL Motif in P. falciparum and the RxLR Motif of AVR3a in P. infestans.

The processing of AVR3a is strikingly similar to the stepwise modification observed for the PEXEL effectors from P. falciparum. However, the PEXEL motif (RxLxE/D/Q) is partially retained, while the RxLR motif is cleaved off completely, although both are acetylated (Ac). The corresponding RxLR-modifying enzymes remain to be investigated. NAT, N-terminal acetyltransferase; PEP, signal peptide peptidase; PMV, plasmepsin V; PTEX, Plasmodium translocon of exported proteins; SP, signal peptide.
different infection structures (intracellular parasitophorous vacuoles for *P. falciparum/T. gondii* versus extracellular haustoria for oomycetes), the stepwise processing of the RxLR motif in oomycetes could act as a signal that facilitates the transport to and secretion of effectors from the haustorial infection structure similarly to PEXEL and TEXEL.

**METHODS**

**Purification of Native AVR3a**

To establish a purification protocol for native AVR3a, initially the optimization was done with *Phytophthora infestans* transfectants carrying a translational AVR3a-mRFP fusion protein (AVR3a::mRFP) as described previously (Whisson et al., 2007). For the purification of native AVR3aEM, 6 liters of a liquid culture of *P. infestans* (isolate 88069) in pea broth was grown in several Petri dishes (each 75 mL) for 22 d to confluence. Mycelium was removed by paper filtration and the supernatant cleared by centrifugation (15 min, 15,000g) and an additional filtration step (0.45 μm, nitrocellulose). The supernatant was supplemented with 2 mM EDTA and a protease inhibitor mix (two tablets/3 liters; Roche). Afterwards, the protein suspension was loaded onto a SO₃⁻ column (Fractogel-EMD-SO₃⁻; Merck) for initial concentration. The column was washed once with 25 mM NaPi buffer, pH 7.3, and once more with the same buffer containing 80 mM NaCl. Subsequently, bound proteins were eluted with the same buffer supplemented with 1 M NaCl and the protease inhibitor AEBSF. The elution fraction was ammonium sulfate precipitated (80%) for 30 min at room temperature. Pellets obtained from precipitation after centrifugation (20 min, 9000g) were resuspended in 20 mL of 50 mM NaPi buffer, pH 7.3, and additionally dialyzed (1 × 3 liters for 3 h and 1 × 3 liters overnight) against NaPi buffer supplemented with 15 mM MgSO₄. The diazylated protein fractions were resubjected to the SO₃⁻ column to remove remaining contaminants and washed twice with 25 mM NaPi buffer, pH 7.3 (the second one with additional 100 mM NaCl), followed by elution with the same buffer supplemented with 200 mM NaCl. An aliquot was TCA precipitated and prominent bands on SDS-PAGE were analyzed by LC-MS/MS. The results in this study arose from several independent preparations of AVR3a from in total 70 liters of *P. infestans* culture.

**TCA Precipitation**

For 300- to 500-μL sample, 1 mL of 40% TCA was used for protein precipitation overnight at 4°C. Subsequently, samples were centrifuged for 30 min at 16,000g (4°C) and the supernatant was discarded. The remaining pellet was washed with 1 mL acetone and centrifuged again for 20 min at 16,000g (4°C). This step was repeated twice before drying the pellet.

**LC-MS/MS Analysis**

Proteins of interests from SDS-PAGE were trypsin digested, dried, and resuspended in 0.1% formic acid. Dissociated peptides were analyzed using an HCT ultra PTM Discovery System with an electrospray source equipped with a 1-kHz Bruker Smartbeam-II solid-state laser. A 2,5-dihydroxybenzoic acid or a α-cyano-4-hydroxycinnamic acid matrix was used as indicated. Linear mode acquisition was performed with a high resolution in a 3000 to 20,000 m/z range, and the sample rate was 1.00 GS/s with Bruker Protein Calibration I Standard P/N 206355. Between 1000 and 10,000 shots were accumulated manually with Smartbeam laser focus raster width set at 50 μm. Spectra were processed in Compass 1.3 software incorporating Flex Control version 3.3 and Flex Analysis version 3.3 using “centroid” peak picking (80%), smoothing performed using Savitzky-Golay 0.2 m/z for one cycle, and TopHat baseline subtraction for reproducible peak annotation on nonresolved isotope distributions prior to calibration.

**Enzymatic Deglycosylation**

Enzymatic deglycosylation was performed on either lyophilized samples or protein pellets obtained after TCA precipitation. The denaturing reaction conditions protocol provided with the New England Biolabs Protein
Deglycosylation Mix (P6039S) was chosen. Deglycosylation was carried overnight at 37°C.

**Cloning and Mutagenesis**

The sequences encoding AVR3a22-147 and AVR3a48-147 poly-E/Q (E50Q, Cloning and Mutagenesis overnight at 37°C. Deglycosylation Mix (P6039S) was chosen. Deglycosylation was carried overnight at 37°C. Cells were collected, lysed by sonication (60% intensity, 1 min interval at 4°C), and ultracentrifuged at 37°C to OD600 = 0.8, and protein expression was induced with 1 mM IPTG for 6 h at 30°C. Cells were collected (15 min, 4500g, 4°C) and lysed by sonication. The supernatant after a centrifugation step (30 min, 16,100g, 4°C) was analyzed by immunoblot with an α-5xHis antibody (1:10,000; Qiagen; no. 34460).

For another assay, domains of all aspartic proteases from P. infestans were cloned into BL21(DE3)T1r. Cells containing both constructs were incubated in LB medium at 37°C to OD600 = 0.8, and protein expression was induced with 1 mM IPTG for 6 h at 30°C. Cells were collected (15 min, 4500g, 4°C) and lysed by sonication. The supernatant after a centrifugation step (30 min, 16,100g, 4°C) was analyzed by immunoblot with an α-5xHis antibody (1:10,000; Qiagen; no. 34460).

**Expression and Purification of Recombinant AVR3a Proteins**

Recombinant AVR3a protein containing a C-terminal 6x His-tag was obtained as described elsewhere (Wawra et al., 2012b). Briefly, for iso-topically labeled protein, cells from 0.5 liters LB culture (OD600 of 0.8) were transferred to 2 liters M9 minimal medium supplemented with 1 g/L [15N] NH4Cl and/or 0.4% (w/v) [U-13C]glucose and further grown to an OD600 of 0.8. Subsequently, protein expression was induced with 0.2 mM IPTG overnight at 25°C. Cells were collected, lysed by sonication (60% intensity, 4 × 45 s, 1 min interval at 4°C; Sonoplus; Bandelin), and ultracentrifuged (Beckman Coulter; 98,000g, 70 min, 4°C). Subsequently, the supernatant was applied to a Q-Sepharose column (equilibrated with 50 mM KPi buffer, pH 6.5). The flow-through containing AVR3a was applied to a Ni-NTA column equilibrated with 50 mM KPi buffer, pH 6.5, and 20 mM imidazole. After a washing step, proteins were eluted with 300 mM imidazole. Fractons containing the AVR3a proteins were pooled and the imidazole removed by dialysis for NMR spectroscopy. The integrity of each construct was confirmed by SDS-PAGE and MALDI-TOF mass spectrometry.

**Expression and Purification of Recombinant PiAP12**

The domain of PIAP12 containing a C-terminal 6x His-tag was obtained from*Escherichia coli* (BL21(DE3)T1r). Briefly, cells were incubated in 2xYT at 37°C to OD600 = 0.8, and protein expression was induced with 1 mM IPTG for 6 h at 30°C. Cells were collected and lysed by sonication and the supernatant after ultracentrifugation applied to Fractogel EMD TMAE (M) for 6 h at 30°C. The reaction was stopped with SDS sample buffer and subsequent heating up to 90°C for 10 min. Samples were analyzed by SDS-PAGE and immunoblotting (anti-His antibody, 1:10,000; Qiagen; no. 34460). In Vitro Cleavage Assays with AVR3a

Purified PIAP12 (180 μg) was incubated with 5 μg recombinant AVR3a22-147 6xHis containing the N-terminal RxLR motif for 1 h at 37°C. The reaction was stopped with SDS sample buffer and subsequent heating up to 90°C for 10 min. Samples were analyzed by SDS-PAGE and immunoblotting (anti-His antibody, 1:10,000; Qiagen; no. 34460). In Vitro Cleavage Assays with AVR3a

For another assay, domains of all aspartic proteases from P. infestans were cloned into BL21(DE3)T1r. Cells containing both constructs were incubated in LB medium at 37°C to OD600 = 0.8, and protein expression was induced with 1 mM IPTG for 6 h at 30°C. Cells were collected (15 min, 4500g, 4°C) and lysed by sonication. The supernatant after a centrifugation step (30 min, 16,100g, 4°C) was analyzed by immunoblot with an α-5xHis antibody (1:10,000; Qiagen; no. 34460).

**NMR Spectroscopy and Resonance Assignment**

The 2D and 3D spectra were recorded at 25°C on a 700-MHz Ultrashield NMR spectrometer (Bruker) equipped with a triple cryoprobe head (Bruker Biospin). The 3D and 4D spectra were recorded with a Varian VNMRs 700 NMR spectrometer equipped with four RF channels, a Performa-XZY PFG gradient unit, and a room temperature 1H/13C/15N triple-resonance probe head. AVR3a22-147 (300 μM in 50 mM KPi buffer, pH 6.5) was prepared with 10% D2O and 50 μM DSS as a calibration standard. All spectra were analyzed with Sparky 3.113. For assignment, all 4D nonsample (NUS) NMR data sets were used. The process of sequentially specific assignment employing 4D NUS NMR data sets was performed with a 3D HNCO experiment based on NUS (1000 data points, maximal evolution times: 30 ms for 13CO and 28 ms for 15N, acquisition time: 6 h), a 4D HNCOCA NMR spectrum (Zawadzka-Kazimierzuk et al., 2010; correlating interresidual HN(i)-N(i)-CO(i-1)-Cu(i-1) resonances (1400 data points, maximum evolution times of 30 ms for 13CO, 10 ms for 15CO, and 28 ms for 15N, acquisition time: 18 h), the complementary 4D HNCOCA (Zawadzka-Kazimierzuk et al., 2010), correlating both intraresidual HN(i)-N(i)-Cu(i)-CO(i) and interresidual HN(i)-N(i)-Cu(i)-CO(i-1) resonances (2500 data points, maximum evolution times of 20 ms for 13CO, 10 ms for 15CO, and 28 ms for 15N, acquisition time: 31 h), a 4D HBAHCBCANH spectrum correlating H1, 13C, and 15N signals for interresidual [HNI]N(i)-[Cu(i)-HNI][Cu(i)] and interresidual [HNI][Cu(i)-HNI][Cu(i-1)] resonances (2500 data points, maximum evolution times of 10 ms for 13CO, 7 ms for 15N, and 28 ms for 15N, acquisition time: 50 h). Additionally, aliphatic side chain H1 and 13C chemical shifts were assigned with HCCH-COSY, HCCH-TOCSY, HCBHNH, and HBBHCO/NH spectra and aromatic side chain resonances were assigned from two-dimensional COSY, TOCSY, and NOESY spectra. Distance constraints were obtained from two 1H-homonuclear NOESY spectra, one recorded in water, the other recorded in D2O buffer, from the 1H-NOESY-HSQC and the 15N-NOESY-HSQC. The 3D 1H-edited NOESY-HSQC spectra were acquired with a mixing time of 150 ms for aliphatic and aromatic regions on Varian VNMRs 800 NMR spectrometer equipped with four RF channels, a Performa IV PFG module, and cryogenic 1H/13C/15N triple resonance probe head. Processing and evaluation of spectra were performed with Topspin 3.0 (Bruker) or NMRPipe (Delaglio et al., 1995); assignment was done with Sparky.

**Structure Calculation**

15N and 13C resonances were calibrated unidirectionally following the IUPAC-IUB recommended chemical shift referencing ratios (Wishart et al., 1995; Marley et al., 1998). NOE restraints were identified and transformed into distance constraints using the automated standard protocol of Cyana (Güntert et al., 1991); Dihedral angles were calculated using Talos+ (Shen et al., 2009). The structure of AVR3a22-147 was calculated using Cyana 2.1 (López-Méndez and Güntert, 2006) without using a homolog as a guide.
structure. Energy minimization was performed with YASARA 11.12.31 (Krieger and Vriend, 2002).

Molecular Weight Estimation by Measuring Relaxation Constants
For estimation of the molecular weight of the AVR3a protein constructs, different spectra with varying relaxation times \( \tau \) were recorded (20, 100, 150, 200, 300, 500, 750, 1000, and 1400 ms for T1 and 10, 30, 50, 90, 130, 170, 210, and 250 ms for T2). With the different signal intensities, the time constants T1 and T2 were calculated for each amino acid as described by Kay et al. (1992). For analysis, only resonances from amino acids located in secondary structure elements without contribution of chemical exchange processes were chosen (Blackledge et al., 1998). Subsequently, the rotation correlation time \( \tau_r \) and the standard curve for the estimation of the molecular weight were calculated as described elsewhere (Rossi et al., 2010).

SEC
SEC experiments for AVR3a48-147 wild type and the poly-E/Q mutant were performed in 50 mM NaP, 500 mM NaCl, pH 7.0, with Superdex 75 10/300 on a Bio-Rad system at a flow rate of 0.5 mL/min. The column was calibrated under the same conditions with thyroglobulin (670 kD), \( \gamma \)-globuline (158 kD), ovalbumin (44 kD), myoglobin (17 kD), and vitamin B12 (1.35 kD); \( R^2 \) was 0.9199 after linear regression of the calibration standards.

Accession Numbers
Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: AVR3a (AVR3aEM, ADC96691.1; AVR3aKI, ADC96694.1), PiAP01 (HMS88685, corrected; Kay et al., 2011), PiAP02 (XP_002900284.1), PiAP04 (XP_002998816.1), PiAP05 (XP_002902303.1), PiAP06 (XP_002905662.1), PiAP07 (XP_002905687.1), PiAP08 (HMS88686, corrected; Kay et al., 2011), PiAP09 (XP_002904877), PiAP10 (XP_002907533), PiAP11 (XP_002907534), and PiAP12 (XP_002901082). Structural coordinates and NMR shift data were deposited in the RCSB databank (entry ID: rcsb104633), respectively. Structures already published, but used in this study are as follows: AVR3a4 (PDB-ID: 2LC2) and AVR3a11 (PDB-ID: 2ZGK).

Supplemental Data
Supplemental Table 1. Primers and restriction sites used for cloning of the constructs used in this study.

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AUTHOR CONTRIBUTIONS
S.W., F.T., A.M., K.A., C.J.S., P.B., and P.v.W. designed research. S.W., F.T., A.M., K.A., U.I., I.Z., W.K., J.S., and I.D. performed research and analyzed data. S.W., F.T., C.J.S., and P.v.W. wrote the article. All authors have given approval to the final version of the manuscript. Received July 8, 2016; revised April 4, 2017; accepted May 10, 2017; published May 18, 2017.

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