Cleavage of the *Pseudomonas syringae* Type III Effector AvrRpt2 Requires a Host Factor(s) Common among Eukaryotes and Is Important for AvrRpt2 Localization in the Host Cell

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Many phytopathogenic bacteria use a type III secretion system to deliver type III effector proteins into the host plant cell. The *Pseudomonas syringae* type III effector AvrRpt2 is cleaved at a specific site when translocated into the host cell. In this study, we first demonstrate that the factor(s) required for AvrRpt2 cleavage is present in extracts from animal and yeast cells, as well as plant cells. The cleavage factor in animal and plant cell extracts was heat labile but relatively insensitive to protease inhibitors. Second, mutational analysis of AvrRpt2 was applied to identify features important for its cleavage. In addition to two of the amino acid residues in the immediate vicinity of the cleavage site, a large part of the region C-terminal to the cleavage site was required when AvrRpt2 was cleaved in animal cell extract. Most of these features were also important when AvrRpt2 was cleaved in plant cells. Third, we investigated the effect of cleavage in interactions of AvrRpt2 with plant cells. Cleavage of AvrRpt2 appeared to be important for proper interactions with Arabidopsis cells that lack the resistance gene product corresponding to AvrRpt2, RPS2. In addition, removal of the region N-terminal to the cleavage site was important for the correct localization of the C-terminal effector region of the protein in the host cell. We speculate that the virulence function of AvrRpt2 requires removal of the N-terminal region to redirect the effector protein to a specific subcellular location in the host cell after translocation of the protein.

The majority of phytopathogenic gram-negative bacteria require the type III protein secretion system (TTSS) for pathogenicity (Galán and Collmer, 1999). In most cases, the TTSS is also required for the elicitation of strong defense responses, such as the hypersensitive response (HR), in resistant plants. Therefore, genes encoding components or regulators of the TTSS were initially identified as HR and pathogenicity (hrp) mutations (Lindgren et al., 1986). Recently, the *Xanthomonas campestris* AvrBs2 protein was shown to be directly translocated into the host cell via the TTSS (Casper-Lindley et al., 2002). In addition, based on mounting indirect evidence and analogies with the role of the TTSS in some bacterial pathogens of animals, it is generally believed that phytopathogenic bacteria use the TTSS to translocate certain proteins into the host cell (Staskawicz et al., 2001). The bacterial proteins that are secreted or translocated via the TTSS are called type III effectors. It is believed that many type III effectors function as virulence factors in susceptible hosts (Galán and Collmer, 1999).

When a plant exhibits strong resistance to a pathogen, the resistance response is often conditioned by a single avirulence (*avr*) gene in the pathogen and the corresponding resistance (*R*) gene in the plant (Dangl and Jones, 2001). Hence, this type of resistance is called gene-for-gene resistance. When plants carry appropriate *R* genes, some of the translocated bacterial proteins are recognized as Avr proteins. In fact, many type III effectors of phytopathogens were initially identified as *avr* gene products. It is believed that many Avr proteins actually function as virulence factors in susceptible hosts. In fact, the virulence function of AvrRpt2 in *rps2* Arabidopsis plants has been demonstrated (Chen et al., 2000; Guttman and Greenberg, 2001).

In the gene-for-gene relationship, the *Pseudomonas syringae* *avr* gene *avrRpt2* corresponds to the Arabidopsis *R* gene *RPS2* (Kunkel et al., 1993; Yu et al., 1993). AvrRpt2 is a type III effector (Mudgett and Staskawicz, 1999). AvrRpt2 must be translocated into the host cell for RPS2-mediated recognition to occur.

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.103.025999.
(Leister et al., 1996; Wu et al., 2003). The AvrRpt2 protein appears to be specifically cleaved between Gly71 and Gly72 by a presumed plant cytoplasmic protease (Mudgett and Staskawicz, 1999). These lines of evidence strongly suggest that AvrRpt2 is translocated via the TTSS into the plant cell and subsequently modified (cleaved). The region C-terminal to residue 81 of AvrRpt2 is sufficient to elicit the RPS2-dependent HR (Mudgett et al., 2000).

Some type III effectors are activated by host cells in a manner dependent on factors prevalent among eukaryotes but not specific to particular host species. Several P. syringae Avr proteins are myristoylated in the plant cell, and this modification is important for recognition by their corresponding R genes (Nimchuk et al., 2000; Shan et al., 2000; Tampakaki et al., 2002). The Yersinia pestis ser/thr protein kinase YpkA requires actin for its activation (Juris et al., 2000). Both the myristoylation machinery and actin are prevalent among eukaryotes.

Here, we report that the AvrRpt2 protein is cleaved accurately in animal and yeast cell extracts and that therefore the factor(s) required for AvrRpt2 cleavage is not plant specific but present in diverse eukaryotes. We also demonstrate that a large part of AvrRpt2 that is C-terminal to the cleavage site contributes to cleavage. These observations suggest that AvrRpt2 may make a major contribution to its own cleavage and that the eukaryotic factor(s) may play a relatively minor role. The cleavability of AvrRpt2 is important for a proper interaction with host plant cells. We show that cleavage is crucial in subcellular localization of AvrRpt2 in Arabidopsis cells. Cleavage of a type III effector in the host cell might be a common strategy for the redirection of proteins to a specific subcellular site after translocation.

RESULTS

AvrRpt2 Protein Was Cleaved Properly in Rabbit Reticulocyte Lysate

Initially, we intended to characterize AvrRpt2 cleavage to identify the recognition sequence for the presumed plant-specific protease so that we could use the sequence as a biotechnology tool in plant cells. We chose a rabbit reticulocyte lysate-based in vitro translation system coupled with T7 RNA polymerase-based in vitro transcription for the purpose of rapid production of AvrRpt2 derivative proteins. In this way, PCR products encoding \textit{avrRpt2} gene derivatives with the T7 promoter sequence at the 5' ends can be used directly for protein production.

As shown in Figure 1a, when AvrRpt2 was produced and labeled with $^{35}$S-Met using the rabbit reticulocyte lysate in vitro translation system, the major product was approximately 25 kD (lanes 1–3, marked by a black triangle). The intensity of the band corresponding to the full-length size (apparently 32 kD, white triangle) decreased with prolonged incubation, whereas the intensity of the 25-kD band increased. In contrast, when the \textit{P. syringae} effector protein AvrB was expressed, the only product observed corresponded to the expected full-length size of the protein (lanes 9–11, white triangle). When AvrRpt2 is cleaved in plant cells or by plant cell extracts, the approximate size of the C-terminal region of the protein is 25 kD (Mudgett and Staskawicz, 1999).

![Figure 1](image-url). AvrRpt2 can be cleaved in rabbit reticulocyte lysate. a, Smaller products observed are cleaved products of AvrRpt2. AvrRpt2 (lanes 1–3), GST::AvrRpt2 (lanes 4–8), and AvrB (lanes 9–11) were translated and $^{35}$S-labeled using rabbit reticulocyte lysate. The durations of incubations are indicated. In lanes 7 and 8, EDTA was added to a final concentration of 20 mM after a 0.5-h in vitro translation reaction to stop the reaction. The hours indicated are the total of the reaction time and the incubation time following the EDTA addition. The labeled proteins were resolved by denaturing PAGE and were detected by phosphor imaging of the gel. b, AvrRpt2 is cleaved at the correct site in rabbit reticulocyte lysate. The cleaved products of the GST::AvrRpt2 reaction (lane 2) were compared with GST::N71 (lane 3), which corresponds to the N-terminal region expected if the cleavage occurs between G71 and G72, and with GST::AC72 (lane 1), which corresponds to the expected C-terminal region. White triangle, Full-length; black triangle, C-terminal region after cleavage; white circle, N-terminal region after cleavage. The positions of molecular markers are indicated on the right.
Therefore, we speculated that a similar cleavage event occurs in the rabbit reticulocyte lysate. The small size of the N-terminal region of the protein that would result from such a cleavage event would have precluded its detection. Therefore, we fused glutathione S-transferase (GST) to the N terminus of AvrRpt2 (GST::AvrRpt2) to make the N-terminal region longer. When GST::AvrRpt2 was expressed in the in vitro translation system, major bands of 60, 35, and 25 kD, corresponding to the expected sizes of the full-length, N-terminal, and C-terminal regions, respectively, were observed (lanes 4–6, white triangle, white circle, and black triangle, respectively). We do not know the nature of the other minor bands in these lanes. With a longer incubation, the intensity of the 60-kD band decreased, whereas that of the 35- and 25-kD bands increased. The ratio of the intensities between the 35- and 25-kD bands is approximately 2:1, which is expected based on the relative Met contents of the predicted N- and C-terminal regions (10:5). Figure 1b shows that the 35- and 25-kD bands comigrate with the bands corresponding to the expected post-cleavage N- and C-terminal products.

To exclude the possibility that the 35- and 25-kD bands were produced by alternative translation initiation or premature translation termination, EDTA was added to the reaction after 30 min to stop protein synthesis, and the accumulation of the 35- and 25-kD bands was observed (Fig. 1a, lanes 7 and 8). A reduction of the 60-kD band intensity and an increase in the 35- and 25-kD band intensities was evident 1 h after addition of EDTA (lane 7), so the 35- and 25-kD bands must result from cleavage rather than translation. We conclude that AvrRpt2 is cleaved in rabbit reticulocyte lysate and that the cleavage site is very close to, if not exactly the same as, the cleavage site observed in Arabidopsis extracts.

Characterization of the Host Cleavage Factor(s)

Next, we tested whether rabbit reticulocyte lysate can cleave Escherichia coli-produced AvrRpt2 protein. C-terminal (his)₆-tagged AvrRpt2 (AvrRpt2::his) was produced in E. coli and purified using nickel-chelated resin. The protein was incubated with rabbit reticulocyte lysate or Arabidopsis protein extracts and subjected to immunoblot analysis using anti-his antibody. As shown in Figure 2a, both reticulocyte lysate (lanes 2 and 3) and Arabidopsis extracts (lanes 4 and 5) cleaved the E. coli-produced AvrRpt2::his protein to generate a C-terminal product of the expected size. We also reproducibly observed a slightly larger band using Arabidopsis extracts, but we do not know the nature of this band. This larger band was not observed in a previous study (Mudgett and Staskawicz, 1999). The AvrRpt2::his protein was also cleaved when incubated with yeast extracts, although the efficiency of cleavage was not very high (lane 8). Therefore, the factor(s) required for AvrRpt2 cleav-
age is present in plant, animal, and fungal cells and must be prevalent among eukaryotes.

Figure 2b shows that the cleaving activities in reticulocyte lysate and Arabidopsis extracts were heat labile (lanes 1, 2, 5, and 6), which suggests that the cleavage was mediated by a protein factor. The activities were only partially blocked by a cocktail containing protease inhibitors of Ser proteases, Cys proteases, metalloproteases, and calpains (lanes 3, 4, 7, and 8).

Because the cleavage factor appears to be prevalent among eukaryotes, we speculated that AvrRpt2 may have a self-cleavage activity that can be activated by a prevalent eukaryotic factor. The Yersinia type III effector protein kinase YpkA is activated by a eukaryote-specific protein, actin (Juris et al., 2000). Although we did not find a putative actin-binding site in AvrRpt2, we tested a commercially available bovine muscle actin fraction for AvrRpt2::his cleavage (lane 2). The results in Figure 2c show that the actin fraction exhibited a weak cleavage activity. Considering that a large amount of actin fraction was added to the reaction (6 μg in a 20-μL reaction; it is unlikely that 2 μL of reticulocyte lysate, which was used in a 20-μL reaction and gave a better cleavage activity, contains a comparable amount of actin) and that it only gave a weak cleavage activity, it is likely that the required eukaryotic factor is not actin itself but is a contaminant in the fraction. The preparation procedure for the actin fraction includes acetone precipitation and extensive dialysis. Therefore, the required factor is unlikely to be a lipid or a small molecule.

Only Two Amino Acid Residues Near the Cleavage Site Are Required for Cleavage

Taking advantage of the quick cleavage assay using rabbit reticulocyte lysate, we tested the cleavage activity of various mutant versions of AvrRpt2. We first tested a series of mutations in the vicinity of the cleavage site. All of the mutants were generated in the GST::AvrRpt2 context. Table I lists the mutants created and the results of the cleavage assay. The results indicate that the only mutants with a detectable cleavage deficiency are GST::F70A and GST::G71A. Therefore, F70 and G71 are the only two amino acid residues near the cleavage site that are required for cleavage.

A Large Part of the C-Terminal Region of AvrRpt2 Is Required for Cleavage

Because the “FG” sequence appears in many protein sequences and because the cleavage is highly specific, these two residues should not be the sole determinants of the cleavage specificity. Therefore, we studied other parts of the protein, beginning with analysis of four deletion mutants, ΔN56, Δ67–76, Δ87–171, and ΔC172, in the GST fusion context. Figure 3a shows that cleavage does not require the N-terminal 56 amino acid residues (GST::ΔN56; lanes 3 and 4). However, not only are the 10 amino acid residues surrounding the cleavage site (GST::Δ67–76; Fig. 3a, lanes 5 and 6) required, but deletions in the C-terminal region of the protein (GST::Δ87–171; Fig. 3a, lanes 7 and 8; GST::ΔC172; Fig. 3a, lanes 9 and 10) also lost cleavage activity.

Because a large part of the C-terminal region of the protein was required for cleavage, we decided to test other mutants within this region. We generated corresponding mutations based on mutants that had been previously identified (Axtell et al., 2001). These mutants were not recognized by RPS2 when directly expressed in plant cells. Only point mutations (GST::C122Y, GST::G141R, and GST::G194E) and the smallest C-terminal deletion mutation (GST::ΔC228) among them were chosen. One mutant gene identified

Table I. Only two amino acid residues near the cleavage site are important for cleavage in rabbit reticulocyte lysate

<table>
<thead>
<tr>
<th>Name</th>
<th>Amino Acid Residues 61 to 82</th>
<th>Protein Accumulation</th>
<th>Cleavage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>GRHKIEVPAFGGWFKKSSKHE</td>
<td>++ +</td>
<td>+</td>
</tr>
<tr>
<td>S78R</td>
<td>GRHKIEVPAFGGWFKKRSSKHE</td>
<td>++ +</td>
<td>+</td>
</tr>
<tr>
<td>K75 mol&amp;K76 mol</td>
<td>GRHKIEVPAFGGWFLKSSKHE</td>
<td>++ +</td>
<td>+</td>
</tr>
<tr>
<td>W73E&amp;F74M&amp;K75Q</td>
<td>GRHKIEVPAFGGEMQKSSKHE</td>
<td>++ +</td>
<td>+</td>
</tr>
<tr>
<td>W73I&amp;F74Y</td>
<td>GRHKIEVPAFGGIYKKSSKHE</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AvrRpt2myr</td>
<td>GRHKIEVPAFGGCV——SSKHE</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G72A</td>
<td>GRHKIEVPAFGAWFKKSSKHE</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G71A</td>
<td>GRHKIEVPAFGAWFKKSSKHE</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>F70A</td>
<td>GRHKIEVPAAGGWFKKSSKHE</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>A69S</td>
<td>GRHKIEVPSFGWFKKSSKHE</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>V67A&amp;P68A</td>
<td>GRHKIEAAFGGWFKKSSKHE</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>I65A&amp;E66A</td>
<td>GRHKIAVPAGGWFKKSSKHE</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H63A&amp;K64A</td>
<td>GRAIEVPAFGGWFKKSSKHE</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G61A&amp;R62A</td>
<td>AAIEVPAFGGWFKKSSKHE</td>
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<td>+</td>
</tr>
</tbody>
</table>
by Axtell et al. (2001) contained two point mutations (C122Y and G131D). We were unable to create G131D by PCR for an unknown reason, and it was omitted from the assay. Three more mutants were included in the assay (GST::(ΔN56) S78R, GST::(ΔN56) E150S, and GST::(ΔN56) P203R&N204S). These mutants were created previously for unrelated purposes and produced in the Δ1–56 context to facilitate PCR. Figure 3b shows that all the mutants created based on the report by Axtell et al. (2001) were inactive in cleavage, whereas the three mutants that we added were active.

**Important Molecular Features of AvrRpt2 for Cleavage Are Similar in the Reticulocyte Lysate and Plant Cells**

To test whether our findings using reticulocyte lysate correlate with events in the plant cell, we expressed AvrRpt2 mutants in Arabidopsis protoplasts. All of the AvrRpt2 derivatives tested were fused at their C termini to green fluorescent protein (GFP) for stability of the proteins in protoplasts (Leister and Katagiri, 2000) and to a FLAG tag for detection by immunoblotting (AvrRpt2::GFPf). As shown in Figure 4a, the only detectable band for the wild-type AvrRpt2::GFPf comigrated with that of the artificially truncated derivative representing the cleaved size (Fig. 4a, lanes 1 and 2). This indicates that, in agreement with our prior observations, wild-type AvrRpt2::GFPf was efficiently cleaved in protoplasts (Leister and Katagiri, 2000). In contrast, C122Y::GFPf, G141R::GFPf, G194E::GFPf, and Δ67–76::GFPf were not cleaved efficiently (Fig. 4a, lanes 3, 5, 7, and 9). Bands that were larger than the expected cleaved size were observed in these lanes (compare with the artificially truncated derivatives; Fig. 4a, lanes 3, 5, 7, and 9). C122Y::GFPf and G141R::GFPf produced multiple bands, including bands similar in size to the cleaved AvrRpt2::GFPf (Fig. 4a, black triangle), in addition to a band of the full-length size (Fig. 4a, white triangle). However, the sizes of the bands similar to the cleaved AvrRpt2::GFPf do not seem to be exactly the same as the size of the cleaved form. In contrast, G194E::GFPf and Δ67–76::GFPf produced bands that are apparently exactly the same as that of the cleaved AvrRpt2::GFPf, in addition to bands of the full-length size. As shown in Figure 4, b and c, the S78R::GFPf, E150S::GFPf, P203R&N204S::GFPf, G72A::GFPf, G71A::GFPf, and F70A::GFPf mutants were tested similarly. S78R::GFPf, E150S::GFPf, and P203R&N204S::GFPf accumulated well, and only the cleaved size products were observed, consistent with the results using reticulocyte lysate. The G72A::GFPf, G71A::GFPf, and F70A::GFPf mutants did not accumulate well. G72A::GFPf and G71A::GFPf were cleaved efficiently, whereas F70A::GFPf was partially cleaved. We did not detect ΔC228::GFPf (data not shown). With the exception of G71A::GFPf, the effects of the mutations on cleavage were similar in Arabidopsis protoplasts and reticulocyte lysate, although cleavage in planta was generally more efficient.

**Figure 4.** Cleavage of AvrRpt2 derivatives in the plant cell. The indicated AvrRpt2::GFPf derivatives were expressed in Arabidopsis rps2 mutant protoplasts. The extracts from the protoplasts were analyzed by immunoblotting as in Figure 2 except that the anti-FLAG antibody was used. AvrRpt2::GFPf was included in every separate experiment as a positive control. a through c represent results from three different experiments. White triangle, Full-length; black triangle, C-terminal region.
Cleavage of AvrRpt2 Is Important for Its Interaction with the Host Plant Cell

We tested whether cleavage is required for recognition by RPS2 using a transient expression assay (Leister et al., 1996). We also tested 71 amino acid residue deletions from the N terminus (∆N71) in AvrRpt2 derivatives that are deficient in cleavage to examine whether the N-terminal region affects recognition by RPS2. The derivatives used in this experiment were all FLAG-tagged (AvrRpt2::f). Figure 5 shows the results of the transient expression assays with selected mutants. In this assay, gene-for-gene interactions were detected as decrease of a reporter gene activity dependent on both avr and R genes (see the result with AvrRpt2::f). For an unknown reason, many of the AvrRpt2 derivatives tested caused a decrease of the reporter gene activity in an RPS2-independent manner. We did not observe further reduction of the reporter activity in an RPS2-dependent manner. This could be correlated with a loss of recognition by RPS2 because G141R::f and G194E::f, which showed the RPS2-independent reporter activity decrease, were previously shown to be inactive when expressed in plant cells (Axtell et al., 2001). In any case, it is clear that these mutants showing RPS2-independent reporter activity decrease differ from wild-type AvrRpt2 in interactions with rps2 mutant cells. All mutations made in the C-terminal region, except S78R::f, led to the malfunction, and the artificial deletion of the N-terminal 71 amino acid residues did not restore the proper function. None of the mutations near the cleavage sites (G72A::f, G71A::f, and F70A::f led to a loss of recognition (data not shown). G72A and G71A were completely cleaved, and F70A was partially cleaved in planta (Fig. 4, b and c, lanes 13, 18, and19). Furthermore, whereas the portion of the protein C-terminal to amino acid residue 82 is sufficient for recognition by RPS2 (Mudgett et al., 2000), the cleavage deficient mutant ∆67–76::f did not function properly. These results suggest that the cleavage is necessary, but not sufficient, for a proper interaction with plant cells lacking RPS2.

Targeting of AvrRpt2 to the Plasma Membrane Does Not Affect Recognition by RPS2

Several P. syringae type III effectors, including AvrRpm1 and AvrB, are myristoylated at their N termini in the plant cell, whereas AvrRpt2 is not. Myristoylation of AvrRpm1 and AvrB is required for their plasma membrane localization and quantitatively important for recognition by the corresponding R gene product, RPM1 (Nimchuk et al., 2000). As shown below, an AvrRpt2::GFP fusion does not appear to be targeted to the plasma membrane. We were interested in testing whether an AvrRpt2 derivative with a canonical myristoylation site can be localized to the plasma membrane and whether such a protein would be recognized more efficiently by RPS2. The observation that F70 and G71 are the only residues in the immediate vicinity of the cleavage site that are essential for cleavage in the reticulocyte lysate (Table I) allowed us to engineer a new post-cleavage N terminus. The amino acid sequence immediately after the cleavage site was changed to the AvrB myristoylation site (AvrRpt2myr: from G(72) WFKKKSSKH to G(72) CVSSKH). AvrRpt2myr::GFPf was cleaved efficiently in the plant cell (Fig. 4, lane 10). The localization of AvrRpt2myr::GFPf was tested by transient expression of the construct in Arabidopsis protoplasts followed by observation under a laser confocal microscope. Figure 6 shows that AvrRpt2myr::GFPf, like AvrB::GFP, appears to be largely localized in the plasma membrane (Fig. 6, c and d). The membrane-localization of AvrR::GFP was reported previously (Nimchuk et al., 2000). The localization of AvrRpt2myr::GFPf differs from that of
AvrRpt2::GFPf and GFP::f, which are both distributed in the cytosol with a tendency to accumulate in the nucleus (Fig. 6, a and b). These results suggest that AvrRpt2myr::GFPf is myristoylated and that the myristoylation targets the protein to the plasma membrane. When RPS2-mediated recognition of AvrRpt2myr::f was tested using the transient expression assay, no significant difference compared with AvrRpt2::f was observed (Fig. 5). It appears that forced targeting of AvrRpt2 to the plasma membrane does not improve recognition by RPS2.

Cleavage Is Important for AvrRpt2 Localization in the Host Cell

Does cleavage target AvrRpt2 to a specific site in the host cell? This question can be addressed by observing where AvrRpt2 would be localized in the plant cell if the N-terminal region were not removed. We tested localization of several cleavage-deficient mutants as C-terminal GFP fusions (Fig. 7). In contrast to AvrRpt2::GFPf, which was localized in the cytosol and nucleus and excluded from the chloroplast (Fig. 7b), C122Y::GFPf was mainly localized in the chloroplast (Fig. 7c), whereas Δ67–76::GFPf was strongly localized in the nucleus (Fig. 7d). G141R::GFPf, G194E::GFPf, and ΔC228::GFPf did not accumulate to levels sufficient for observation (data not shown). The localization of S78R::GFPf, E150S::GFPf, P203R&N204S::GFPf, and G72A::GFPf was indistinguishable from that of AvrRpt2::GFPf (data not shown). F70A::GFPf was often detected in the chloroplast in addition to the cytosol and the nucleus (Fig. 7e). The localization of G71A::GFPf was similar to that of AvrRpt2::GFPf, but it was occasionally detected in chloroplasts (data not shown). Considering the low efficiency for detection of GFP in the chloroplast (Reed et al., 2001), this mutant protein may be partitioned in the chloroplast at a significant level. In summary, there is a good correlation between cleavability and subcellular localization similar to that of the wild-type AvrRpt2::GFPf protein.

DISCUSSION

The AvrRpt2 protein is properly cleaved in plant, animal, and yeast cell extracts. Two amino acid residues immediately preceding the cleavage site and a large part of the C-terminal region of AvrRpt2 were required for cleavage. Most of these features of AvrRpt2 were also important when AvrRpt2 was cleaved inside plant cells. Cleavage deficiency altered the way AvrRpt2 interacts with rps2 plant cells. Cleavage also appears to be required for the proper localization of the C-terminal region of the protein in the host cell.

What Is the AvrRpt2 Cleavage Mechanism?

The factor(s) required for AvrRpt2 cleavage is prevalent among eukaryotes. It is unlikely that the specificity of highly selective proteases is conserved among plants, animals, and fungi. In addition, incubation of the eukaryotic cell extracts with a cocktail of protease inhibitors did not exhibit a strong effect on cleavage of AvrRpt2. Therefore, it is likely that the eukaryotic factor is not a specific protease. A large part of the C-terminal region of AvrRpt2 contributes to cleavage. Cleavage specificity could be determined by a protein–protein interaction between relatively large peptides. Alternatively, a large peptide could have self-cleaving activity. It has been shown that AvrPphB is homologous to cysteine proteases and that amino acid residues conserved among cysteine proteases are required for self-cleavage of AvrPphB (Shao et al., 2002). Although we failed to detect any appreciable protease homology in AvrRpt2, one attractive model is that AvrRpt2 has self-cleaving activity that is activated by a eukaryotic factor.
If AvrRpt2 cleaves itself, the reaction appears to occur in an intramolecular manner rather than an intermolecular manner. We used reticulocyte lysate to coexpress each of the cleavage-deficient mutants with mutations in the C-terminal region in the GST fusion context together with the cleavage site mutant GST::Δ67–76. We did not detect “trans”-cleavage of any of the cleavage-deficient mutants (data not shown).

AvrRpt2 may still be able to cleave other proteins, and cleavage of other plant proteins may be central in how AvrRpt2 affects plant defense. Recently, it was reported that RIN4, a protein involved in RPS2- and RPM1-mediated recognition, rapidly disappears in the presence of AvrRpt2 (Axtell and Staskawicz, 2003; Mackey et al., 2003). The AvrRpt2 mutants that we demonstrated to be cleavage deficient, C122Y, G141R, and G194E, did not cause the disappearance of RIN4 (Axtell and Staskawicz, 2003). It will be interesting to see whether AvrRpt2 in the presence of an appropriate eukaryotic factor can directly digest RIN4.

Identification of the required eukaryotic factor will provide insight into how the C-terminal region of AvrRpt2 functions in cleavage. Our characterization of the factor indicates that it is heat labile, acetone precipitable, and not removed by extensive dialysis. These observations strongly suggest that the factor is a protein.

Cleavage Is Important for Proper Interaction with Host Cells

We characterized three different classes of AvrRpt2 loss-of-function mutants. The first class contains E150S and P203R\&N204S, which are cleaved well in the plant cell but exhibit an altered interaction with rps2 plant cells in the transient assay. The second class is composed of Δ67–76, which is deficient in both cleavage and interaction with rps2 plant cells, but appears to recover proper interactions with both RPS2 and rps2 plant cells when the N-terminal region is artificially removed (the portion C-terminal to amino acid residue 82 retains the ability to be recognized by RPS2 [Mudgett et al., 2000]). The third class contains C122Y, G141R, and G194E, which are deficient in both cleavage and interaction with rps2 plant cells, and in which artificial removal of the N-terminal region does not restore proper interactions with plant cells. Therefore, these residues are required for both cleavage and proper interactions with plant cells. All of these classes of mutants are consistent with the notion that the cleavage is necessary but not sufficient for proper AvrRpt2 function.
This notion suggests that the N-terminal region of the protein has an adverse effect on the AvrRpt2 function because the C-terminal region of AvrRpt2 is the effector part of the protein. As discussed below, the adverse effect of the N-terminal region may be related to improper localization of the C-terminal region. Alternatively, cleavage may lead to a conformational change of the C-terminal region of the protein.

**Where Is the Site of Action for AvrRpt2 in the Host Cell?**

In general, defense responses induced by the AvrRpt2-RPS2 interaction are weaker/slower than those induced by the AvrRpm1/AvrB-RPM1 interaction, although the RPS2- and RPM1-mediated pathogen recognition mechanisms appear to share components (Ritter and Dangl, 1996; Leister and Katagiri, 2000; Axtell and Staskawicz, 2003; Mackey et al., 2003). The myristoylation and consequential plasma membrane-localization of AvrRpm1 and AvrB quantitatively contributes to the ability of those proteins to be recognized by RPM1 (Nimchuk et al., 2000). Therefore, the RPM1-mediated recognition mechanism is thought to operate at the plasma membrane (Boyce et al., 1998). Does the RPS2-mediated recognition mechanism also operate at the plasma membrane? It was of interest to see whether myristoylation and membrane localization of AvrRpt2myr would enhance RPS2 recognition. We did not observe any appreciable increase in the ability of AvrRpt2myr to be recognized by RPS2 (Fig. 5). Therefore, our observations do not appear to support the idea that RPS2 recognition occurs at the plasma membrane.

Axtell and Staskawicz (2003) recently demonstrated that when AvrRpt2 is expressed at a low level in Arabidopsis, it is associated with the membrane fraction. This and our observations appear to contradict each other. However, they are easy to reconcile if we assume that the membrane association of AvrRpt2 is mediated by a limited number of specific binding sites in the membrane. In this way, when AvrRpt2 is overexpressed, as in our experimental setup, the AvrRpt2-binding sites in the membrane would be quickly saturated, and the majority of AvrRpt2 protein would remain in the cytosol. Forming a complex with the binding site in the membrane could be important for AvrRpt2 recognition mediated by RPS2. If this is the case, forced targeting of excessive AvrRpt2 to the membrane by AvrRpt2myr would not significantly affect the level of the interaction between AvrRpt2 and RPS2.

**Is Cleavage a Mechanism to Redirect an Avr Protein after Translocation?**

Various N-terminal signal peptides are known to target proteins in the eukaryotic cell, such as target-

![Diagram](https://example.com/diagram.png)
signal peptides are similar to plastid-targeting signal peptides. At least one cleavage-deficient mutant (C122Y::GFP) was mainly localized in the chloroplast. Two mutants with limited cleavability (G71A::GFP and F70A::GFP) appeared partially localized to the chloroplast. Another cleavage mutant (∆67-76::GFP) was localized in the nucleus. It is not clear why this mutant is localized in the nucleus. We did not inadvertently create an obvious nuclear localization signal when we made the mutation. In any case, the inappropriate localization of the cleavage-deficient mutant proteins suggests that cleavage is crucial for proper localization of the C-terminal region of AvrRpt2.

Many type III secretion signal peptides of P. syringae are similar to plastid-targeting signal peptides, and it has been suggested that the site of action for many type III effectors may be more broadly used by type III effectors. Cleavage of the N-terminal region of AvrRpt2 appears to remove the putative signal for plastid targeting. Some other Avr proteins are myristoylated at their N termini, leading to membrane localization (Nimchuk et al., 2000). It is possible that these proteins would otherwise be targeted to the plastid, which is not their presumed site of action. AvrPpHB is both cleaved and myristoylated (Nimchuk et al., 2000; Shao et al., 2002). These observations raise questions such as how common it is for cleavage of N-terminal signal sequences to target type III effectors for redirection in host cells. Because signal peptides for various target sites in eukaryotic cells are commonly located at the N terminus of a protein (Emanuelsson and von Heijne, 2001), the process of exposing a new targeting signal peptide by cleaving off the N-terminal region of a protein could enable the targeting of proteins to a wide variety of intracellular sites in the host cell. This type of mechanism may be more broadly used by type III effectors than we currently realize.

MATERIALS AND METHODS

Plant Materials

Three types of Arabidopsis plants were used, all are the Columbia ecotype: RPM1 RPS2 (Col-0, wild type), RPM1 rps2 (rps2-101C1 Mindrinos et al., 1994), and rpm1 RPS2 (rps3-1; Innes et al., 1993). Plants were grown in Metro-Mix 200 soil (The Scotts Company, Marysville, OH) at 22°C and 65% relative humidity with a 12-h-light/12-h-dark cycle in approximately 80% relative humidity with a 12-h-light/12-h-dark cycle in environment-controlled growth chambers.

Plasmids

The pEX4avr and pEXavrRpt2 constructs have been described previously (Mindrinos et al., 1994; Leister et al., 1996; Tao et al., 2000). The PCR products for GST::AvrRpt2 and its derivatives were amplified from a GST::AvrRpt2::MaIE construct made in the pGEX-5X-3 vector (Pharmacia, Uppsala). Note that the MaIE part was not included in the PCR products and is irrelevant to this study. The junction sequence between GST and AvrRpt2 in the construct is GGATCCCCATGG (GGATCC is the BamHI site in the vector, and ATG is the start codon for AvrRpt2). The pET-avrRpt2-his construct has been described (Wu et al., 2003). AvrRpt2 derivatives were amplified by PCR from the templates used in the in vitro transcription/translation system with primers that incorporated a BamHI site at the 5′ end of the gene and a KpnI site at the 3′ end. The products were inserted into the vector pKEx-CFL (for in-frame C-terminal fusion to the FLAG epitope tag) or pKEx-GFPFL (for in-frame C-terminal fusion to GFP and the FLAG epitope tag). Both of these vectors are derived from pKEx4avr, with the FLAG and GFPFL inserts cloned between KpnI and NotI. In pKEx-CFL, two amino acids GT were added between the last amino acid of AvrRpt2 and the FLAG tag. In pKEx-GFPFL, two amino acids GT were added between the last amino acid of AvrRpt2 and GFP. Details of the cloning procedures are available upon request.

In Vitro Translation with Rabbit Reticulocyte Lysate

The templates for in vitro transcription were generated by PCR SOEing (Horton et al., 1990) in which the final 3′ primer contains the T7 promoter sequence. AVRT3 was used as the final 3′ primer. The primer pairs used in SOEing to create mutations are supplied in supplemental materials. Three mutant versions were cloned into a plasmid vector first for the purpose of unrelated experiments and then amplified from the plasmids. For in vitro translation coupled with in vitro transcription, the TNT T7 Quick for PCR DNA kit (Promega, Madison, WI) was used according to the supplier’s instructions. The proteins were labeled with [35S]Met and resolved by denaturing PAGE. The gels were dried and subjected to phosphor imaging (Molecular Dynamics, Sunnyvale, CA).

Cleavage of Escherichia coli Produced Protein

Production and purification of AvrRpt2 from E. coli was performed as described (Wu et al., 2003). For preparation of Arabidopsis extracts, one well-expanded leaf from a 4-week-old Col-0 wild-type plant was ground in 100 μL of extraction buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 0.1% [v/v] Triton X-100), and the extracts were collected after centrifugation at 16,000g for 2 min at 4°C. Yeast cell extracts were prepared from Brewer’s yeast (Saccharomyces cerevisiae strain NAV203a) as follows. Yeast cells were grown in 5 mL of yeast peptide dextrose medium overnight at 30°C. Cells were harvested and disrupted by vortexing in breaking buffer (0.1 mL Tris-HCl, pH 8, 20% [w/v] glycerol, and 1 mL diethiothreitol) with glass beads (PN. G8772, Sigma-Aldrich, St. Louis). The cleared lysate was used as the yeast extract. The E. coli-produced AvrRpt2 protein (2 μg) was incubated with either 2 μL of T7 TNT lysate (rabbit reticulocyte lysate), 3 μL of Arabidopsis extracts, 6 μL of yeast cell extracts, or 5 μL of 2 mg mL−1 bovine muscle actin (PN. A3653, Sigma-Aldrich) in total volume of 20 μL in 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 5 mM MgCl2, at 30°C for the indicated duration of time. Then proteins in 3 μL of the mixture were resolved by denaturing PAGE and blotted onto a nitrocellulose membrane by semidry blotting (Bio-Rad Laboratories, Hercules, CA). (His)6-tagged proteins were detected by immunoblot analysis using the anti-(his)6 antibody (Qiagen USA, Valencia, CA) as the primary antibody and the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) for detection, according to the manufacturer’s instructions. The chemiluminescent image was captured by a ChemiImager (Alpha Innotech, San Leandro, CA).

AvrRpt2 Expression in Protoplasts

GFP and FLAG-tagged AvrRpt2 derivatives were expressed in Arabidopsis protoplasts by transient expression as previously described (Leister and Katagiri, 2000). Approximately 1.2 × 105 protoplasts were lysed in 30 μL of lysis solution (1 × Tris-buffered saline, 0.5% [w/v] sodium deoxycholate, and 1% [v/v] IGEPA). After an 18-h incubation, the lysate was subjected to immunoblot analysis as described above, except that the anti-FLAG M2 antibody (Sigma-Aldrich) was used as the primary antibody.

Biologic Transient Assay for RPS2 Recognition of AvrRpt2

Biotic transient assay for RPS2 recognition of AvrRpt2 was performed as previously described (Leister et al., 1996).
Microscopy
Confocal microscopy was performed using a Fluoview 5 confocal microscope (FV5-LSM, Olympus, Melville, NY).

ACKNOWLEDGMENTS
We thank Jane Glazebrook for critical reading of the manuscript and Kim Campbell for helping to care for the plants.

Received April 24, 2003; returned for revision June 1, 2003; accepted August 4, 2003.

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