A Role in Immunity for Arabidopsis Cysteine Protease RD21, the Ortholog of the Tomato Immune Protease C14

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Abstract

Secrated papain-like Cys proteases are important players in plant immunity. We previously reported that the C14 protease of tomato is targeted by cystatin-like EPIC proteins that are secreted by the oomycete pathogen Phytophthora infestans (Pinf) during infection. C14 has been under diversifying selection in wild potato species coevolving with Pinf and reduced C14 levels result in enhanced susceptibility for Pinf. Here, we investigated the role C14-EPIC-like interactions in the natural pathosystem of Arabidopsis with the oomycete pathogen Hyaloperonospora arabidopsidis (Hpa). In contrast to the Pinf-solanaceae pathosystem, the C14 orthologous protease of Arabidopsis, RD21, does not evolve under diversifying selection in Arabidopsis, and rd21 null mutants do not show phenotypes upon compatible and incompatible Hpa interactions, despite the evident lack of a major leaf protease. Hpa isolates express highly conserved EPIC-like proteins during infections, but it is unknown if these HpaEPICs can inhibit RD21 and one of these HpaEPICs even lacks the canonical cystatin motifs. The rd21 mutants are unaffected in compatible and incompatible interactions with Pseudomonas syringae pv. tomato, but are significantly more susceptible for the necrotrophic fungal pathogen Botrytis cinerea, demonstrating that RD21 provides immunity to a necrotrophic pathogen.

Introduction

Papain-like Cys proteases (PLCPs) are important players in plant immunity. PLCPs participate in immune responses and are targeted by pathogen-derived inhibitors. Cathepsin-B, for example, is a PLCP necessary to mount the full hypersensitive response (HR) in Nicotiana benthamiana [1]. Furthermore, RCR3 is a PLCP of tomato required to trigger HR in tomato plants carrying the Cf-2 resistance gene, when infected by the fungal tomato pathogen Cladosporium fulvum producing Avr2 [2]. Similarly, PLCP RD19 is required in Arabidopsis for RRS1-R-mediated resistance against the bacterial pathogen Ralstonia solanacearum producing effector PopP2 [3].

A crucial role of PLCPs in disease immunity is also indicated by the observation that many pathogens produce effectors that manipulate these observations. For instance, the PopP2 effector interacts with RD19 and causes the protease to relocalize from vesicles to the nucleus [3]. Furthermore, RCR3 is inhibited by Avr2, a small secreted protein produced by C. fulvum during infection of tomato [4]. Avr2 also inhibits PIP1, a closely related PLCP from tomato [5,6]. Similarly, RCR3 and PIP1 are also inhibited by EPIC1 and EPIC2B, two closely related cystatin-like proteins, produced by the oomycete pathogen Phytophthora infestans during infection [7,8].

We recently demonstrated that apart from RCR3 and PIP1, EPICs have an even higher affinity to the C14 proteases of tomato and potato [9]. This C14 protease has also been named TDI-65, CYP1 or SENU2 [10–13]. Silencing of a C14-like protease in N. benthamiana increases susceptibility for P. infestans, showing that this C14-like protease is indeed a bona fide target during infection [9]. Interestingly, these effector-targeted proteases have been under diversifying selection, indicating that they are part of an ongoing arms race at the plant-pathogen interface. Both RCR3 and PIP1, for example, carry a significant number of variant amino acids at the surface where Avr2 or EPICs are presumed to interact, and at least one of these variant residues can suppress inhibition by Avr2 [5]. Furthermore, C14 exhibits a pattern of diversifying selection in wild potato, which are the natural hosts of P. infestans [9], illustrating that diversifying selection correlates with coevolving plant-pathogen interactions. Interestingly, some of the variant residues in potato C14 seem to locate at the predicted EPIC interaction surface in 3D modelling studies [14]. Similar traces of molecular arms races have been described for other enzyme-inhibitor interactions at the plant-pathogen interface [15].

In this study, we studied the role of the C14-like protease in Arabidopsis thaliana and investigated if an interaction orthologous to EPIC-C14 would exist in the model pathosystem Hyaloperonospora arabidopsidis (Hpa) and A. thaliana. Hpa is an obligate biotrophic oomycete pathogen that causes downy mildew on Arabidopsis [16]. As a natural pathogen, Hpa has co-evolved with Arabidopsis and exists as ecotype-specific isolates [17]. The genome sequence of Hpa revealed the loss of genes that encode hydrolytic enzymes or proteins triggering host cell death, consistent with the lifestyle of Hpa as a stealthy pathogen that does not kill host cells [18]. During infection, Hpa grows in the leaf apoplast and employs haustoria to feed from parenchyma cells [16]. Since C. fulvum and P. infestans
both colonize the host apoplast and encounter similar apoplastic Cys proteases, we investigated the role of EPIC-C14-like interactions in the Hpa-Arabidopsis pathosystem. In addition, we tested the role of the C14-like protease in other pathogensof Arabidopsis.

**Results**

Arabidopsis RD21 is the ortholog of tomato C14

To identify the ortholog of tomato C14 in the Arabidopsis genome, we performed a BLASTP search of the C14 protein sequence to the Arabidopsis protein TAIR10 database and found that RD21 (encoded by RD21A gene, At1g47120) is the closest ortholog of tomato C14 (E-value e-162). Phylogenetic analysis of the pr- and protease domains of Arabidopsis PLCPs demonstrate that not only the presence of the granulin domain, but also the proprotease of RD21 is most closely related to tomato C14 (Figure 1A). The protein alignment shows that RD21 and C14 are 58%, 76% and 61% identical in the prodomain, protease domain and granulin domain, respectively (Figure 1B). Similar to tomato C14, Arabidopsis RD21A is highly expressed in leaf tissue and is upregulated during senescence [19]. The closest homolog of RD21 in Arabidopsis is RD21B (At3g13060), which also carries a granulin domain but the corresponding gene is transcriptionally less expressed in leaves when compared to RD21A (Figure 1C) and has not been detected in leaf extracts by protease activity profiling [6,20]. We therefore focussed our studies on RD21 (At1g47120) as the ortholog of tomato C14.

To evaluate whether selective forces are acting on the RD21A gene similar to potato C14, we performed population genetic analyses with 80 RD21A alleles extracted from genome sequences of 80 Arabidopsis thaliana accessions [21]. When combined, the 80 RD21A sequences contain 30 single nucleotide polymorphisms (SNPs), which affect 27 codons in the 1389 bp long open reading frame of RD21A (Figure 1C and Figure S2). Only ten SNPs affect the encoded amino acid but most of these variant residues are similar (Figure 1C and Figure S2). The nonsynonymous nucleotide diversity (\( \pi_s = 0.00041 \)) is lower when compared to the synonymous nucleotide diversity (\( \pi_s = 0.0054 \)) and the \( \pi_s/\pi_s \) ratio is 0.077, which indicates that RD21A is under purifying selection.

We also calculated Tajima’s D (\( D_T \)) and Fu and Li’s D (\( D_F \)), which we used to evaluate the site frequency spectrum of SNPs and can indicate selective pressures or demographic effects acting on RD21A. Both values are significantly negative compared to the expectation under neutrality (\( D_T = -1.912, P<0.05 \) and \( D_F = -3.22, P<0.02 \)). This negative deviation from neutrality is caused by an excess of SNPs that occur with low frequencies (Figure 1C and Figure S3). In fact, the majority (seven of the ten) of the nonsynonymous mutations occur in only one RD21A sequence (Figure 1C and Figure S3). Even though these data do not exclude that the observed pattern is caused by demography, these findings indicate that natural variation at the RD21A gene is not maintained by balancing selection.

To extend the analysis of SNPs we also included the RD21-encoding sequence of Arabidopsis lyrata (gi|297852301). A. lyrata RD21A differs at 54 SNPs from A. thaliana RD21A Col-0, which affect 52 codons (Figure S4). Importantly, 39 of these altered codons encode an invariant amino acid, and only 5 codons affect the amino acid code significantly, of which four are located in the signal peptide and prodomain (Figure S4). The ratio of nonsynonymous to synonymous divergence Ks/K, of this comparison is 0.007. This result confirms the conservation of RD21, even in comparisons between species.

EPIC orthologs are present in Hpa isolate Emoy2

To identify EPIC orthologs from Hpa, we performed a BLASTP search with PToEPICs on the genome database of Hpa isolate Emoy2 at the VBI Microbial Database [18] and identified three EPIC-like proteins, called HpaEPIC-A, -B and -C. Phylogenetic analysis of the Pto and Hpa cystatins showed that HpaEPIC-A is most closely related to Pto EPIC4, and that HpaEPIC-B and -C are more similar to Pto EPIC1, -2B and -3 (Figure 2A). Thus, HpaEPIC-B and -C are the most likely orthologs of PtoEPIC1 and -2B, and were selected for further studies.

Alignment of HpaEPIC-B and -C with PtoEPIC1, -2B and -3 shows that HpaEPIC-B contains the classical conserved cystatin features: a conserved glycine in the N-terminus; a conserved QxVxG motif in the middle, and a conserved tryptophane in the C-terminus (Figure 2B). Thus, HpaEPIC-B is likely to have cystatin activity. In contrast, HpaEPIC-C carries a QxVxG instead of the QxVxG motif, and lacks the conserved glycine and tryptophane in the N- and C-termini, respectively (Figure 2B).

The striking disruption of the three key cystatin motifs is likely to render HpaEPIC-C inactive as a Cys protease inhibitor.

HpaEPICs are conserved and expressed in other Hpa isolates

To investigate if HpaEPIC-B and -C are also present in other Hpa isolates and to determine natural variation in these genes, HpaEPIC-B and -C were amplified and sequenced from various isolates using PCR on genomic DNA, isolated from infected plants. Isolates Noco2 and Emwa1 contain an HpaEPIC-B that is identical to that of the Emoy2 isolate, whereas the HpaEPIC-B of isolates Cala2, Make9 and Waco3 carry a single nucleotide polymorphism resulting in a V131I substitution (Figure 2C and Figure S5). HpaEPIC-C of Emwa1 is identical to that of Emoy2, and differs at three sites from that of the Noco2 isolate, causing two silent mutations and one amino acid substitution (E134G), located in the C-terminus of HpaEPIC-C (Figure 2C and Figure S6). Importantly, the disrupted cystatin motifs in HpaEPIC-C are conserved among the Hpa isolates. The low diversity in HpaEPICs suggests strong conservation of these proteins in the investigated Hpa isolates.

To detect the expression of HpaEPICs during infection, RNA was isolated from Arabidopsis plants infected with isolates Emwa2, Noco2 and Cala2, and used as template for RT-PCR using HpaEPIC-specific primers. Both HpaEPIC-B and HpaEPICB-C were detected in the infected material and not in non-infected plants (Figure 2D), indicating that both genes are expressed during infection. Poor amplification of HpaEPIC-C suggests that transcript levels of this gene are relatively low when compared to that of HpaEPIC-B.

Characterization of Arabidopsis rd21 mutant lines

To investigate the role of RD21A in the Arabidopsis-Hpa interaction, we characterized the rd21-1 knockout line [22], and selected a second, independent knockout line, rd21-2. The rd21-1 and rd21-2 lines carry T-DNA insertions in the third and first introns of RD21A, respectively (Figure 3A). The RD21A gene encodes a pre-pro-protease carrying a C-terminal granulin domain (Figure 3B). RD21 matures in several steps, resulting in a 40 kDa intermediate RD21 (iRD21) and 30 kDa mature RD21 (mRD21) [23]. Both iRD21 and mRD21 are active proteases and only iRD21 carries the C-terminal granulin domain. Western blot analysis of leaf extracts from these plants shows signals at 30 and 40 kDa, representing mRD21 and iRD21, respectively (Figure 3C, [23]). Both 30 and 40 kDa RD21 signals are absent
in the rd21-1 and rd21-2 mutants (Figure 3C and Figure S7), demonstrating that these mutants are null mutants. Protease activity profiling using the biotinylated DCG-04 probe [20,24] shows that the 40 kDa signal and the upper 30 kDa signals in the activity profile are absent in the rd21 mutants (Figure 3C and Figure S7), indicating that RD21 is one of the major Cys protease in leaf extracts and that no compensatory Cys protease activities are detectable in these knock-out lines. Importantly, despite severe defects in Cys protease activities, no macroscopic phenotypes were observed for the rd21 mutants grown in the greenhouse.

Lack of RD21 does not affect Hpa infections

To investigate the role of RD21 in the Hpa-Arabidopsis interactions, (mutant) Arabidopsis plants were infected with Hpa isolate Noco2, which is virulent on Arabidopsis ecotype Col-0. Unexpectedly, infection on rd21 lines occurs indistinguishable

Figure 1. Arabidopsis RD21 is the ortholog of tomato C14. A, Phylogenetic tree of protein sequences of Arabidopsis papain-like Cys proteases (black) and tomato C14, RC3 and PIP1 (grey). This tree was obtained using the neighbor joining method with bootstrap support. Arabidopsis CTB3 was used as outgroup and CTB1 and CTB2 were not included in the analysis. B, Alignment of Arabidopsis RD21 with tomato C14 protein sequences. *, catalytic residues; #, putative glycosylation site; C, cysteine; =>, transition between protein domains. C, Natural variation of RD21A in 80 Arabidopsis accessions. The position of the single nucleotide polymorphisms (SNPs) in the RD21 open reading frame are indicated on top, and their distribution in the ecotype sequences are indicated in the matrix. Nonsynonymous SNPs are indicated in black and synonymous SNPs in grey, respectively. The affected amino acids are summarized on the bottom. See Figure S2 for more details.

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from that of the Col-0 control and spore counts at 7 days-post-inoculation (dpi) repeatedly revealed no significant differences of infection of \textit{rd21} mutant lines when compared to the Col-0 control (Figure 4A). In contrast, \textit{eds1-2} mutant facilitates significant growth of \textit{Hpa} \textit{Noco2}, as reported previously (Figure 4A).

Since RD21 is an abundant vacuolar protease [23,25], and the vacuolar content is released into the apoplast during the hypersensitive response (HR) [26], we anticipated that RD21 might be acting during incompatible interactions. We therefore infected the (mutant) plants with \textit{Hpa} isolate \textit{Emwa1}, which triggers \textit{RPP4}-dependent HR on Col-0 [17]. Spore counts at 7 dpi showed no significant differences between \textit{rd21} mutant plants and \textit{Col-0} wild-type plants, whereas the controls (\textit{eds1-2} and ecotype \textit{Ws}), are susceptible (Figure 4B). Taken together, we observed no significant phenotype for \textit{rd21} mutants upon infection with virulent and avirulent \textit{Hpa} isolates.

\textbf{Mutant \textit{rd21} plants show no phenotype in \textit{P. syringae} infections}

Having the \textit{rd21} mutant lines at hand, we decided to test if the plants show any phenotype with other pathogens. We first tested infection by the virulent \textit{Pseudomonas syringae} \textit{pv. tomato DC3000} (\textit{Pto DC3000}). The \textit{sid2-2} mutant line was included in this assay as positive control since this line supports enhanced bacterial growth.
due to its incapability to accumulate inducible salicylic acid (SA) [27]. The rd21 mutant supports PtoDC3000 growth undistinguishable from wild-type plants, whereas the sid2-2 mutant is more susceptible (Figure 5A).

To test if RD21 plays a role during infection with avirulent bacteria, we challenged the (mutant) plants with PtoDC3000 expressing avrRpm1. Again, bacterial populations were indistinguishable between rd21 mutant and wild-type lines, whereas bacterial growth was enhanced in the sid2-2 mutant (Figure 5B). In conclusion, no bacterial growth phenotypes were observed for rd21 mutant lines upon infection with virulent or avirulent PtoDC3000 bacteria.

Lack of RD21 increases susceptibility for *Botrytis cinerea*

To test if RD21 is involved in immunity against necrotrophic pathogens, we performed infection assays with the necrotrophic pathogen *Botrytis cinerea*. The pad3 mutant, which is deficient in camalexin production [28], was included in these assays as a positive control for enhanced susceptibility. Leaves of (mutant) plants were inoculated with spore-containing droplets and scored for expanding lesions at 5–7 dpi. Importantly, both rd21 mutants displayed more expanding lesions when compared to the wild-type control plants (Figure 6A). Quantification of the frequency of expanding lesions show a significantly enhanced frequency of lesion expansion for rd21 mutant plants when compared to wild-type controls, though still reduced when compared to the pad3 mutant (Figure 6B). These data demonstrate that RD21 contributes to immunity to *B. cinerea* infection.

**Discussion**

In this study, we set out to investigate the role of EPIC-C14 interactions in the *Hpa*-Arabidopsis pathosystem. Our journey identified Arabidopsis RD21 as the likely C14 ortholog, and EPIC homologs from *Hpa*. However, in contrast to earlier studies in the *Pto*-solanaceae pathosystem, RD21 is conserved in *A. thaliana* and rd21 mutants are not affected in compatible and incompatible interactions with *Hpa*, despite the evident lack of Cys protease activities in the leaf. However, the rd21 knock-outs are significantly more susceptible for *Botrytis cinerea*, and not for virulent and avirulent strains of PtoDC3000. These data demonstrate that RD21 is involved in immunity to the necrotrophic fungal pathogen *Botrytis cinerea*.

Are there EPIC-C14-like interactions in the *Hpa*-Arabidopsis pathosystem?

At this stage, it remains unknown if EPIC-C14-like interactions might exist in the *Hpa*-Arabidopsis pathosystem. We believe we have identified the right EPIC and C14 orthologs from *Hpa* and Arabidopsis, respectively. HpaEPIC-B and HpaEPIC-C are both expressed during infection and are likely secreted into the apoplast. Arabidopsis RD21 is the closest ortholog of C14 and is a very abundant Cys protease in leaves.

Despite the presence of EPIC and C14 orthologs, our further studies do not support the role of *Hpa*EPIC-RD21 interactions. First, *Hpa*EPIC-C lacks key cystatin-like motifs and is therefore unlikely to be an inhibitor of Cys proteases. The specific disruption of key cystatin motifs and the fact that this is conserved in different *Hpa* isolates is remarkable, and indicates that a different role has
evolved for HpaEPIC-C. HpaEPIC-B, however, carries all essential cystatin-like motifs and is therefore a likely Cys protease inhibitor.

A second aspect is that we expected the Hpa-Arabidopsis pathosystem to be co-evolving in nature, similar to the Pinf-potato pathosystem, where the pattern of diversifying selection at the C14 gene could have been caused by coevolution with the corresponding effectors [9]. We found, however, that RD21 is conserved in A. thaliana. This situation is similar to that of C14 in tomato, which is nevertheless targeted by PspEPICs [9]. Diversifying selection may not be essential for antagonistic protein-protein interactions at the plant-pathogen interface.

There are different explanations for the observed conservative selection of RD21 in A. thaliana. Nucleotide diversity in A. thaliana is generally low and it has been suggested that this overall pattern is due to purifying selection acting on the A. thaliana genome and/or a recent population expansion [29–32]. However, the pattern of strong protein conservation we observed for the RD21A gene is slightly stronger than this overall pattern in the species, suggesting that RD21A is under purifying selection. Some caution is needed interpreting these data since we did not calculate the variation in the genomic background for the same dataset. Nevertheless, we can conclude that selective pressures acting on potato C14 and Arabidopsis RD21A differ substantially. These data indicate that RD21 is strongly conserved because of a pivotal process in plants, that remains to be identified. Meanwhile, it is possible that RD21 is targeted by pathogen-derived effectors, but environmental, spatial or temporal conditions of host-pathogen interactions do not promote rapid diversification of this protease beyond the internal constraints on RD21 function.

A third distinct difference to the EPIC-C14 interaction is that the rd21 mutant plants behave indistinguishable from wild-type plants in compatible and incompatible interactions with Hpa. This is in strong contrast to C14-silenced N. benthamiana plants, which are significantly more susceptible for infection by P. infestans [9]. The absence of a phenotype in Hpa assays is remarkable since the rd21 mutant plants are clearly impaired in Cys protease activities. However, the absence of phenotypes for rd21 mutants does not imply that the HpaEPIC-RD21 interactions do not exist. In fact, effective inhibition of RD21 by HpaEPICs or other ways of manipulation might rather make Hpa insensitive for the presence or absence of RD21. In this case, the role of RD21 would only be revealed in the Hpa-Arabidopsis interactions in the absence of Hpa-derived RD21 inhibitors. This paradox is similar to that of showing the role of PAMP-triggered immunity (PTI) when pathogens have evolved mechanism to suppress PTI [33].

There are two more issues unresolved for possible HpaEPIC-RD21 interactions. First, it is unknown if HpaEPICs and RD21

Figure 5. Mutant rd21 lines are not compromised in interactions with PstDC3000. A, Compatible Pst interactions. (Mutant) Arabidopsis plants were spray-inoculated with PstDC3000 (vir) and bacterial populations were measured at 0 and 3 dpi. Error bars represent SD of 5 independent bacterial extractions. This experiment was repeated three times with similar results. B, Incompatible Pst interactions. (Mutant) Arabidopsis plants were spray-inoculated with PstDC3000 avrRpm1 (avr) and bacterial populations were measured at 0 and 3 dpi. Error bars represent SD of 5 independent bacterial extractions. This experiment was repeated three times with similar results.

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Figure 6. Mutant rd21 lines have increased susceptibility for Botrytis cinerea. A, Infection of (mutant) Arabidopsis plants with Bc. Representative photographs were taken at six days after droplet inoculation. B, Frequency of lesion expansion. (Mutant) Arabidopsis plants were droplet-inoculated with spores of Bc and expansion of necrotic lesions was scored at 7 dpi. Error bars represent SD of three samples of 10 leaves each. This assay was repeated five times with similar results. P-values of Students t-test when compared to the Col-0 control are: rd21-1: 0.0414*; rd21-2: 0.0151**; and pad3: 0.008**.

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would colocalize in the apoplast. We presume that HpaEPICs are secreted during infection into the apoplast. Arabidopsis RD21 is present in vesicles and the vacuole [23,25,34], and was not detected in leaf apoplastic fluids [35]. A second unresolved issue is whether or not HpaEPICs physically interact with Arabidopsis RD21. We were unable to express and purify HpaEPIC-B and HpaEPIC-C proteins in sufficient amounts for inhibition and interaction studies. However, we did find that Arabidopsis RD21 can be inhibited by PlantEPIC1 and PlantEPIC2 (Figure S8), demonstrating that RD21 can be inhibited by EPIC-like proteins.

The role of rd21 in other pathosystems

The rd21 mutants are unaffected in infection with virulent and avirulent PtoDC3000 strains. The absence of phenotypes for rd21 mutants in these pathassays and during normal growth is remarkable given the fact that rd21 mutants lack one of the major Cys protease activities in leaves. More specifically, since RD21 is and we have identified an inhibitor of RD21 from the RD21 activity can be suppressed by mutants in these pathassays and during normal growth is demonstrating that RD21 can be inhibited by EPIC-like proteins. Hpa can be inhibited by interaction studies. However, we did find that Arabidopsis RD21 that the genome of be exposed to large amounts of vacuolar RD21 during host cell

Materials and Methods

Bioinformatics

To identify the ortholog of tomato C14 in the Arabidopsis genome, we performed a BLASTP search to the Arabidopsis TAIR10 database using the C14 protein sequence as a template. E-values of the BLAST search were used to define the closest ortholog to tomato C14. For phylogenetic assessment of the relationship of C14 to Arabidopsis proteases sequences of all Arabidopsis PLCPs and tomato C14, RCR3 and PIP1 were obtained from TAIR and GenBank [accession numbers: At1g02300, At3g48340, At3g48350, At1g06260, At3g43960, At1g09850, At1g20850, At1g29080, At1g29090, At1g47128, At2g21430, At2g27420, At2g34800, At3g19390, At3g19400, At3g53130, At3g54950, At3g54940, At4g11310, At4g11320, At4g16190, At4g23220, At4g35350, At4g36800, At4g39090, At5g30600, At5g45990, At5g0260, At3g60360 and At4g01610]. Sequences were aligned using BioEdit 7.0.9 (Ibis Biosciences, Carlsbad, CA, USA) and Mesquite [39]. The first 66 amino acids at the N-terminus of the proteins, which exhibited only low homology between taxa, were removed from the alignment prior phylogenetic analyses. The phylogenetic relationships between the sequences were determined using PAUP v. 4.0b10 (Sinauer Associates). Arabidopsis Cathepsin B (CTB3) was used as outgroup to root the tree. Maximum parsimony and neighbor-joining methods were performed and these methods yielded similar topologies. To identify the ortholog of PlantEPICs in the Hpa genome, we performed a BLASTP search on the genome database of Hpa isolate EmoY2 at the VBI Microbial Database using the PlantEPICs as templates. For phylogenetic assessment of the relationship between the PlantEPICs and Hpa EPICs amino acid sequences of these proteins were obtained from the VBI Microbial Database (605925, 603437, and 603430) and the GenBank (XP_002903480, XP_002903482, AXY21194, and XP_002900692). Sequence alignment and phylogenetic analyses on the full-length alignment were performed as described above. Cystatin from Allium tubbaucii (GenBank accession number CCA25330) was used as outgroup to root the tree.

We performed population genetic analyses with RD21 sequences from 80 Arabidopsis individuals [21]. The RD21 sequence of A. lyrata (NM_103612) was obtained from GenBank and used as outgroup. The standard summary statistics including π, divergence, Tajima’s D (D), Fu and Li’s D (D) test statistics were calculated using DnaSP v. 5.10 [40]. The site frequency spectrum of mutations was determined using SITES [41].

DNA work

For sequencing HpaEPIC-encoding genes from other Hpa isolates, genomic DNA was isolated by grinding infected leaf material in liquid nitrogen with sand using mortar and pestle. The leaf material was mixed in 200 mM Tris pH 7.5, 250 mM NaCl, 0.5% SDS, 25 mM EDTA and centrifuged (5 minutes 16,000 g). DNA was precipitated from the supernatant by adding one volume isopropanol and centrifuging (5 minutes 16,000 g). The pellet was dissolved in 30 mM Tris pH 8 EDTA by heating at 65°C for 5 minutes. HpaEPIC genes were amplified by PCR using primers that anneal in the 5’ and 3’ untranslated regions (Table S1). PCR fragments were sequenced from both sides. Forward and reverse sequences were aligned and observed nucleotide differences were verified in the trace data. For RT-PCR, RNA was isolated from Hpa-infected tissues at 5 dpi using RNeasy plant mini kit (Qiagen). Transcripts were amplified by semi-quantitative RT-PCR using primers summarized in Table S1.

Protein work

DCG-04 labeling was performed on leaf extracts, generated by grinding an Arabidopsis leaf in 1 mL water in a 1.5 mL tube, and clearing by centrifugation for 5 minutes at 16,000 g. 100 μL of leaf extract was labeled with 2 μM DCG-04 in the presence of 1 mM DTT and 25 mM NaAc pH6, in a total volume of 500 μL for 5 hours at 22°C. Proteins were precipitated by adding 1 mL ice-cold acetone and the pellet was dissolved in 50 μL gel-loading buffer. Proteins were separated on 12% SDS-PAGE gels and transferred to PVDF membranes (Immobilon-P, Biorad), and detected using anti-FLAG antibody (Sigma-Aldrich), or streptavi-
Disease assays

Plants were grown in a growth cabinet at 22°C at a 10-hour light regime and used for three different pathogen assays. Infection with Hpa by spray inoculation was performed on 3-week-old plants as described previously [42]. The numbers of spores at 7 dpi was counted by measuring the weight of infected plants, vortexing in water and counting released spores using a haemocytometer. Infection with Bc (strain B-169558) was performed on 5-week-old plants by inoculating each leaf with a 5 μL droplet of a 10^8 spores/mL LB spore suspension. The plants were kept at high humidity and the percentage of leaves showing spreading lesions was scored at 5-7 dpi. Infections with PdDC3000 were performed by spray inoculation of 5-week-old plants with 5 × 10^8 bacteria in 10 mM MgCl2 0.05% Silwet L77 and bacterial growth was measured at 0 and 3 dpi by plating dilution series of extracts on solid selection medium and counting colonies.

Supporting Information

Figure S1 Expression of RD21A and RD21B during Arabidopsis development. Data were extracted from Genevestigator.
(PDF)

Figure S2 Overview of polymorphisms in the RD21A gene in 80 individuals of A. thaliana. The amino acid sequence at polymorphic positions is given in the one-letter code of amino acids using the ecotype Col-0 as a reference. Identical amino acids encoded by identical codons are indicated with dots, synonymous polymorphisms are labelled with grey boxes and nonsynonymous polymorphisms are labelled with red boxes. Polymorphic positions in the same codon are indicated by black lines on top.
(PDF)

Figure S3 The site frequency spectrum of 80 RD21A alleles reveals an excess of polymorphisms in low frequency. The frequency of synonymous (light blue) and nonsynonymous (dark blue) mutations occurring at a certain number in the dataset (mutational class) is plotted for each mutational class. The red line indicates the expectation under complete neutrality.
(PDF)

Figure S4 Polymorphism in RD21-encoding sequences of A. lyrata and thaliana. Single nucleotide polymorphisms are indicated in grey on sequences of thaliana Col-0 (black) and lyrata (gi|297052301, red). Variant codons encode identical amino acids (light grey), similar amino acids (dark grey) and non-similar amino acids (red). The protease domain is printed in bold amino acids.
(PDF)

Figure S5 Sequences and alignments of HpaEPIC-B from various isolates.
(PDF)

Figure S6 Sequences and alignments of HpaEPIC-C from various isolates.
(PDF)

Figure S7 Both rd21-1 and rd21-2 are null mutants. Leaf extracts of Col-0 and rd21 mutant plants were labelled with DCG-04 in the presence or absence of an excess E-64 and separated on protein gels. Proteins were detected with RD21 antibody (A) and streptavidin-HRP (B), and coomassie staining (C).
(PDF)

Table S1 Sequences of primers used in this study.
(PDF)

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Author Contributions

Conceived and designed the experiments: TS JM-V. Analyzed the data: TS AH RvdH. Contributed reagents/materials/analysis tools: JS. Wrote the paper: RvdH.

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