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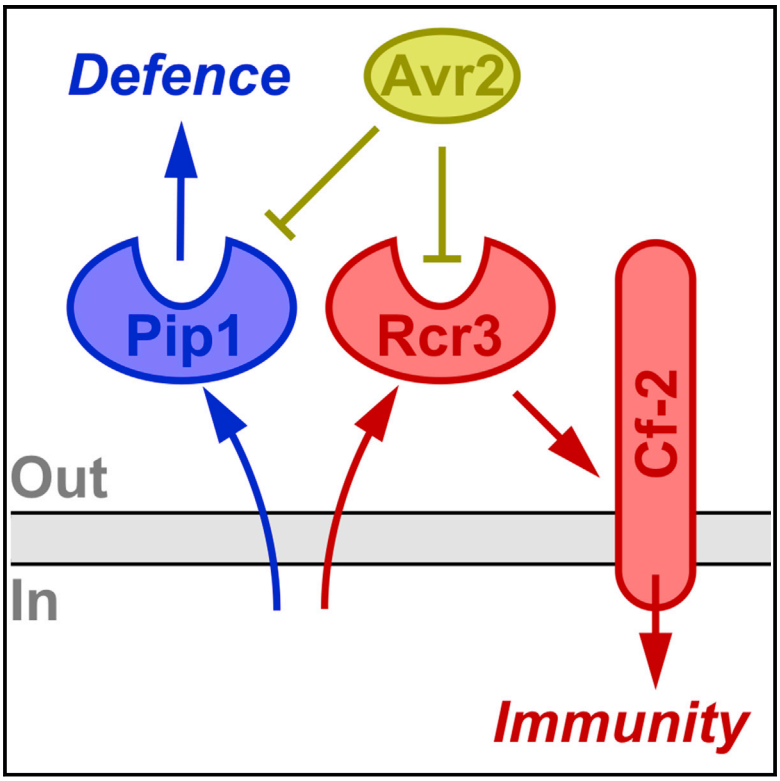
Current Biology

Functional Divergence of Two Secreted Immune Proteases of Tomato

Graphical Abstract



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In Brief

Ilyas et al. show that Rcr3 and Pip1 are paralogous proteases that differ in their expression levels and exposed surface and diverged >36 mya. Pip1 is an important immune protease acting against unrelated apoplastic pathogens, while Rcr3 is a co-receptor for pathogen-derived inhibitors, playing a minor role in the absence of immune receptor Cf-2.

Highlights

- Rcr3 and Pip1 are paralogous secreted tomato proteases that diverged >36 mya
- Rcr3 and Pip1 differ in expression levels and solvent-exposed residues
- Pip1 depletion increases susceptibility to unrelated apoplastic pathogens
- Without Cf-2, Rcr3 depletion increases susceptibility only to oomycete infection

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Functional Divergence of Two Secreted Immune Proteases of Tomato

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SUMMARY

Rcr3 and Pip1 are paralogous secreted papain-like proteases of tomato. Both proteases are inhibited by Avr2 from the fungal pathogen *Cladosporium fulvum*, but only Rcr3 acts as a co-receptor for Avr2 recognition by the tomato Cf-2 immune receptor [1–4]. Here, we show that Pip1-depleted tomato plants are hyper-susceptible to fungal, bacterial, and oomycete plant pathogens, demonstrating that Pip1 is an important broad-range immune protease. By contrast, in the absence of *Cf-2*, Rcr3 depletion does not affect fungal and bacterial infection levels but causes increased susceptibility only to the oomycete pathogen *Phytophthora infestans*. *Rcr3* and *Pip1* reside on a genetic locus that evolved over 36 million years ago. These proteins differ in surface-exposed residues outside the substrate-binding groove, and Pip1 is 5- to 10-fold more abundant than Rcr3. We propose a model in which Rcr3 and Pip1 diverged functionally upon gene duplication, possibly driven by an arms race with pathogen-derived inhibitors or by coevolution with the Cf-2 immune receptor detecting inhibitors of Rcr3, but not of Pip1.

RESULTS AND DISCUSSION

Rcr3 and *Pip1* Reside on an Ancient Gene Cluster and Differ in Relative Expression Levels and Surface-Exposed Residues

Analysis of the tomato genome sequence [5] revealed that *Rcr3* and *Pip1* reside on a 70-kb genetic cluster on the short arm of chromosome 2 in the tomato genome (Figure 1A). The *Rcr3*/*Pip1* locus contains two more predicted genes encoding papain-like Cys proteases, which we named *Rfp1* and *Pfp1*, for *Rcr3*- and *Pip1*-flanking proteases. The gene cluster is interspersed by an array of five predicted genes encoding GDSL lipases (*Lip1-5*). The locus is flanked by predicted genes encoding a member of the unidentified protein family 52 (*UPF52*) and an *RPW8*-like atypical resistance protein [6]. Most *Rfp1* transcripts carry an unspliced intron absent in the other protease genes (Figure S1A). Analysis of the open reading frames of the four tomato protease genes indicates that all these genes encode intact proteins containing a signal peptide, a prodomain with ERFNIN motif, and a protease domain with an intact Cys-His-Asn catalytic triad and three disulphide bridges (Figure S1B). All four proteases belong to the SAG12 subfamily [7].

Analysis of RNA sequencing data [8] showed that all 11 genes except *Lip3* are transcribed in leaves but with different relative expression levels (Figures 1B and S1C). *Pip1* transcripts are most abundant in leaves, followed by *Pfp1* and *Rcr3*. Inoculation with the fungal pathogen *Cladosporium fulvum* (both virulent and avirulent races) and a pathogenic strain of the bacterial pathogen *Pseudomonas syringae* causes significant increases in transcript levels of only *Rcr3* and *Pip1* in both Money Maker (MM) and Rio Grande 76S (RG) cultivars (Figures 1B and 1C) [8]. Furthermore, quantitative proteomic analysis only detected Rcr3 and Pip1 proteins in leaf apoplast isolated from tomato leaves, and treatment with the salicylic acid analog benzothiadiazole (BTH) increased Pip1 protein levels (Figure 1D). These data indicate that Pip1 protein is over 10-fold more abundant than Rcr3 in tomato apoplast. Consistently, *Pip1* transcript levels were 5- to 10-fold higher than *Rcr3* levels in cultivars MM, RG, M82, and Heinz and in wild tomato relatives *S. pimpinellifolium* and *S. pennellii* (Figure S1D) [5,9].

Analysis of the genome sequences of potato [10] and pepper [11, 12] showed that orthologs of *Rcr3* and *Pip1* reside at syntenic gene clusters on the short arm of chromosome 2 (Figure 1A). The gene clusters are larger in potato and pepper due to gene duplication and larger intergenic regions, but the order and direction of the genes is similar. Both *Rfp1* and *Rcr3* have duplicated several times in the genomes of potato and pepper, resulting in mixed *Rfp1-Rcr3* clusters of six and five genes, respectively. A *Pip1-Pfp1* gene pair is present in potato and pepper downstream of the lipase gene cluster, and *Pip1* is duplicated in the potato genome sequence. As in tomato, *Pip1* transcript levels are 5- to 10-fold higher than *Rcr3* in potato and pepper leaves (Figure S1D).

The protease genes fall into four well-supported phylogenetic clades separating the paralogs from each other (Figure 1E). Consistent with their taxonomic relation, pepper genes are more diverged from the tomato and potato orthologs. By contrast, the lipase-encoding genes are all closely related (Figure S1E). The phylogenetic clades indicate that the proteases have evolved independently from each other, despite residing at the same locus, where homogenization by recombination and/or intragenic gene conversion is common. The *Cf* resistance genes, for example, also reside in clusters that showed clear evidence of sequence exchange between paralogs, which is thought to generate novel resistance specificities [13, 14]. However, nucleotide identity between the protease genes is <62% (Table S1), which is below the >80% identity required for homogenization [15, 16], and alignments of tomato and potato *Rcr3* and *Pip1* did not display any larger stretch of shared polymorphisms (Figure S1F), indicating that *Rcr3* and *Pip1* evolved independently at least since the speciation of tomato and potato, ~7.3 million years ago [5].

Comparison of structural models generated for tomato and potato Rcr3 and Pip1 proteins, based on the castor bean endoprotease (1s4v [17]), displayed the amino acid differences between the Rcr3 and Pip1 proteases (Figures 1F and S1G). The substrate-binding groove and interior of Rcr3 and Pip1 are similar, and the differences between the two proteases reside mostly on the solvent-exposed protein surface (Figures 1F and S1H). This indicates that Rcr3 and Pip1 may have similar interactions with substrates that target the substrate-binding groove but may interact with different proteins in the remainder protein surface.

In the Absence of *Cf-2*, *Rcr3* Does Not Affect Fungal or Bacterial Infection but Suppresses Oomycete Infection

To investigate the role of *Rcr3* in the absence of the *Cf-2* resistance gene, we crossed MM-Cf0 (carrying $Rcr3^{lyc}$ but lacking *Cf-2*) with MM-Cf2 (carrying $Rcr3^{pim}$ and *Cf-2*) and with a mutant MM-Cf2 line carrying the $rcr3\text{-}3^{pim}$ allele with a premature translation stop codon [1] (Figure 2A). Two homozygous lines of both MM-Cf0/$Rcr3^{pim}$ (lines #A and #B) and MM-Cf0/$rcr3\text{-}3^{pim}$ (lines #C and #D) were selected and confirmed by PCR using gene-specific primers and sequencing (Figure S2B). As expected for unlinked genes, the segregation of both *Cf-2* and *Rcr3* was Mendelian and independent in both crosses (Figure S2A). Necrotic phenotypes caused by the interaction between $Rcr3^{lyc}$ (the *Rcr3* from cultivated tomato) and *Cf-2* (introgressed from *S. pimpinellifolium*) were also observed in these crosses. Notably, the necrotic phenotype of $Rcr3^{lyc}$ is dominant over $rcr3\text{-}3^{pim}$ but recessive over $Rcr3^{pim}$ (Figures 2A and S2A) [1]. These data support a biochemical competition model in which $Rcr3^{lyc}$ protein interacts with Cf-2 but that $Rcr3^{pim}$ protein (which appears to have co-evolved with Cf-2) can outcompete $Rcr3^{lyc}$ and prevent necrotic responses (Figure S2C). Sequencing *Rcr3* transcripts in the F1 hybrids revealed that $Rcr3^{lyc}$ transcript levels are always 4-fold lower when compared to $Rcr3^{pim}$ (Figure S2D), which is not inconsistent with the biochemical competition model.

We performed disease assays to study the role of *Rcr3* in the absence of *Cf-2*. Quantitative disease assays with *C. fulvum* and *P. syringae* did not reveal any significantly altered resistance or susceptibility in the *Cf0/rcr3-3* lines when compared to the *Cf0*/$Rcr3^{pim}$ control lines (Figures 2B and 2C). Previous work demonstrated that MM-Cf2/$rcr3\text{-}3^{pim}$ plants are more susceptible to the oomycete pathogen *Phytophthora infestans* than MM-Cf2/$Rcr3^{pim}$ plants [18]. Notably, disease assays also revealed that MM-Cf0/$rcr3\text{-}3^{pim}$ plants are slightly but still significantly more susceptible to *P. infestans* when compared to MM-Cf0/$Rcr3^{pim}$ plants (Figure 2D), indicating that $Rcr3^{pim}$ contributes to resistance to *P. infestans* independently of the *Cf-2* resistance gene.

Pip1 Is a Major Immune Protease against Various Apoplastic Pathogens

To study the role of *Pip1* in immunity, MM-Cf0 plants were transformed with an antisense *Pip1* construct, and two independent transformants were selected for further analysis (Figure 3A). qRT-PCR analysis revealed a selective transcript reduction of *Pip1*, but not of *Rcr3* or *C14* protease genes (Figure 3B). Likewise, western blot analysis of apoplastic proteomes did not display Pip1 protein in MM-Cf0/*asPip1* plants, whereas C14 protein levels remained unaltered (Figure 3C). Protease activity profiling of these apoplastic proteomes revealed that a 25-kDa signal is missing in MM-Cf0/*asPip1* plants, whereas the absence of *Rcr3* does not affect the activity profile (Figure 3D). Strongly reduced *Pip1* levels are not associated with defects in growth and development (Figure S3A).

Infection assays with *C. fulvum* revealed that disease symptoms developed faster and sporulation was increased on MM-Cf0/*asPip1* plants, as compared to MM-Cf0, and qRT-PCR analysis confirmed a 5-fold increased fungal biomass accumulation (Figure 3E). The MM-Cf0/*asPip1* plants are also more susceptible for *P. syringae* pv. *tomato* DC3000 (PtoDC3000), supporting a 10-fold increased bacterial growth when compared to MM-Cf0 (Figure 3F). A similar increase in bacterial growth was observed upon inoculation with the less virulent *ΔavrPto*/*ΔavrPtoB* double mutant of PtoDC3000 (Figure S3B). Finally, lesions caused by *P. infestans* on MM-Cf0/*asPip1* plants grow 4-fold faster when compared to MM-Cf0 control plants (Figure 3G). These data demonstrate that *Pip1* is an important contributor to broad-range immunity against three unrelated pathogens that colonize the apoplast of tomato leaves.

The important role of Pip1 explains why this protease is targeted by unrelated pathogen-derived inhibitors [3, 19]. The role of Pip1 in immunity is presumably dependent on proteolytic activity and can be direct (inactivating pathogen proteins) or indirect (releasing elicitors from host or pathogen). Further studies on the substrates of Pip1 are required to understand how it confers immunity and how it may discriminate between self and non-self proteins in the apoplast.

We conclude that in the absence of *Cf-2*, *Rcr3* does not significantly contribute to immunity against *C. fulvum* or *P. syringae*, whereas *Pip1* does. By contrast, both *Rcr3* and *Pip1* contribute to immunity against *P. infestans* in the absence of *Cf-2*, but *Pip1* seems to contribute significantly more to immunity than *Rcr3*. These findings suggest that *Rcr3* plays different roles in different pathosystems. There are several possibilities to explain these different roles: (1) Rcr3 and Pip1 proteases might act on different substrates and thereby affect different pathogens in different

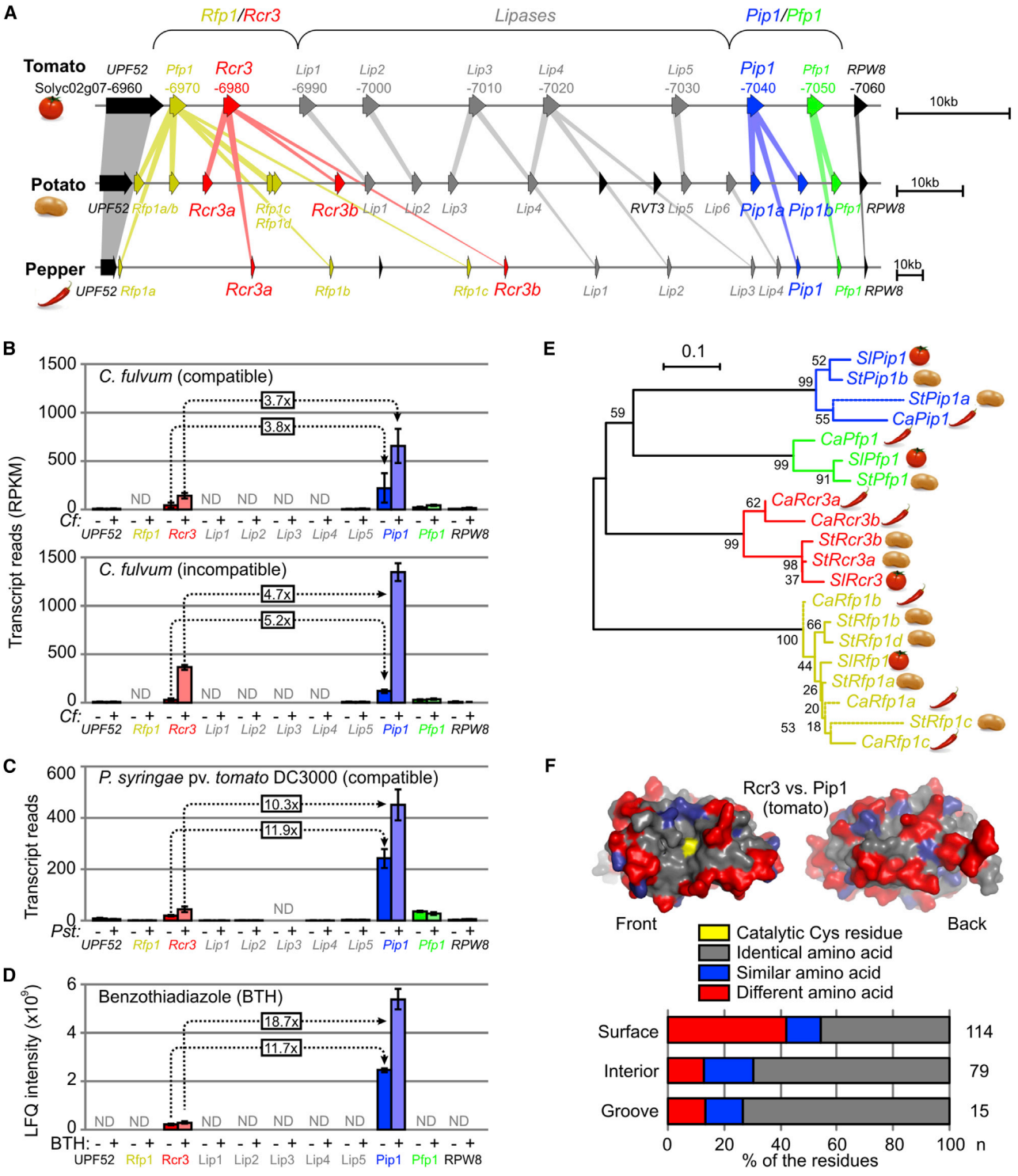

Figure 1. Analysis of *Rcr3*/*Pip1* Gene Clusters, Expression Levels, and Structural Models of Rcr3 and Pip1

(A) Synteny of the *Rcr3*/*Pip1* gene clusters in tomato, potato, and pepper. The closest homologs between the different genome sequences are indicated with connecting lines. The different 10-kb scales on the right indicate the extent of expansion of the different *Rcr3*/*Pip1* loci. Color codes: *Rfp1* (yellow), *Rcr3* (red), *Pip1* (blue), and *Pfp1* (green).

(B) Transcript level of genes of the *Rcr3*/*Pip1* locus of susceptible (MM-Cf0) and resistant (MM-Cf4) tomato inoculated with or without *C. fulvum* race 5. 4-week-old plants were spray inoculated with 1×10^6 conidia/ml or mock inoculated, and RNA was extracted 6 days later. The transcriptome was sequenced, and the reads per kilobase per million (RPKM values) were extracted and plotted for each gene of the *Rcr3*/*Pip1* locus. The error bars represent the SE of three biological replicates. Only upregulation of *Rcr3* and *Pip1* was over 2-fold and statistically significant ($p < 0.01$).

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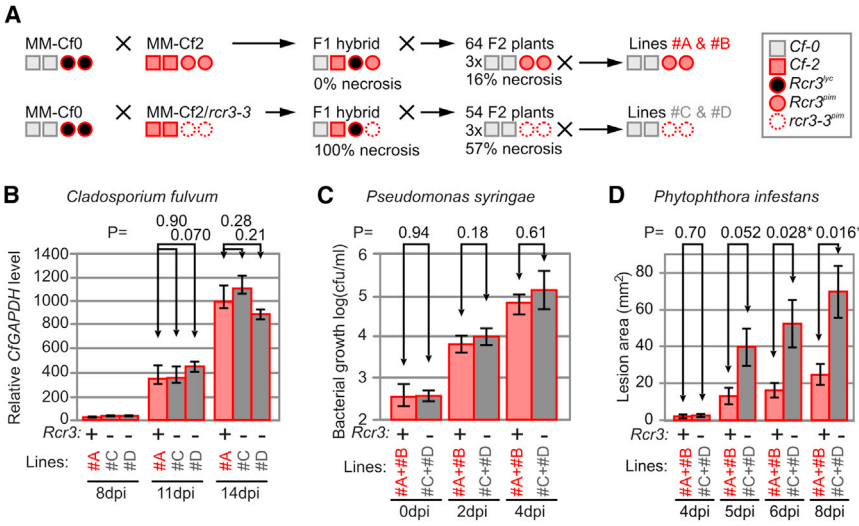

Figure 2. In the Absence of *Cf-2*, *Rcr3* Contributes to Resistance to *P. infestans*, but Not to *C. fulvum* or to *P. syringae*

(A) Generation of tomato lines carrying $Rcr3^{pim}$ (lines #A and #B) and $rcr3\text{-}3^{pim}$ (lines #C and #D) in the absence of *Cf-2* by crossing. The occurrence of plants with necrotic phenotypes due to the combination of $Rcr3^{lyc}$ with *Cf-2* is indicated.

(B) In the absence of *Cf-2*, $Rcr3^{pim}$ does not significantly affect susceptibility to *C. fulvum* race 5. Tomato plants (lines #A, #C, and #D) were spray inoculated with spores, and fungal growth was measured at 8, 11, and 14 days post inoculation (dpi) using qRT-PCR using primers for fungal *GAPDH* and tomato *Actin*. Error bars represent SEM (n = 15). The experiment was repeated three times with similar results.

(C) In the absence of *Cf-2*, $Rcr3^{pim}$ does not significantly affect susceptibility to *P. syringae* pv. *tomato* DC3000 (PtoDC3000). Tomato plants (mix of lines #A + #B and of #C + #D) were spray inoculated with PtoDC3000, and bacterial growth in colony forming units (cfu) was measured at 0, 2, and 4 dpi. Error bars represent SEM (n = 3). The experiment was repeated three times with similar results.

(D) In the absence of *Cf-2*, $Rcr3^{pim}$ contributes to resistance against *P. infestans*. Detached leaves from MM-Cf0 carrying $Rcr3^{pim}$ (mix of lines #A + #B) or MM-Cf0 carrying $rcr3\text{-}3^{pim}$ (mix of lines #C + #D) were droplet inoculated with spores of *P. infestans* 88069, and the lesion area was measured from pictures taken at 4, 5, 6, and 8 days post-inoculation. Error bars represent SEM (n = 6–8). The experiment was repeated twice with similar results.

ways. The high similarity of the putative substrate-binding groove of Rcr3 and Pip1 would not support this hypothesis, but this remains to be verified with detailed substrate studies. (2) *Rcr3* still functions in *P. infestans* recognition even in the absence of *Cf-2*. Notably, there are two *Cf-2*-like genes (*Hcr2-0A* and *Hcr2-0B*) located on the allelic locus in MM-Cf0 [14, 20]. It is possible that their products could interact with Rcr3 and perceive *P. infestans* infection, perhaps by detecting *P. infestans* effectors other than EpiCs. (3) Rcr3 and Pip1 contribute to defense to the same extent as their relative abundance, and the observed effect against *P. infestans* is because this pathogen is more sensitive to apoplastic proteases.

Cf-2/Rcr3 is an important example of indirect perception mechanisms in plants, which has been interpreted by the Guard Model [21, 22]. The Guard Model predicts that resistance (R) proteins act by monitoring (guarding) the manipulation of host targets by pathogen-derived effector proteins. This Guard Model, however, implies a fitness benefit for manipulation of the guardee (Rcr3) by the effector protein (Avr2 from *C. fulvum*) in the absence of the R protein (Cf-2). The discovery that Avr2 also targets the more abundant Pip1 [3] led us previously to propose the Decoy Model, which predicts that the guarded host target only acts as co-receptor in the presence of the R proteins but has no role in its absence. This Decoy Model has become an important concept explaining many observations in indirect perception mechanisms including Pto, PBS1, the promoter of *Bs3* [23], and more recently, the pseudokinases ZED1 and RKS1, receptor-like protein RFO2, and integrated decoy RRS1 [24–28].

Our data on Rcr3 support both the Guard Model and the Decoy Model, depending on the pathogen. The Decoy Model is supported by the fact that in the absence of *Cf-2*, *Rcr3* does not significantly contribute to defense against *C. fulvum*. However, the Guard Model is supported by the finding that *Rcr3* contributes to defense against *P. infestans* in the absence of *Cf-2*, though its contribution seems relatively minor when compared to that of *Pip1*. Decoys are expected to have lost some of their biochemical activities. For instance, ZED1 and RKS1 are inactive pseudokinases [24, 26]. However, Rcr3 is still an active protease that appears to function as a decoy in some plant-pathogen interactions, but not in others.

Based on these observations we propose evolutionary models for Rcr3 and Pip1 (Figure 4). We anticipate that secreted

(C) Transcript level of genes of the *Rcr3/Pip1* locus of susceptible tomato plants (Rio Grande 76S) inoculated with *Pseudomonas syringae* pv. *tomato* DC3000 or mock-inoculated plants. Expression data as RPKM were extracted from post-infection transcriptome data in [8].

(D) Label free quantification (LFQ) intensities indicate higher Pip1 than Rcr3 protein accumulation levels. Equal volumes of apoplastic proteomes from water- or BTH-treated MM-Cf0 tomato plants were separated on protein gel, and the 20–30 kDa region was excised, treated with trypsin, and subjected to mass spectrometry. ND, not detected.

(E) Neighbor-joining tree of the protease-encoding open reading frames of the *Rcr3/Pip1* locus of potato, tomato, and pepper. Labels at the nodes indicate bootstrap support.

(F) Pairwise comparison of the surface of the protease model of tomato Rcr3 and Pip1 shown from the front and the back. Top: differences between Rcr3 and Pip1 of tomato were plotted onto a structural model of tomato Rcr3 and Pip1 modeled on the 1s4v crystal structure. Residues are indicated in colors as shown in the legend. Bottom: differences reside on the surface of Rcr3/Pip1 outside the substrate-binding groove. Residues in the Rcr3 protein model were divided into surface-exposed, internal, or substrate-binding grove using PyMol. The frequency of residues that are different, similar, or identical between Rcr3 and Pip1 in tomato was counted for each of these three positions.

See Figure S1 for more information.

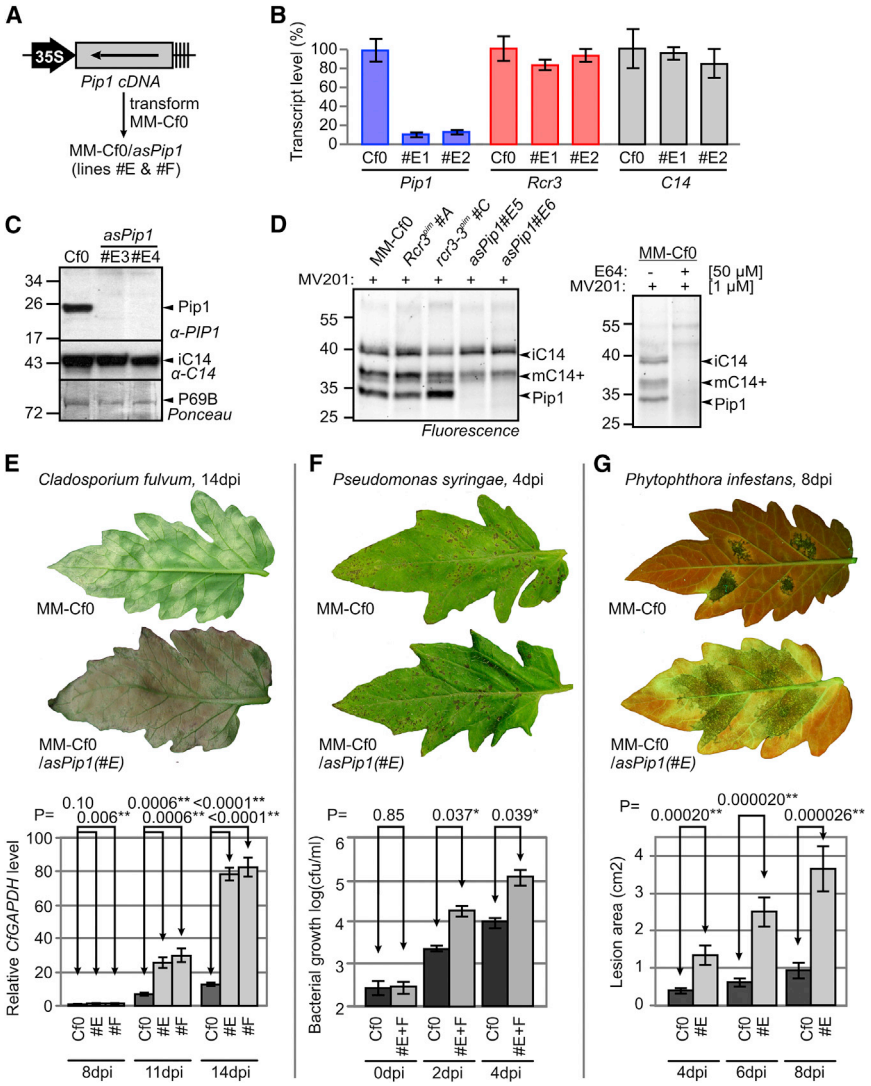

Figure 3. Antisense *Pip1* Tomato Plants Are Hyper-susceptible to Pathogens

(A) A cDNA for *Pip1*, driven by the CaMV 35S promoter in reverse orientation, was transformed into MM-Cf0 plants, resulting in independent transformant lines #E and #F.

(B) Reduced transcript levels in MM-Cf0/*asPip1* tomato plants of *Pip1*, but not of *Rcr3* or *C14*. mRNA was isolated from two 2-week-old F2 MM-Cf0/*asPip1* tomato plants (line #E) or the recipient MM-Cf0 control and used for qRT-PCR using gene-specific primers. Transcript levels were normalized against transcript levels of tomato *Actin*, and the expression level of the untransformed control plants was set to 100%. The error bars represent the SE of nine biological replicates. The experiment was repeated three times with similar results. Similar results were obtained for plants of line #F.

(C) Reduced Pip1 protein levels in MM-Cf0/*asPip1* tomato plants. Apoplastic proteomes were isolated from 4-week-old MM-Cf0 and two MM-Cf0/*asPip1* (line #E) tomato plants and analyzed on protein blot using anti-Pip1 and anti-C14 antibodies and Ponceau staining. Similar results were obtained for plants of line #F.

(D) Reduced protease activity in MM-Cf0/*asPip1* tomato plants. Equal volumes of apoplastic fluids isolated from 4-week-old MM-Cf0 and two MM-Cf0/*asPip1* plants (line #E) and plants of lines #A and #C were pre-incubated with or without 50 μM E-64 and labeled with 1 μM MV201. Labeled proteins were detected by fluorescence scanning. Similar results were obtained for plants of line #F.

(E) MM-Cf0/*asPip1* plants are hyper-susceptible to *C. fulvum*. MM-Cf0 and MM-Cf0/*asPip1* plants of lines #E and #F having reduced *Pip1* transcript levels were spray inoculated with conidia of *C. fulvum* race 5. Top: pictures of the lower side of infected leaves are representative and were taken at 14 dpi. Bottom: RNA was isolated at 8, 11, and 14 dpi and used for qRT-PCR using specific fungal *GAPDH* and tomato *Actin* primers. Error bars represent SEM (n = 15). The experiment was repeated twice with similar results.

(F) MM-Cf0/*asPip1* plants are more susceptible to *P. syringae* (PtoDC3000). Wild-type plants and MM-Cf0/*asPip1* plants (mix of both lines #E and #F) having reduced *Pip1* transcript levels were spray inoculated with 10^8 PtoDC3000 bacteria/ml. Top: pictures of the upper side of infected leaves are representative and were taken at 4 dpi. Bottom: bacterial populations were determined at 0, 2, and 4 dpi. Error bars represent SEM (n = 3). The experiment was repeated three times with similar results.

(G) MM-Cf0/*asPip1* tomato plants are hyper-susceptible to *P. infestans*. MM-Cf0 and MM-Cf0/*asPip1* plants (line #E) having reduced *Pip1* transcript levels were droplet inoculated with *P. infestans* isolate 88069. Top: pictures of the lower side of infected leaves are representative and were taken at 8 dpi under UV irradiation. Bottom: lesion areas of three leaves of three plants were quantified at 4, 6, and 8 dpi using imaging software. Error bars represent SEM (n = 9). The experiment was repeated three times with similar results.

proteases might have been employed early in evolution as a component of an ancestral immune response (Figure 4A). Their exclusive role in extracellular defense allowed these proteases to target non-self proteins and become stress inducible, while the secreted plant proteome may have been under selection to prevent accidental processing. Second, pathogens have adapted to this harmful proteolytic environment by secreting inhibitors that inactivate these defense proteases (Figure 4B). Indeed, unrelated effector proteins (Avr2, EpiCs, and Vap1) target secreted tomato proteases [2, 19, 29]. This process is associated with the accumulation of variant residues on the surface of Rcr3 and Pip1, some of which affect inhibitor affinity [3, 29–31]. Next, gene duplication and diversification of the duplicates occurred, probably driven by the arms race with pathogen-derived inhibitors (Figure 4C). Finally, a surface receptor (a Cf-2 progenitor) evolved that perceives protease-inhibitor complexes and triggers immune responses (Figure 4D). This order implies that *Cf-2* is younger than *Rcr3*, and this is supported by the findings that *Cf* gene clusters evolve fast by recombination [13, 14] and *Cf-2* itself evolved recently because its open reading frame contains nearly identical nucleotide repeats [20].

Alternatively, a Cf-2 progenitor evolved first, before protease gene duplication (Figure 4E). We anticipate that a single guarded immune protease would be under opposite selection forces

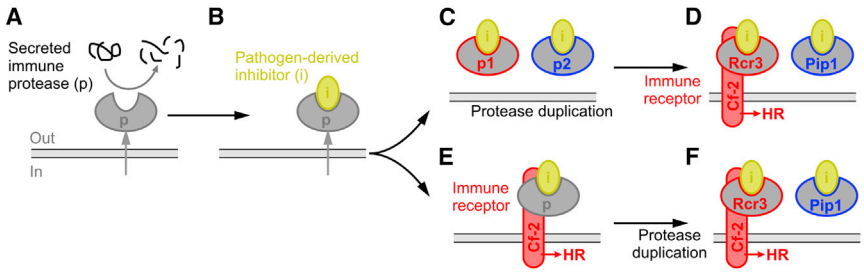

Figure 4. Proposed Model for Functional Diversification of Rcr3 and Pip1

(A) The Rcr3/Pip1 ancestor is a secreted immune protease (p).

(B) Immune proteases are targeted by pathogen-derived inhibitors (i).

(C) Protease gene duplication and diversification is driven by an arms race with pathogen-derived inhibitors.

(D) A receptor-like protein (Cf-2) evolved that recognizes the protease-inhibitor complex and triggers a hypersensitive response (HR).

(E and F) Alternatively, the surface receptor evolved first (E), and opposite selective forces on the immune protease gene were relaxed by gene duplication and diversification (F).

acting on both the level of protein-protein interactions and transcriptional activity. Gene duplication would relax this evolutionary constraint and allow the evolution of (1) a co-receptor protease with reduced expression levels and increased affinity for pathogen-derived inhibitors and (2) a defense-related protease that retained high expression levels and evolves avoiding interactions with pathogen-derived inhibitors (Figure 4F). This second evolutionary scenario cannot be excluded until we study the history of functional Cf-2-like proteins in Solanaceae [32] in more detail. These observations trigger further research into the evolution, molecular mechanism, and occurrence of this intriguing indirect pathogen recognition system in Solanaceous plants.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.07.030.

AUTHOR CONTRIBUTIONS

M.I., A.C.H., M.H.A.J.J., S.K., and R.A.L.v.d.H. designed experiments. M.I. performed experiments. M.I., T.O.B., and K.B. performed *P. infestans* infections. A.C.H., H.A.v.d.B., and R.A.L.v.d.H. performed bioinformatic analysis. F.K. and M.K. generated mass spectrometry data. M.H.A.J.J. provided RNA-seq data and supported *C. fulvum* inoculations. M.S. transformed MM-Cf0 tomato lines with *asPip1*. R.A.L.v.d.H. wrote the paper with input from all authors.

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REFERENCES

1. Krüger, J., Thomas, C.M., Golstein, C., Dixon, M.S., Smoker, M., Tang, S., Mulder, L., and Jones, J.D.G. (2002). A tomato cysteine protease required for *Cf-2*-dependent disease resistance and suppression of autonecrosis. Science *296*, 744–747.
2. Rooney, H.C., Van't Klooster, J.W., van der Hoorn, R.A.L., Joosten, M.H.A.J., Jones, J.D.G., and de Wit, P.J.G.M. (2005). *Cladosporium* Avr2 inhibits tomato Rcr3 protease required for *Cf-2*-dependent disease resistance. Science *308*, 1783–1786.
3. Shabab, M., Shindo, T., Gu, C., Kaschani, F., Pansuriya, T., Chintha, R., Harzen, A., Colby, T., Kamoun, S., and van der Hoorn, R.A.L. (2008). Fungal effector protein AVR2 targets diversifying defense-related cys proteases of tomato. Plant Cell *20*, 1169–1183.
4. van Esse, H.P., Van't Klooster, J.W., Bolton, M.D., Yadeta, K.A., van Baarlen, P., Boeren, S., Vervoort, J., de Wit, P.J.G.M., and Thomma, B.P.H.J. (2008). The *Cladosporium fulvum* virulence protein Avr2 inhibits host proteases required for basal defense. Plant Cell *20*, 1948–1963.
5. Tomato Genome Consortium (2012). The tomato genome sequence provides insights into fleshy fruit evolution. Nature *485*, 635–641.
6. Xiao, S., Ellwood, S., Calis, O., Patrick, E., Li, T., Coleman, M., and Turner, J.G. (2001). Broad-spectrum mildew resistance in *Arabidopsis thaliana* mediated by *RPW8*. Science *291*, 118–120.
7. Richau, K.H., Kaschani, F., Verdoes, M., Pansuriya, T.C., Niessen, S., Stüber, K., Colby, T., Overkleeft, H.S., Bogyo, M., and Van der Hoorn, R.A. (2012). Subclassification and biochemical analysis of plant papain-like cysteine proteases displays subfamily-specific characteristics. Plant Physiol. *158*, 1583–1599.
8. Rosli, H.G., Zheng, Y., Pombo, M.A., Zhong, S., Bombarely, A., Fei, Z., Collmer, A., and Martin, G.B. (2013). Transcriptomics-based screen for genes induced by flagellin and repressed by pathogen effectors identifies a cell wall-associated kinase involved in plant immunity. Genome Biol. *14*, R139.
9. Koenig, D., Jiménez-Gómez, J.M., Kimura, S., Fulop, D., Chitwood, D.H., Headland, L.R., Kumar, R., Covington, M.F., Devisetty, U.K., Tat, A.V., et al. (2013). Comparative transcriptomics reveals patterns of selection in domesticated and wild tomato. Proc. Natl. Acad. Sci. USA *110*, E2655–E2662.
10. Xu, X., Pan, S., Cheng, S., Zhang, B., Mu, D., Ni, P., Zhang, G., Yang, S., Li, R., Wang, J., et al.; Potato Genome Sequencing Consortium (2011). Genome sequence and analysis of the tuber crop potato. Nature *475*, 189–195.
11. Kim, S., Park, M., Yeom, S.I., Kim, Y.M., Lee, J.M., Lee, H.A., Seo, E., Choi, J., Cheong, K., Kim, K.T., et al. (2014). Genome sequence of the hot pepper provides insights into the evolution of pungency in Capsicum species. Nat. Genet. *46*, 270–278.
12. Qin, C., Yu, C., Shen, Y., Fang, X., Chen, L., Min, J., Cheng, J., Zhao, S., Xu, M., Luo, Y., et al. (2014). Whole-genome sequencing of cultivated

and wild peppers provides insights into *Capsicum* domestication and specialization. Proc. Natl. Acad. Sci. USA *111*, 5135–5140.

13. Parniske, M., Hammond-Kosack, K.E., Golstein, C., Thomas, C.M., Jones, D.A., Harrison, K., Wulff, B.B., and Jones, J.D.G. (1997). Novel disease resistance specificities result from sequence exchange between tandemly repeated genes at the Cf-4/9 locus of tomato. Cell *91*, 821–832.
14. Dixon, M.S., Hatzixanthis, K., Jones, D.A., Harrison, K., and Jones, J.D.G. (1998). The tomato *Cf-5* disease resistance gene and six homologs show pronounced allelic variation in leucine-rich repeat copy number. Plant Cell *10*, 1915–1925.
15. Harris, S., Rudnicki, K.S., and Haber, J.E. (1993). Gene conversions and crossing over during homologous and homeologous ectopic recombination in Saccharomyces cerevisiae. Genetics *135*, 5–16.
16. Mansai, S.P., Kado, T., and Innan, H. (2011). The rate and tract length of gene conversion between duplicated genes. Genes (Basel) *2*, 313–331.
17. Than, M.E., Helm, M., Simpson, D.J., Lottspeich, F., Huber, R., and Gietl, C. (2004). The 2.0 A crystal structure and substrate specificity of the KDEL-tailed cysteine endopeptidase functioning in programmed cell death of Ricinus communis endosperm. J. Mol. Biol. *336*, 1103–1116.
18. Song, J., Win, J., Tian, M., Schornack, S., Kaschani, F., Ilyas, M., van der Hoorn, R.A.L., and Kamoun, S. (2009). Apoplastic effectors secreted by two unrelated eukaryotic plant pathogens target the tomato defense protease Rcr3. Proc. Natl. Acad. Sci. USA *106*, 1654–1659.
19. Tian, M., Win, J., Song, J., van der Hoorn, R., van der Knaap, E., and Kamoun, S. (2007). A Phytophthora infestans cystatin-like protein targets a novel tomato papain-like apoplastic protease. Plant Physiol. *143*, 364–377.
20. Dixon, M.S., Jones, D.A., Keddie, J.S., Thomas, C.M., Harrison, K., and Jones, J.D.G. (1996). The tomato *Cf-2* disease resistance locus comprises two functional genes encoding leucine-rich repeat proteins. Cell *84*, 451–459.
21. Van der Biezen, E.A., and Jones, J.D.G. (1998). Plant disease-resistance proteins and the gene-for-gene concept. Trends Biochem. Sci. *23*, 454–456.
22. Dangl, J.L., and Jones, J.D.G. (2001). Plant pathogens and integrated defence responses to infection. Nature *411*, 826–833.
23. van der Hoorn, R.A.L., and Kamoun, S. (2008). From Guard to Decoy: a new model for perception of plant pathogen effectors. Plant Cell *20*, 2009–2017.
24. Lewis, J.D., Lee, A.H., Hassan, J.A., Wan, J., Hurley, B., Jhingree, J.R., Wang, P.W., Lo, T., Youn, J.Y., Guttman, D.S., and Desveaux, D. (2013). The Arabidopsis ZED1 pseudokinase is required for ZAR1-mediated immunity induced by the Pseudomonas syringae type III effector HopZ1a. Proc. Natl. Acad. Sci. USA *110*, 18722–18727.
25. Shen, Y., and Diener, A.C. (2013). *Arabidopsis thaliana* resistance to fusarium oxysporum 2 implicates tyrosine-sulfated peptide signaling in susceptibility and resistance to root infection. PLoS Genet. *9*, e1003525.
26. Huard-Chauveau, C., Perchepied, L., Debieu, M., Rivas, S., Kroj, T., Kars, I., Bergelson, J., Roux, F., and Roby, D. (2013). An atypical kinase under balancing selection confers broad-spectrum disease resistance in Arabidopsis. PLoS Genet. *9*, e1003766.
27. Le Roux, C., Huet, G., Jauneau, A., Camborde, L., Trémousaygue, D., Kraut, A., Zhou, B., Levaillant, M., Adachi, H., Yoshioka, H., et al. (2015). A receptor pair with an integrated decoy converts pathogen disabling of transcription factors to immunity. Cell *161*, 1074–1088.
28. Sarris, P.F., Duxbury, Z., Huh, S.U., Ma, Y., Segonzac, C., Sklenar, J., Derbyshire, P., Cevik, V., Rallapalli, G., Saucet, S.B., et al. (2015). A plant immune receptor detects pathogen effectors that target WRKY transcription factors. Cell *161*, 1089–1100.
29. Lozano-Torres, J.L., Wilbers, R.H.P., Gawronski, P., Boshoven, J.C., Finkers-Tomczak, A., Cordewener, J.H.G., America, A.H.P., Overmars, H.A., Van 't Klooster, J.W., Baranowski, L., et al. (2012). Dual disease resistance mediated by the immune receptor Cf-2 in tomato requires a common virulence target of a fungus and a nematode. Proc. Natl. Acad. Sci. USA *109*, 10119–10124.
30. Hörger, A.C., Ilyas, M., Stephan, W., Tellier, A., van der Hoorn, R.A.L., and Rose, L.E. (2012). Balancing selection at the tomato *RCR3* Guardee gene family maintains variation in strength of pathogen defense. PLoS Genet. *8*, e1002813.
31. Dong, S., Stam, R., Cano, L.M., Song, J., Sklenar, J., Yoshida, K., Bozkurt, T.O., Oliva, R., Liu, Z., Tian, M., et al. (2014). Effector specialization in a lineage of the Irish potato famine pathogen. Science *343*, 552–555.
32. Caicedo, A.L., and Schaal, B.A. (2004). Heterogeneous evolutionary processes affect R gene diversity in natural populations of Solanum pimpinellifolium. Proc. Natl. Acad. Sci. USA *101*, 17444–17449.

