Apoplastic effectors secreted by two unrelated eukaryotic plant pathogens target the tomato defense protease Rcr3

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Current models of plant–pathogen interactions stipulate that pathogens secrete effector proteins that disable plant defense components known as virulence targets. Occasionally, the perturbations caused by these effectors trigger innate immunity via plant disease resistance proteins as described by the "guard hypothesis." This model is nicely illustrated by the interaction between the fungal plant pathogen *Cladosporium fulvum* and tomato. *C. fulvum* secretes a protease inhibitor Avr2 that targets the tomato cysteine protease Rcr3^{pim}. In plants that carry the resistance protein Cf2, Rcr3^{pim} is required for resistance to *C. fulvum* strains expressing Avr2, thus fulfilling one of the predictions of the guard hypothesis. Another prediction of the guard hypothesis has not yet been tested. Considering that virulence targets are important components of defense, different effectors from unrelated pathogens are expected to evolve to disable the same host target. In this study we confirm this prediction using a different pathogen of tomato, the oomycete *Phytophthora infestans* that is distantly related to fungi such as *C. fulvum*. This pathogen secretes an array of protease inhibitors including EPIC1 and EPIC2B that inhibit tomato cysteine proteases. Here we show that, similar to Avr2, EPIC1 and EPIC2B bind and inhibit Rcr3^{pim}. However, unlike Avr2, EPIC1 and EPIC2B do not trigger hypersensitive cell death or defenses on Cf-2/Rcr3^{pim} tomato. We also found that the *rcr3–3* mutant of tomato that carries a premature stop codon in the *Rcr3* gene exhibits enhanced susceptibility to *P. infestans*, suggesting a role for Rcr3^{pim} in defense. In conclusion, our findings fulfill a key prediction of the guard hypothesis and suggest that the effectors Avr2, EPIC1, and EPIC2B secreted by two unrelated pathogens of tomato target the same defense protease Rcr3^{pim}. In contrast to *C. fulvum*, *P. infestans* appears to have evolved stealthy effectors that carry inhibitory activity without triggering plant innate immunity.

Inhibitors | plant-microbe interactions

Plant pathogens secrete effectors into the apoplast and cytoplasm of plants to facilitate colonization and to suppress host defenses (1, 2). Although effectors are known to positively contribute to virulence, in some plant varieties, effectors can also be recognized by disease resistance (R) proteins, resulting in programmed cell death, a defense response known as the hypersensitive response (HR). In such cases, the effectors are said to have an avirulence (Avr) activity, and their interactions with the R proteins typically follow the gene-for-gene model, with resistance occurring only when matching pairs of R and Avr proteins are produced (1, 2).

Recognition of Avr proteins by R proteins can be direct, and binding between matching R-Avr pairs has been reported in bacterial and fungal pathosystems (3, 4). In many other instances, however, there is no evidence of a direct interaction between the R and Avr proteins; and pathogen recognition by the plant is thought to be indirect, involving at least one additional plant component (5, 6). According to the "guard hypothesis," this component is a host target (virulence target) that is manipulated or altered by the pathogen effector to contribute to the pathogen success on susceptible host plants (5–7). On resistant plants, these perturbations of the host target by the effector activate the cognate R protein, resulting in the HR and resistance. The R protein is therefore viewed as "guarding" the host target from manipulation by pathogen effectors.

There are several examples of R-Avr interactions that are consistent with the "guard hypothesis" (2, 5, 6). One of the classical examples is the interaction between the fungal plant pathogen *Cladosporium fulvum* and its host tomato (*Solanum lycopersicum*). Consistent with the gene-for-gene model, tomato lines that contain the *R*-gene *Cf-2* exhibit resistance only to *C. fulvum* strains that carry the avirulence gene *avr2*. Mutational analysis of tomato genes required for *Cf-2* function identified *Rcr3^{pim}* (required for *Cladosporium fulvum* resistance 3), a gene specifically required for *Cf-2*-mediated resistance (8). The *Rcr3^{pim}* gene was introgressed into tomato from *Solanum pimpinellifolium* along with Cf-2 and encodes a papain-like cysteine protease that is inhibited by Avr2 (9). The role of Rcr3^{pim} in perception of Avr2 by Cf-2 is consistent with the guard hypothesis in that the Rcr3-Avr2 complex, not Avr2 alone or other Avr2-protease complexes, specifically activates Cf-2 (9). Inhibition of Rcr3 protease activity is not sufficient for activation of Cf-2 suggesting that some Avr2 mediated alteration in the conformation of Rcr3 is recognized by Cf-2 (9).

One prediction of the guard hypothesis is that host (virulence) targets are important components of basal defense. Consequently, effectors that disable the same host target are predicted to evolve independently (2). One example is the three different *Pseudomonas syringae* type III secretion system effectors that affect the host target RIN4 of *Arabidopsis thaliana* (2). Two of these effectors, AvrRpm1 and AvrB, induce phosphorylation of RIN4 (10), whereas AvrRpt2 is a protease that cleaves RIN4 at two sites (11, 12). Similarly, the tomato serine-threonine protein kinase Pto is targeted by two unrelated *P. syringae* effectors, AvrPto and AvrPtoB (2, 13). In this study, we further support the predictions of the guard hypothesis by demonstrating that the

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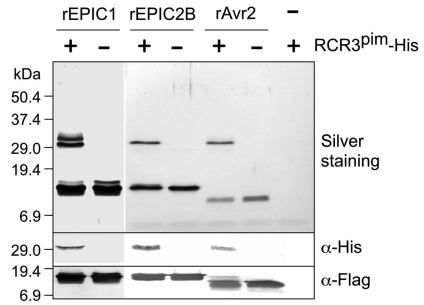

Fig. 1. Like Avr2, EPIC1 and EPIC2B were observed to physically interact with Rcr3^{pim}. coimmunoprecipitations of rEPIC1, rEPIC2B, and rAvr2 with Rcr3^{pim}-His using FLAG antisera. Eluates from coimmunoprecipitations of rEPICs or rAvr2 with proteins in apoplastic fluids from *N. benthamiana* leaves infiltrated with *Agrobacterium tumefaciens* carrying the binary vector pCB302-Rcr3^{pim}-His (Rcr3^{pim}-His +) or pCB302–3 (Rcr3^{pim}-His-) were separated on SDS/PAGE gel followed by silver staining and Western blot. The + and – signs refer to the presence or absence of Rcr3^{pim}-His, respectively. Western blot with anti-FLAG showed the recovery of rEPICs and rAvr2 from the elution fractions, whereas the blot with anti-His revealed the presence of the Rcr3^{pim}-His in the elution fractions. The ≈30-kDa band that was pulled down with rEPICs or rAvr2 corresponds to Rcr3^{pim}-His. The size (kDa) of the molecular weight markers is shown on the left.

virulence target Rcr3^{pim} is affected by distinct effectors from two phylogenetically unrelated pathogens. Besides Avr2 of the fungus *C. fulvum*, we show that the secreted effectors EPIC1 and EPIC2B of the oomycete pathogen *Phytophthora infestans* also bind and inhibit Rcr3^{pim}. Unlike Avr2, EPIC1 and EPIC2B did not trigger hypersensitivity in Cf-2/Rcr3^{pim} tomato plants. However, Rcr3^{pim} contributed to defense against *P. infestans* strains that express EPIC1 and EPIC2B. These results provide a compelling example of effectors that evolved in two phylogenetically unrelated pathogens to target the same host target, thereby fulfilling a key prediction of the guard hypothesis.

Results

Like Avr2, EPIC1 and EPIC2B Physically Interact with Rcr3^{pim}. We previously showed that the potato and tomato late blight pathogen *P. infestans* secretes a family of cystatin-like cysteine protease inhibitors, named EPICs (14). Annotation of the recently completed genome sequence of *P. infestans* strain T30–4 (GenBank accession no. AATU01000000) revealed 6 *epiC* genes, four of which are clustered within a 70-kb region in supercontig 14 and are unique to *P. infestans* relative to the sequenced genomes of *Phytophthora sojae* and *Phytophthora ramorum* [(supporting information (SI) Fig. S1)]. Among the products of these four genes, EPIC2B but not EPIC1 was shown to physically interact with and inhibit a tomato protease PIP1 (*Phytophthora* inhibited protease 1) that is related to Rcr3 (14). We therefore hypothesized that Rcr3^{pim} is also targeted by EPIC2B and possibly EPIC1. Co-immunoprecipitation was used to investigate the interaction between the EPICs and Rcr3^{pim}. We expressed His tagged Rcr3^{pim} in *N. benthamiana* apoplast using *Agrobacterium tumefaciens*-mediated transient protein expression (agroinfiltration), while recombinant FLAG tagged EPIC1, EPIC2B, and Avr2 proteins (rEPIC1, rEPIC2B and rAvr2, respectively) were expressed and purified from *Escherichia coli* supernatants (see Materials and Methods). FLAG antibody agarose bead immunoprecipitations resulted in the recovery of rEPIC1, rEPIC2B, and rAvr2 proteins in all samples except the negative controls (Fig. 1). In addition, a 30-kDa protein corresponding to Rcr3^{pim}-His was pulled down only in the presence of rEPICs/rAvr2 (Fig. 1). Western blot analyses showed that α-His antisera interacts with the 30-kDa bands (Fig. 1), suggesting physical interaction between Rcr3^{pim} and rEPIC1, rEPIC2B, or rAvr2. No degradation of the EPICs during incubation with Rcr3^{pim} was evident indicating that like Avr2, the EPICs are not a substrate for Rcr3^{pim}.

Like Avr2, EPIC1 and EPIC2B Inhibit Rcr3^{pim}. Because rEPIC1 and rEPIC2B physically interacts with Rcr3^{pim}, we tested whether they inhibit Rcr3^{pim} using the DCG-04 protease profiling assay (9, 14). DCG-04 is a biotinylated probe that covalently binds to cysteine proteases of the papain family and can be detected by streptavidin-horseradish peroxidase (HRP)(15, 16). E-64, an irreversible cysteine protease inhibitor (17), is used here as a positive inhibition control. Protease activity profiling with 220 nmol/l DCG-04 was performed in the absence of inhibitors or in the presence of rEPIC1 (2 μmol/l), rEPIC2B (2 μmol/l) or rAvr2 (2 μmol/l). E-64 (1 μmol/l) and the *P. infestans* serine protease inhibitor EPI1 (2 μmol/l) were used as positive and negative controls, respectively. Detection with streptavidin-HRP revealed that labeling of Rcr3^{pim} by DCG-04 was strongly reduced in the presence of the rEPICs, whereas Rcr3^{pim} was not biotinylated in the presence of E-64 or rAvr2 (Fig. 2*A* and Fig. S2). This indicates that rEPIC1 and rEPIC2B inhibit Rcr3^{pim} cysteine protease activity, although to a lesser degree than E-64 and rAvr2.

To further examine the dynamics of Rcr3^{pim} inhibition by the EPICs, we performed a time course of DCG-04 protease activity profiling. Acetone was added after 0, 10, 20, 30, 60, or 120 minutes after DCG-04 labeling to stop the labeling reaction and to precipitate the protein complexes. In the absence of inhibitors, biotinylated Rcr3^{pim}-His became clearly detectable 10 minutes after addition of DCG-04 and reached a maximal level at 120 minutes (Fig. 2*B*). In contrast, preincubation with rEPIC2B blocked DCG-04 labeling of Rcr3^{pim}-His at 30 minutes and reduced labeling at 60–120 minutes, confirming that Rcr3^{pim} inhibition by rEPIC2B is weaker than inhibition by rAvr2 and E-64.

Modification of Rcr3^{pim} During Infection by *P. infestans*. Experiments toward confirmation of Rcr3 inhibition during infection of tomato by *P. infestans* were unsuccessful because Rcr3 is present in low levels in the tomato apoplast. We therefore elected to exploit the *N. benthamiana* agroinfiltration assay to assess the extent to which Rcr3 is modified during infection by *P. infestans*. We expressed HA tagged Rcr3^{pim} (18) in *N. benthamiana* apoplast as described above. Two days after agroinfiltration, leaves were inoculated with *P. infestans* zoospores. Apoplastic fluids were harvested over 3–5 days and analyzed by Western blots hybridized with HA antiserum. In noninfected control leaves, Rcr3^{pim}-HA appeared as two bands at ≈25–30 kDa in sodium dodecyl sulfate-polyacrylamide gel eletrophoresis (SDS/PAGE) and as one major immunoreactive band in native PAGE (Fig. 2*C*). However, as infection progressed from 3 to 5 days postinoculation, two higher–molecular-weight Rcr3^{pim}-HA bands appeared in the SDS/PAGE Westerns, suggesting that Rcr3^{pim} is modified during interaction with *P. infestans*. Remarkably, although Rcr3^{pim}-HA was readily detectable in native PAGE of apoplastic extracts from noninfected leaves, a significantly weaker Rcr3^{pim}-HA band was observed at 3 days postinoculation; and, at 4–5 days postinoculation, Rcr3^{pim}-HA did not enter the gel. Overall, these results suggest that Rcr3^{pim}-HA is modified during *P. infestans* infection, possibly by engaging in high–molecular-weight complexes involving inhibitor proteins such as the EPICs.

Unlike Avr2, EPIC1 and EPIC2B Do Not Trigger the Hypersensitive Response on Cf-2/Rcr3^{pim} Tomato. To investigate whether the EPICs trigger the HR on Cf-2/Rcr3^{pim} tomato, we infiltrated leaves of tomato plants with purified rEPIC1 and rEPIC2B side by side with purified rAvr2. Unlike rAvr2, the rEPICs did not trigger

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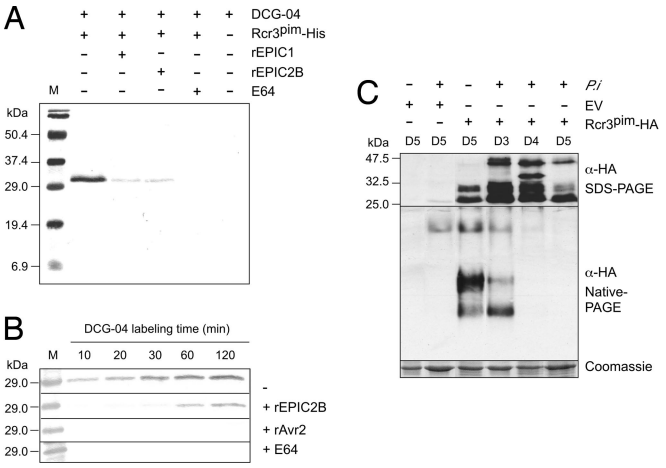

Fig. 2. Like Avr2, EPIC1 and EPIC2B inhibit Rcr3^{pim}. (*A*) EPIC1 and EPIC2B inhibit Rcr3^{pim}. Inhibition of Rcr3^{pim} produced in *N. benthamiana* apoplast by rEPIC1 and rEPIC2B. Apoplastic fluid was isolated from *N. benthamiana* expressing Rcr3^{pim}-His. Protease activity profiling with 220 nmol/l DCG-04 was performed in the absence (–) of inhibitor or in the presence (+) of E-64, FLAG-EPIC1 (rEPIC1), or FLAG-EPIC2B (rEPIC2B). Rcr3^{pim}-His were captured (pulled down) by Ni-NTA beads, electrophoresed on an SDS gel, and detected with streptavidin-HRP. The DCG-04 labeling reactions were stopped at 30 minutes by adding ice-cold acetone into the reaction mix. Detection with streptavidin-HRP revealed that Rcr3^{pim}-His was not biotinylated in the presence of E-64, whereas biotinylation of Rcr3^{pim} occurred without inhibitor. The biotinylation was significantly reduced in samples containing rEPICs, indicating that both rEPIC1 and rEPIC2B inhibited Rcr3^{pim} cysteine protease activity. Approximate molecular weights of the labeled Rcr3^{pim}-His proteins are shown on the left side. (*B*) Unlike Avr2, EPIC2B was shown to be a weak inhibitor of Rcr3^{pim}. The time course of labeling with DCG-04 was used to analyze the inhibition of Rcr3^{pim} by rEPIC2B, rAvr2, or E-64. Protease activity profiling with 220 nmol/l DCG-04 was performed in the absence (−) of inhibitor or in the presence (+) of E-64, FLAG-Avr2 (rAvr2), or FLAG-EPIC2B (rEPIC2B). Rcr3^{pim}-His was captured (pulled down) by Ni-NTA beads, electrophoresed on an SDS gel, and detected with streptavidin-HRP for biotinylation by DCG-04. Acetone was added after 0, 10, 20, 30, 60, or 120 minutes after adding DCG-04 to stop the labeling reaction and to precipitate the protein complex. Approximate molecular weights of the labeled Rcr3^{pim}-His proteins are shown on the left side. (*C*) Modification of Rcr3^{pim} during infection by *P. infestans*. Rcr3^{pim}-HA was transiently expressed in *N. benthamiana* leaves by agroinfiltration. The leaves were inoculated with *P. infestans* zoospores 2 days postinfiltration, and apoplastic fluids were harvested 3–5 days after infection. Similar amounts of proteins were separated by SDS- and native PAGE and were blotted onto nitrocellulose membranes. Rcr3^{pim}-HA proteins were probed with HA antibody followed by secondary antibody attached to HRP, which produced chemiluminescence upon exposure to luminol and H_2O_2 substrates. Approximate molecular weights are shown on the left. Rcr3^{pim}-HA luminescent bands were detected on x-ray films. The full length of the gel is shown for the native PAGE. Coomassie Blue staining was used to ascertain the equal loading of the gels. *P.i* = *P. infestans*, EV = empty vector infiltrations, D3-D5 = 3–5 days after infection.

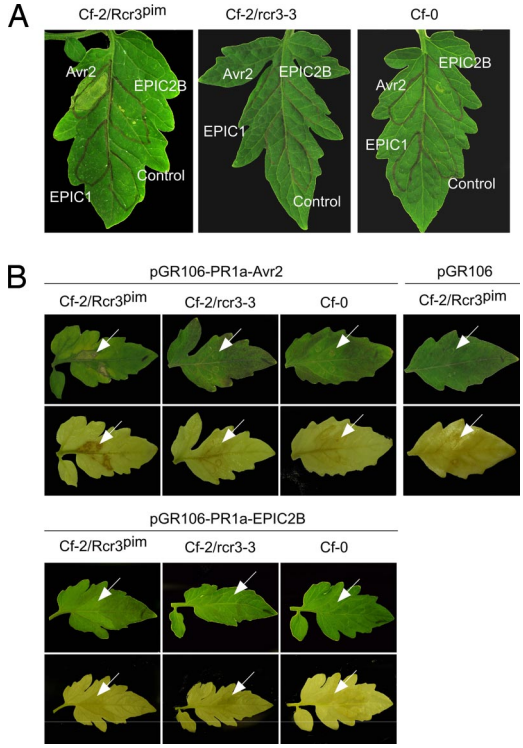

Fig. 3. Unlike Avr2, EPIC1 and EPIC2B were found not to trigger the hypersensitive response on Cf-2/Rcr3^{pim} tomato. (*A*) EPIC1 and EPIC2B proteins did not trigger the hypersensitive response on Cf-2/Rcr3^{pim} tomato. Purified proteins rAvr2, rEPIC1, and rEPIC2B were desalted and diluted to concentration of 0.4 μmol/l and then were infiltrated into tomato leaves. rAvr2 triggered the hypersensitive response (HR) on Cf-2/Rcr3^{pim} but not on Cf-2/rcr3–3 or Cf-0 tomato plants. rEPIC1 and rEPIC2B did not trigger the HR on any of these tomato plants. Buffer solution with neither rAvr2 nor rEPIC proteins was used as a negative control, and the experiment was repeated three times with the same results. (*B*) Potato virus X assays confirmed that EPIC2B does not trigger the hypersensitive response on Cf-2/Rcr3^{pim} tomato. Hypersensitive cell death (*top panels*) and hydrogen peroxide production (*bottom panels*) were detected on tomato leaves inoculated with *Potato virus X* expressing Avr2 or EPIC2B. Leaves of Cf-2/Rcr3^{pim}, Cf-2/rcr3–3, and Cf-0 tomato plants were infiltrated with *Agrobacterium tumefaciens* GV3101 carrying the PVX constructs pGR106-PR1a-Avr2, pGR106-PR1a-EPIC2B, or pGR106 (vector control). The HR and hydrogen peroxide accumulation, visualized by 3, 3′-diaminobenzidine (DAB) staining, were detected only on Cf-2/Rcr3^{pim} leaves inoculated with the Avr2 construct.

visible cell death symptoms on Cf-2/Rcr3^{pim} tomato plants (Fig. 3*A*), did not trigger autofluorescence typical of the HR (Fig. S3), and did not induce the expression of tomato *PR1a* gene (Fig. S4). Assays with high concentrations (2 μmol) of purified rEPIC1 and rEPIC2B also failed to trigger the HR on Cf-2/Rcr3^{pim} tomato (data not shown). These results indicate that although the EPICs inhibit Rcr3^{pim}, they do not activate Cf2-mediated hypersensitivity in sharp contrast to Avr2. To independently validate the finding that the EPICs do not activate Cf2 hypersensitivity, we cloned the inhibitors in the *Potato virus X* vector pGR106, which enables high expression levels *in planta* (19, 20). Constructs pGR106-PR1a-EPIC2B and pGR106-PR1a-Avr2, carrying a fusion between the signal peptide of the tobacco PR1a protein and the mature portion of the inhibitor proteins, were used in agroinfiltration assay on tomato. Consistent with the protein infiltration results, the EPIC2B construct failed to trigger cell death symptoms or the accumulation of hydrogen peroxide, whereas Avr2 triggered hydrogen peroxide production in Cf-2/Rcr3^{pim} but not in Cf-2/rcr3–3 or Cf-0 tomato leaves (Fig. 3*B*).

Tomato *Rcr3^{pim}* Mutant Shows Enhanced Susceptibility to *P. infestans*. To test whether *Rcr3^{pim}* contributes to defense against *P. infestans*, we used a standard zoospore inoculation assay on detached leaves from tomato Cf-2/Rcr3^{pim} and Cf-2/rcr3–3, an isogenic mutant that carries a premature stop codon in the *Rcr3^{pim}* gene (8, 9). Lesions were measured at 3, 4, and 5 days postinoculation. Lesion growth rate (mm/day) from two time periods (days 3–4 and days 4–5) was used to quantify the susceptibility of the different tomato lines to *P. infestans*. Difference in the lesion growth rate between the Cf-2/Rcr3^{pim} and Cf-2/rcr3–3 was statistically significant at the $P < 0.00001$ level (Fig. 4), suggesting that tomato plants carrying the *rcr3–3* mutation show enhanced susceptibility to *P. infestans* compared with the *Rcr3* wild type (Fig. 4). Similar results were obtained in four independent

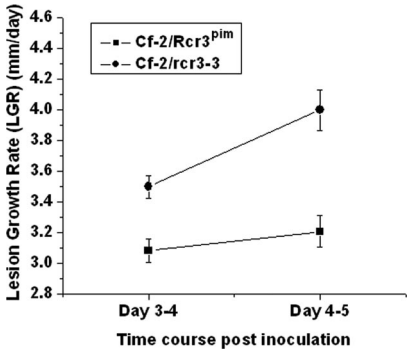

Fig. 4. Rcr3^{pim} contributed to defense against *P. infestans*. Graphic view of lesion growth rates deducted from an infection time course revealed that Cf-2/rcr3–3 plants are more susceptible to *P. infestans* than Cf-2/Rcr3^{pim}. Lesions were measured at 3–5 days postinoculation. Lesion growth rate (mm/day) was used to quantify the susceptibility of the different tomato genotypes. Data were analyzed by Microsoft Excel and Origin 7.0. The graph depicts a representative experiment among six repeats.

experiments with strain 90128 and in two experiments with strain 88069.

Discussion

The current view of molecular plant–pathogen interactions is that pathogens secrete effector proteins to disable plant defense components known as virulence (effector) targets. Occasionally, the perturbations caused by these effectors trigger innate immunity via plant disease resistance proteins as described by the "guard hypothesis" (2). This model predicts that the host targets of pathogen effectors are important components of basal defense; and, as a result, effectors that disable the same host target are expected to evolve independently (2). Our findings satisfy some predictions of the guard hypothesis and expand on previous reports that different *P. syringae* effectors target the same host proteins. Here we show that two phylogenetically unrelated pathogens of tomato, the fungus *C. fulvum* and the oomycete *P. infestans*, evolved unrelated apoplastic effectors (Avr2 and EPIC1/EPIC2B, respectively) to target the same defense-related protease Rcr3^{pim}. Like Avr2, EPIC1 and EPIC2B bind and inhibit Rcr3^{pim}. However, unlike Avr2, EPIC1 and EPIC2B are weak inhibitors of Rcr3^{pim} and do not trigger hypersensitive cell death on Cf-2/Rcr3^{pim} tomato. Furthermore, the *rcr3–3* null mutant of tomato showed enhanced susceptibility to *P. infestans*, suggesting that Rcr3^{pim} contributes to defense against *P. infestans*.

Our findings led us to suggest a model illustrating the targeting and inhibition of a common tomato defense protease by different effectors from two unrelated pathogens. We propose that Avr2, EPIC1, and EPIC2B target Rcr3^{pim} to suppress a possible defense activity of this protease. In tomato plants carrying the resistance protein Cf-2, the Avr2-Rcr3^{pim} complex activates hypersensitivity and innate immunity resulting in resistance against *C. fulvum*. On the other hand, the EPICs inhibit Rcr3^{pim} without activating Cf-2, possibly because, unlike Avr2, EPIC1 and EPIC2B are weak inhibitors of Rcr3^{pim}. Thus, compared with *C. fulvum*, *P. infestans* has evolved stealthy effectors that inhibit the virulence target in the host but avoid being recognized by the innate immunity surveillance system of the plant. However, it remains to be determined whether Cf2 recognizes alleles of the *epiC* genes other than *epiC1* and *epiC2B*.

The molecular mechanism of the differential recognition of the Rcr3-inhibitor complexes by Cf-2 remains unknown. On one hand, weaker protease-inhibitor interactions might result in lower concentration of the protease-inhibitor complexes that are perceived by Cf-2, thus repressing EPIC recognition when compared with Avr2. On the other hand, EPIC-Rcr3 complexes are probably different from Avr2-Rcr3 complexes, and EPICs might even cause different conformational changes in Rcr3 than Avr2. The three-dimensional structures of the tomato protease Rcr3^{pim} as well as the effector Avr2 are unknown even though the structures of the EPICs and Rcr3 can be predicted by comparative modeling (21) (Fig. S1). Future elucidation of the three dimensional structures of Rcr3^{pim} in complex with Avr2 or EPICs will help to determine whether conformational changes in Rcr3 form the basis of recognition by Cf-2.

We reported previously that EPIC2B but not EPIC1 binds and inhibits PIP1, a salicylic acid–induced, papain-like protease of tomato that is related to Rcr3 (14). Recently, Avr2 was shown to inhibit PIP1 in addition to Rcr3 (21, 22). The finding that the EPICs and Avr2 target various apoplastic cysteine proteases provides an example of effectors that manipulate several host targets (2, 23). The next question is to determine which of the proteases targeted by Avr2 and the EPICs are the operative targets, e.g., the targets that when inhibited result in enhanced pathogen fitness (23). Interestingly, Rcr3 may not contribute to defense against *C. fulvum* (8) although it plays a role in defense against *P. infestans* (Fig. 4). These findings raise questions about whether Rcr3 is truly a "decoy" that is detected by Cf2 to activate resistance but with no function in host defense or susceptibility in the absence of its cognate R protein (23). The degree to which the EPICs contribute to virulence remains to be determined. Attempts to knock down the *epiC* genes have been unsuccessful so far and are further complicated by the occurrence of multiple paralogs in the *P. infestans* genome (Fig. S1).

Infection assays with the rcr3–3 mutant indicated that Rcr3^{pim} clearly contributes to defense against *P. infestans*. However, we were unable to test whether the defense function of Rcr3^{pim} requires Cf-2, because of the unavailability of tomato lines that vary in the presence of *Cf-2* but still retain *Rcr3^{pim}* (*Cf-2*/*Rcr3^{pim}* vs. -/*Rcr3^{pim}*). Both the *Cf-2* and *Rcr3^{pim}* genes were cointrogressed in tomato from the wild relative *S. pimpinellifolium* because *Rcr3^{esc}*, the tomato allele of *Rcr3^{pim}*, confers an autonecrosis phenotype in the presence of Cf-2 (*Rcr3^{esc}* is also known as *ne*, *necrosis*) (25). Thus, it remains possible that the EPIC-Rcr3^{pim} complexes weakly activate Cf-2, resulting in partial resistance to *P. infestans*. However, this is doubtful in light of our finding that, unlike Avr2, EPIC2B does not induce the expression of the defense gene *PR1a* in *Cf-2*/*Rcr3^{pim}* tomato (Fig. S4).

Materials and Methods

Plants, Bacterial Strains, and Plasmids. Tomato (*Solanum lycopersicum*) isogenic lines Cf-0 (Moneymaker), Cf-2/Rcr3^{pim}, and Cf-2/rcr3–3 (rcr3 premature stop mutant) (8, 25), as well as and *Nicotiana benthamiana* were grown at 25 °C, 60% humidity, and under 16 hours light/8 hours dark cycles. *E. coli* DH5α, BL21, and *Agrobacterium tumefaciens* GV3101 were routinely grown in LB medium (26) at 37 °C and 28 °C, respectively. pCB302-Rcr3^{pim}-His was generated in the *A. tumefaciens* binary vector pCB302–3 (27) using SpeI and XbaI anchored primers (RCR3Spe: 5′-GCGACTAGTATGGCTATGAAAGTTGATTTGATG - 3′ and RCR3Xba_R: 5′-GCGTCTAGATTAgtgatggtgatggtgatgCGCTATGTTTGGATAAGAAGAC - 3′) and the pMWBinRcr3^{pim}: His: HA plasmid as template (9). Plasmid pFLAG-Avr2 was constructed by cloning overlap PCR products corresponding to an *E. coli* codon optimized Avr2 into the vector pFLAG-ATS (Sigma). *A. tumefaciens* binary *Potato virus X* (PVX) plasmids pGR106-PR1a-EPIC2B and pGR106-PR1a-Avr2, carrying a fusion between the signal peptide of the tobacco PR1a protein and the mature portion of the inhibitor proteins, were constructed by overlap PCR as described previously (28). The recombinant proteins used in this study are shown in Fig. S5.

Protein Expression and Purification. Expression and purification of FLAG-EPIC1 (rEPIC1), FLAG-EPIC2B (rEPIC2B), FLAG-Avr2 (rAvr2), and FLAG-EPI1 (rEPI1) in *E. coli* using plasmids pFLAG-EPIC1, pFLAG-EPIC2B, pFLAG-Avr2, and pFLAG-EPI1 were conducted as described earlier (29, 30). Protein concentrations were determined using the BioRad protein assay (BioRad Laboratories). To assess purity, eluted fractions of purified protein were subjected to a SDS/PAGE gel followed by silver nitrate staining (31). (See also Fig. S6.)

PLANT BIOLOGY

Transient *In Planta* Expression. *In planta* transient expression by agroinfiltration was performed according to methods described elsewhere (19, 25). *A. tumefaciens* strains GV3101 carrying plasmids pCB302-Rcr3^{pim}-His, empty vector pCB302–3 (27) and pJL3-p19–55 (provided by Dr. John Lindbo, Campbell's Seeds) were used. pJL3-p19–55 is a construct expressing the P19 protein of tomato bushy stunt virus (TBSV), a suppressor of posttranscriptional gene silencing in *N. benthamiana* (32). Overnight, *A. tumefaciens* cultures were harvested by centrifugation at 2000 *g* for 20 minutes and resuspended in 10 mmol/l $MgCl_2$, 10 mmol/l Mes (pH 5.6), and 150 μmol/l acetosyringone. Resuspended *A. tumefaciens* cells with an optical density (OD_{600}) of 1.0 were mixed with equal volume of *A. tumefaciens* carrying pJL3-p19–55. The mixtures were kept at room temperature for 3 hours and then infiltrated into leaves of 6-week-old *N. benthamiana* plants. Apoplastic fluids from infiltrated leaves were isolated 4 days after infiltration as described elsewhere (14, 30), filter sterilized (0.45 μmol/l), and used immediately or stored at −80 °C. Agroinfiltration assays on tomato were performed using *A. tumefaciens* strains carrying *Potato virus X* vector pGR106 as described elsewhere (19, 33).

SDS/PAGE and Western Blot Analysis. Proteins were subjected to 15% SDS/PAGE. Afterward, gels were either stained with Coomassie Brilliant Blue or transferred to nitrocellulose membrane (BioRad Laboratories) using a Mini transBlot apparatus (BioRad Laboratories). Western blotting was used to detect the protein with the alkaline phosphatase kit (BioRad Laboratories). Anti-His alkaline phosphatase–conjugated antisera and anti-FLAG antisera were purchased from Sigma.

Coimmunoprecipitations. Coimmunoprecipitation of rEPICs/rAvr2 and proteins present in *N. benthamiana* intercellular fluids (apoplastic extracts) was performed using the FLAG-tagged protein immunoprecipitation kit (Sigma) as described previously (14, 30). An 800-pmol quantity of purified rEPIC2B was preincubated with 500 μl of *N. benthamiana* intercellular fluids expressing Rcr3^{pim}-His for 30 minutes at 25 °C. A 40-μl quantity of anti-FLAG M2 resin was added and incubated at 4 °C for 2 hours with gentle shaking. The precipitated protein complexes were eluted in 60 μl with 150 ng/μl FLAG peptide solution and were analyzed by SDS/PAGE and Western blot.

DCG-04 Activity Profiling Assays. Activity profiling of Rcr3^{pim} in *N. benthamiana* intercellular fluids was performed as described elsewhere (9, 34) using DCG-04, a biotinylated analog of the irreversible cysteine protease inhibitor E-64 (15, 34). A 50-μl quantity of intercellular fluids from *N. benthamiana* expressing Rcr3^{pim}-His was diluted 10-fold in DCG-04 assay buffer (50 mmol/l sodium acetate, 10 mmol/l L-cysteine, pH 5.0) to a final volume of 500 μl. The diluted samples were preincubated with an excess of E-64 (1020 nmol/l; Sigma) or rEPICs (2 μmol/l), rAvr2 (2 μmol/l), or the serine protease inhibitor EPI1 (2 μmol/l) (30) for 1 hour at room temperature. DCG-04 (220 nmol/l) was then added to each reaction and the mixtures were incubated for 30 minutes at room temperature. Proteins were precipitated by adding 1 ml ice-cold acetone (−20 °C) followed by centrifugation at 13,000 × *g* for 30 minutes at 4 °C. The pellets were washed with ice-cold 70% (wt/wt) acetone, air dried, and resuspended in 500 μl Tris-buffered saline (TBS) buffer (50 mmol/l Tris/HCl, 150 mmol/l NaCl, and pH 7.5). To capture the biotinylated proteins, 20 μl Ni-NTA magnetic beads (Qiagen) were added to each reaction and incubated for 16 hours at 4 °C. The Ni-NTA beads were washed with 1 ml TBS buffer using a magnetic stand (Promega) to remove nonspecifically bound proteins. Biotin labeled proteins were harvested by boiling the magnetic beads in 25 μl Laemmli SDS loading buffer and subjected to SDS/PAGE (12%) and Western blotting. Biotin-labeled proteins were detected by probing the membrane with streptavidin-HRP polymers (Sigma) followed by color development using the substrate 3,3′-diaminobenzidine (Sigma). For the time course assay, rEPIC2B (2 μmol/l), Avr2 (2 μmol/l), and E-64 (1 μmol/l, Sigma) were preincubated with 10 times DCG-04 assay buffer that was diluted in *N. benthamiana* apoplastic fluids containing Rcr3^{pim}-His for half an hour at room temperature. DCG-04 (220 nmol/l) was then added to each reaction to label the cysteine proteases with biotin. Ice-cold acetone was added at 0, 10, 20, 30, 60, or 120 minutes after DCG-04 labeling to stop the labeling reaction and to precipitate the protein complexes. Further procedures and detection were as described above.

Detection of Rcr3 During *P. infestans* Infection. We transiently expressed HA tagged Rcr3^{pim} (18) in *N. benthamiana* apoplast as described above. Two days after agroinfiltration, leaves were inoculated with *P. infestans* zoospores (10^5 ml^{-1}, as outlined below) and apoplastic fluids were harvested over 3–5 days as the pathogen fully colonized the leaves. Proteins were separated on SDS/PAGE and native PAGE and were analyzed by Western blots using HA antiserum as probe. Native PAGE (12% acrylamide) was performed following the standard methods for SDS/PAGE except that SDS and reducing agents were omitted and samples were not boiled.

Hypersensitive Response Assays. Affinity purified rEPIC1, rEPIC2B, and rAvr2 proteins were cleaned and concentrated using Microcon YM-3 Centrifugal Filter Unit (Millipore; 3 kDa cutoff) and diluted to 0.4 μmol/l final concentration in double distilled water before infiltration into tomato leaves. Hydrogen peroxide accumulation was visualized using 3, 3′- diaminobenzidine (DAB) as the substrate. Leaves were collected and floated in solutions containing 1 mg/ml DAB for 5–8 hours at 25 °C. The treatment was terminated and the leaves were bleached by immersion in boiling ethanol (95%) for 3 minutes. RNA isolation and reverse transcription polymerase chain reaction (RT-PCR) were performed as previously described (14, 21). Primers were PR1-f: 5′-ATAGACGATACTTTAATAAGGACGTTCTCC-3′; PR1r 5′-TTGTTACTCACTTGTCT-CATGGTATTAGCC-3′actin-f 5′-ATGAAGCTCAATCCAAGAGGGGTATC-3′; and actin-r 5′-CTCCTGCTCATAGTCAAGAGCCAC-3′.

Virulence Assays. *P. infestans* strains 90128 and 88069 that express the EPICs were cultured on rye sucrose medium at 18 °C in the dark for 2 weeks before zoospores were collected. Plates were then flooded with chilled ddH_2O and incubated at 4 °C for at least 2 hours to release zoospores. The suspension medium was then measured for spore concentration using a hemacytometer and the concentration was adjusted to 10^5 zoospores per milliliter. Droplets (10 μl) were used to inoculate the underside of detached tomato leaves. Leaves were incubated in moist trays at 18 °C. Disease lesions size (mm) were measured at 3, 4, and 5 days after inoculation, and the lesion growth rate was calculated for days 3–4 and days 4–5. All disease score data were subjected to statistical analysis using the Origin 7.0 software and one-way analysis of variance.

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1. Chisholm ST, Coaker G, Day B, Staskawicz BJ (2006) Host-microbe interactions: Shaping the evolution of the plant immune response. *Cell* 124:803–814.
2. Jones JDG, Dangl JL (2006) The plant immune system. *Nature* 444:323–329.
3. Deslandes L, Olivier J, Peeters N, Feng DX, Khounlotham M, *et al.* (2003) Physical interaction between RRS1-R, a protein conferring resistance to bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus. *Proc Natl Acad Sci USA* 100:8024–8029.
4. Dodds PN, Lawrence GJ, Catanzariti AM, Teh T, Wang CI, *et al.* (2006) Direct protein interaction underlies gene-for-gene specificity and coevolution of the flax resistance genes and flax rust avirulence genes. *Proc Natl Acad Sci USA* 103:8888–8893.
5. Dangl JL, Jones JDG (2001) Plant pathogens and integrated defence responses to infection. *Nature* 411:826–833.
6. Innes RW (2004) Guarding the goods. New insights into the central alarm system of plants. *Plant Physiol* 135:695–701.
7. Van der Biezen EA, Jones JDG (1998) Plant disease resistance proteins and the gene-for-gene concept. *Trends Plants Sci* 23:454–456.
8. Dixon MS, Golstein C, Thomas CM, van Der Biezen EA, Jones JD (2000) Genetic complexity of pathogen perception by plants: The example of. Rcr3, a tomato gene required specifically by *Cf-2. Proc Natl Acad Sci USA* 97:8807–8814.
9. Rooney HCE, van't Klooster JW, van der Hoorn RAL, Joosten MHAJ, Jones JDG, de Wit PJGM (2005) *Cladosporium* Avr2 inhibits tomato Rcr3 protease required for Cf-2-dependent disease resistance. *Science* 308:1785–1786.
10. Mackey D, Holt BF, Wiig A, Dangl JL (2002) RIN4 interacts with *Pseudomonas syringae* type III effector molecules and is required for RPM1 mediated disease resistance in *Arabidopsis. Cell* 108:743–754.
11. Kim HS, Desveaux D, Singer AU, Patel P, Sondek J, Dangl JL (2005) The *Pseudomonas syringae* effector AvrRpt2 cleaves its C-terminally acylated target, RIN4, from *Arabidopsis* membranes to block RPM1 activation. *Proc Natl Acad Sci USA* 102:6496–6501.
12. Chisholm ST, Dahlbeck D, Krishnamurthy N, Day B, Sjolander K, Staskawicz BJ (2005) Molecular characterization of proteolytic cleavage sites of the *Pseudomonas syringae* effector AvrRpt2. *Proc Natl Acad Sci USA* 102:2087–2092.

13. Abramovitch RB, Martin GB (2005) AvrPtoB: A bacterial type III effector that both elicits and suppresses programmed cell death associated with plant immunity. *FEMS Microbiol Lett* 245:1–8.
14. Tian M, Win J, Song J, van der Hoorn R, van der Knaap E, Kamoun S (2007) A *Phytophthora infestans* cystatin-like protein targets a novel tomato papain-like apoplastic protease. *Plant Physiol* 143:364–377.
15. Greenbaum D, Medzihradszky KF, Burlingame A, Bogyo M (2000) Epoxide electrophiles as activity-dependent cysteine protease profling and discovery tools. *Chem Biol* 7:569–581.
16. Greenbaum D, Arnold W, Lu F, Hayrapetian L, Baruch A, *et al.* (2002) Small molecule affinity fingerprinting: A tool for enzyme family subclassification, target identification, and inhibitor design. *Chem Biol* 9:1085–1094.
17. Barrett AJ, Kembhavi AA, Brown MA, Kirschke H, Knight CG, *et al.* (1982) L-trans-epoxysuccinyl-leucylamido(4-guanidino butane (E-64) and its analogues as inhibitors of cysteine proteinases including cathepsins B, H and L. *Biochem J* 201:189–198.
18. Lindbo J (2007) TRBO: A high-efficiency tobacco mosaic virus RNA-based overexpression vector. *Plant Phys* 145:1232–1240.
19. Huitema E, Bos JIB, Tian M, Win J, Waugh ME, Kamoun S (2004) Linking sequence to phenotype in *Phytophthora*-plant interactions. *Trends Microbiol* 12:193–200.
20. Lu R, Malcuit I, Moffett P, Ruiz MT, Peart J, *et al.* (2003) High throughput virus-induced gene silencing implicates heat shock protein 90 in plant disease resistance. *EMBO J* 22:5690–5699.
21. Shabab M, Shindo T, Gu C, Kaschani F, Pansuriya T, *et al.* (2008) Fungal effector protein AVR2 targets diversifying defense-related Cys proteases of tomato. *Plant Cell* 20:1169–1183.
22. van Esse HP, Van't Klooster JW, Bolton MD, Yadeta KA, van Baarlen P, *et al.* (2008) The *Cladosporium fulvum* virulence protein Avr2 inhibits host proteases required for basal defense. *Plant Cell* 20:1948–1963.
23. van der Hoorn RAL, Kamoun S (2008) From guard to decoy: A new model for perception of plant pathogen effectors. *Plant Cell* 20:2009–2017.
24. Rzychon M, Chmiel D, Stec-Niemczyk J (2004) Modes of inhibition of cysteine proteases. *Acta Biochim Pol* 51:861–873.
25. Kruger J, Tomas CM, Golstein C, Dixon MS, Smoker M, *et al.* (2002) A tomato cysteine protease required for Cf-2-dependent disease resistance and suppression of autonecrosis. *Science* 296:745–747.
26. Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning*: *A Laboratory Manual*, *Ed 2.* Cold Spring Harbor Laboratory Press (Cold Spring Harbor, NY).
27. Xiang C, Han P, Lutziger I, Wang K, Oliver DJ (1999) A mini binary vector series for plant transformation. *Plant Mol Biol* 40:711–717.
28. Kamoun S, Honee G, Weide R, Lauge R, Kooman-Gersmann M, *et al.* (1999) The fungal gene *Avr9* and the oomycete gene *inf1* confer avirulence to potato virus X on tobacco. *Mol Plant-Microbe Interact* 12:459–462.
29. Kamoun S, van West P, de Jong AJ, de Groot K, Vleeshouwers V, Govers F (1997) A gene encoding a protein elicitor of *Phytophthora infestans* is down-regulated during infection of potato. *Mol Plant-Microbe Interact* 10:13–20.
30. Tian M, Huitema E, Da Cunha L, Torto-Alalibo T, Kamoun S (2004) A Kazal-like extracellular serine protease inhibitor from *Phytophthora infestans* targets the tomato pathogenesis-related protease P69B. *J Biol Chem* 279:26370–26377.
31. Leck JR, Wiese TJ (2004) Purification and characterization of the L-fucose transporter. *Protein Expr Purif* 37:288–293.
32. Voinnet O, Rivas S, Mestre P, Baulcombe D (2003) An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *Plant J* 33:949–956.
33. Torto T, Li S, Styer A, Huitema E, Testa A, *et al.* (2003) EST mining and functional expression assays identify extracellular effector proteins from *Phytophthora. Genome Res* 13:1675–1685.
34. van der Hoorn RA, Leeuwenburgh MA, Bogyo M, Joosten MH, Peck SC (2004) Activity profiling of papain-like cysteine proteases in plants. *Plant Physiol* 135:1170–1178.