Dual disease resistance mediated by the immune receptor Cf-2 in tomato requires a common virulence target of a fungus and a nematode

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Plants lack the seemingly unlimited receptor diversity of a somatic adaptive immune system as found in vertebrates and rely on only a relatively small set of innate immune receptors to resist a myriad of pathogens. Here, we show that disease-resistant tomato plants use an efficient mechanism to leverage the limited nonself recognition capacity of their innate immune system. We found that the extracellular plant immune receptor protein Cf-2 of the red currant tomato (*Solanum pimpinellifolium*) has acquired dual resistance specificity by sensing perturbations in a common virulence target of two independently evolved effectors of a fungus and a nematode. The Cf-2 protein, originally identified as a monospecific immune receptor for the leaf mold fungus *Cladosporium fulvum*, also mediates disease resistance to the root parasitic nematode *Globodera rostochiensis* pathotype Ro1-Mierenbos. The Cf-2–mediated dual resistance is triggered by effector-induced perturbations of the apoplastic Rcr3^{pim} protein of *S. pimpinellifolium*. Binding of the venom allergen-like effector protein Gr-VAP1 of *G. rostochiensis* to Rcr3^{pim} perturbs the active site of this papain-like cysteine protease. In the absence of the Cf-2 receptor, Rcr3^{pim} increases the susceptibility of tomato plants to *G. rostochiensis*, thus showing its role as a virulence target of these nematodes. Furthermore, both nematode infection and transient expression of *Gr-VAP1* in tomato plants harboring *Cf-2* and *Rcr3^{pim}* trigger a defense-related programmed cell death in plant cells. Our data demonstrate that monitoring host proteins targeted by multiple pathogens broadens the spectrum of disease resistances mediated by single plant immune receptors.

parasitism | secretions | SCP/TAPS proteins | hypersensitive response

Dominant disease resistance genes encode highly specific immune receptors that offer plants protection against strains of pathogens carrying matching effector genes (1, 2). This phenomenon was captured in the "gene-for-gene" model of recognition specificity in disease resistance genes 60 y ago and still forms the basis of disease resistance breeding in major food crops (3). Plant pathogens secrete effectors into the apoplastic space and the cytoplasm of host cells to suppress the cell-autonomous defense responses of the plant and to cause disease by targeting specific host cell components (4). Plant immune receptors can activate effector-triggered immunity upon direct recognition of unique nonself signatures in these pathogen-derived effector molecules (5–11). However, as opposed to directly recognizing effectors, most plant immune receptors are thought to act as sensors of disease-signaling perturbations in the virulence targets of pathogen effectors (2, 12).

Unlike most vertebrates, plants lack the seemingly infinite receptor diversity of a somatic adaptive immune system to detect effectors of invasive pathogens (13). The molecular mechanisms by which plants are nonetheless able to fend off a myriad of attackers with a limited innate receptor repertoire are largely unknown (2). Recent studies show that independently evolving effectors of different plant pathogens interact with common apoplastic and cytoplasmic host proteins involved in defense-related signaling networks (14, 15). It is speculated that indirectly perceiving pathogen effectors via their actions on common virulence targets enable plants to leverage the limited recognition capacity of their immune receptor repertoire (16, 17). Here we show that the plant immune receptor protein Cf-2 of the currant tomato (*Solanum pimpinellifolium*) has acquired dual resistance specificity by sensing perturbations in a common virulence target of two different plant pathogens.

Tomato cultivars (*Solanum lycopersicum*) harboring the Cf-2 immune receptor originating from currant tomato are resistant to particular strains of *Cladosporium fulvum* secreting the effector protein Avr2 (18, 19). The *C. fulvum* effector Avr2 interacts with the extracellular papain-like cysteine protease Rcr3 of tomato, which is required for Cf-2–mediated fungal resistance (18, 19). It is thought that specific perturbations of Rcr3 by Avr2 activate Cf-2 function in immune signaling cascades, resulting in effector-triggered immunity to the fungus. Tomato cells secrete a range of papain-like cysteine proteases into the apoplast, at least two of which are inhibited by Avr2 [i.e., Rcr3 and PIP1 (20, 21)]. Here, we report on an effector (hereafter named Gr-VAP1) of the obligate plant-parasitic nematode *Globodera rostochiensis* that interacts among others with a papain-like cysteine protease highly similar to Rcr3 and PIP1 in a yeast two-hybrid screen of tomato root cDNA. Gr-VAP1 belongs to a class of secreted venom allergen-like proteins that occur in all plant and animal-parasitic nematodes (22) and that share no sequence similarity with the effector Avr2 of *C. fulvum*. This prompted us to investigate whether Gr-VAP1–induced perturbations of Rcr3 can nonetheless activate Cf-2–

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AJ536826 (Gr-VAP1) and JX100461 (interacting yeast two-hybrid clone INT9)].

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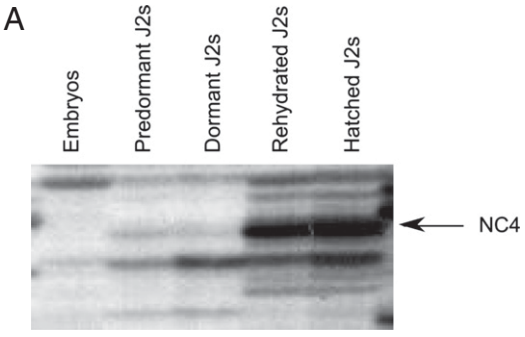
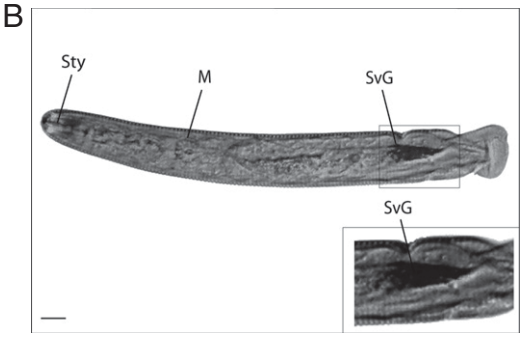
mediated resistance to *G. rostochiensis* in tomato plants. We found that *Cf-2*, which has been used for decades in tomato as a resistance gene against the leaf mold fungus *C. fulvum* (18), also confers resistance to the potato cyst nematode *G. rostochiensis*.

Results and Discussion

***G. rostochiensis* Secretes a Venom Allergen-Like Protein.** The expression of effectors in the potato cyst nematode *G. rostochiensis* is turned on when the obligate dormant period ends and the nematode begins to hatch from eggs in the soil. To identify effectors of *G. rostochiensis*, we conducted a cDNA–amplified fragment length polymorphism (AFLP)-based transcriptome analysis during this transition in metabolic activity of the nematodes (23). Our analysis revealed, among others, a transcript-derived fragment of 176 base pairs named NC4, which was strongly up-regulated in infective juveniles (Fig. 1*A*). Rapid amplification of the 5′ and 3′ ends of a matching expressed-sequence tag (GenBank accession no. AW506232) resulted in a full-length cDNA sequence of 926 base pairs, encoding a predicted secreted protein of 21,900 Da. The predicted protein sequence showed a significant similarity with Secreted Cysteine-rich Protein domains (SCP; GenBank accession no. CD05380, with E-value $2e^{-28}$), which are found in all members of the CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 proteins) protein superfamily. The NC4 protein is most similar to so-called Venom Allergen-like Proteins (VAPs) from plant- and animal-parasitic nematodes that collectively constitute a monophyletic clade within the CAP superfamily (E-values of $7.6e^{-85}$ and higher in BLASTP). In accordance with a recently proposed nomenclature (22), we formally designate the NC4 protein as Nem-*Gro*-SCP/TAPS-1a, but for the remainder of this article it is referred to as Gr-VAP1.





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Protein	GenBank Accession	Number of matching peptides
Venom allergen-like protein 1	AEL16453	2
Endoglucanase 1	CAB42452	7
Endoglucanase 2	CAB42451	6
β-expansin B2	ACV31370	24
Pectate lyase 2	ACO37153	9

Fig. 1. Secreted venom allergen-like protein of *G. rostochiensis*. (*A*) Differential display of the transcript-derived fragment NC4 in embryos and second-stage juveniles (J2s) of *G. rostochiensis* at different phases of dormancy and hatching. (*B*) In situ localization of transcripts encoding the venom allergen-like protein (Gr-VAP1) in the subventral esophageal gland (SvG) of a preparasitic J2 of *G. rostochiensis* (Sty, stylet; M, metacorpus). (Scale bar, 10 μm.) (*C*) Numbers of peptides matching Gr-VAP1 and several cell wall-modifying proteins identified with LC-MS/MS in a tryptic digest of collected stylet secretions from preparasitic J2s of *G. rostochiensis*.

Effectors of plant-parasitic nematodes are produced in three single-celled esophageal glands that are connected to a hollow protusible oral stylet. To investigate whether *Gr-VAP1* is expressed in the esophageal glands of *G. rostochiensis*, we used whole-mount in situ hybridization microscopy on infective juveniles. Antisense probes amplified from *Gr-VAP1* cDNA uniquely hybridized to the subventral esophageal glands, which are particularly active during host invasion and the early stages of feeding site formation (Fig. 1*B*). The complementary sense probe showed no hybridization. Effectors produced in the esophageal glands of *G. rostochiensis* are delivered to the apoplast and cytoplasm of host cells through the stylet. To investigate whether Gr-VAP1 is secreted by the nematodes, we analyzed collected stylet secretions of *G. rostochiensis* using liquid chromatography–mass spectrometry (LC-MS/MS). Two peptides perfectly matching Gr-VAP1 (i.e., SVLACHNNY and YSTSAETTAQNWANGCSMAHSSSSSR) were identified in a tryptic digest of secretions released by nematodes incubated for 24 h in potato root exudates. Furthermore, the same tryptic digest included peptides matching plant cell wall-modifying proteins previously identified in stylet secretions of *G. rostochiensis* (Fig. 1*C*). These plant cell wall-modifying proteins are also produced in the subventral esophageal glands (24–26), and we therefore concluded that Gr-VAP1 is most likely secreted into the apoplast of host cells along with plant cell wall-modifying proteins.

***G. rostochiensis* Effector Gr-VAP1 Interacts with the Apoplastic Cysteine Protease $Rcr3^{pim}$ of Tomato.** To identify host targets of Gr-VAP1, a tomato root cDNA library (*S. lycospersicum* cultivar GCR161) was screened in a yeast two-hybrid analysis using Gr-VAP1 as bait. One of the 42 tomato cDNA clones interacting with Gr-VAP1 in yeast (Fig. S1*A*) included a partial cDNA sequence encoding a protein with high similarity to the apoplastic papain-like cysteine proteases Rcr3 and PIP1 (20, 21). The specificity of this interaction was confirmed by introducing the plasmid containing tomato cDNA into yeast cells together with the bait vector containing human lamin C or the empty bait vector (Fig. S1*A*). Possible autonomous transcriptional activation of reporter genes by Gr-VAP1 in yeast was excluded by cotransforming yeast cells with the bait vector harboring *Gr-VAP1* and the empty prey vector (Fig. S1*B*).

The Cf-2–mediated fungal resistance in tomato specifically requires binding of Avr2 to the protein encoded by the *S. pimpinellifolium* allele of Rcr3 [$Rcr3^{pim}$ (18, 19)]. We therefore examined whether affinity-tagged Gr-VAP1 also binds to recombinant $Rcr3^{pim}$ in an in vitro coimmunoprecipitation assay (Fig. 2*A*). $Rcr3^{pim}$-His-HA, but not the affinity-tagged product of the Rcr3 allele of *S. lycopersicum* ($Rcr3^{lyc}$-His-HA; Fig. 2*B*), was pulled down by His-FLAG-Gr-VAP1 on agarose beads. By contrast, affinity-tagged Avr2 (His-FLAG-Avr2) pulled down both $Rcr3^{pim}$-His-HA (Fig. 2*A*) and $Rcr3^{lyc}$-His-HA (Fig. 2*B*). Notably, the coimmunoprecipitation of Avr2 and $Rcr3^{lyc}$-His-HA was only detectable with anti-HA serum after an extended exposure of the Western blot, which suggests a weaker interaction between Avr2 and $Rcr3^{lyc}$. A similarly tagged bacterial alkaline phosphatase of *Escherichia coli* (FLAG-BAP) did not pull down $Rcr3^{pim}$ or $Rcr3^{lyc}$ (Fig. 2 *A* and *B*), demonstrating that the interaction between Gr-VAP1 and $Rcr3^{pim}$ is specific. E-64 is a protease inhibitor that irreversibly

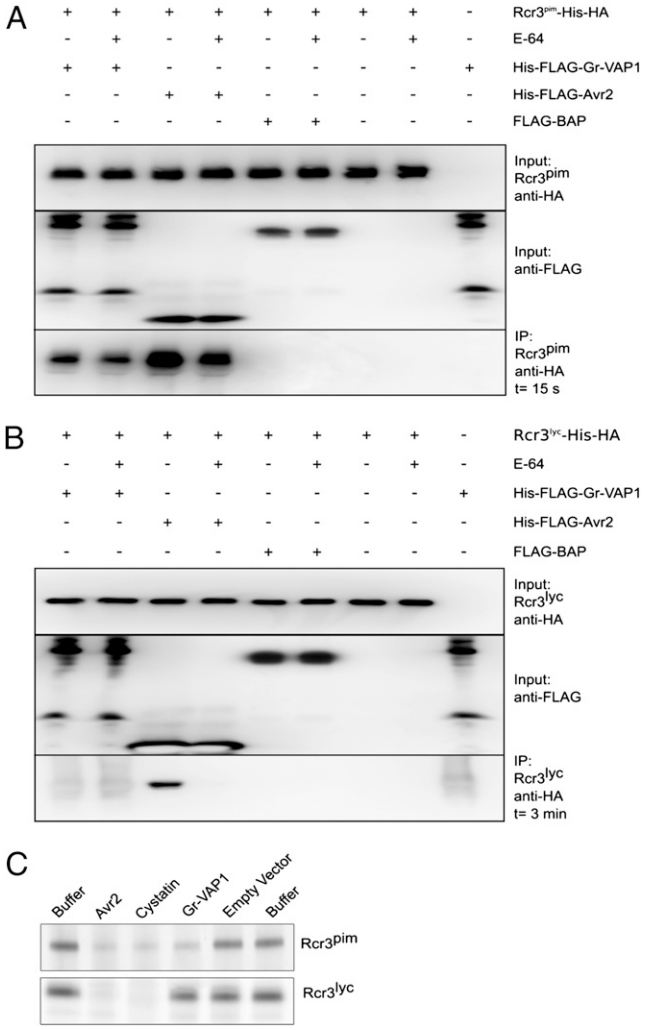

Fig. 2. *G. rostochiensis* effector Gr-VAP1 interacts with the apoplastic papain-like cysteine protease $Rcr3^{pim}$ of tomato. Coimmunoprecipitation of $Rcr3^{pim}$ ($Rcr3^{pim}$-His-HA in *A*) and $Rcr3^{lyc}$ ($Rcr3^{lyc}$-His-HA in *B*), with and without prior treatment with the protease inhibitor E-64, by either Gr-VAP1 of *G. rostochiensis* (His-FLAG-Gr-VAP1), Avr2 of *C. fulvum* (His-FLAG-Avr2), or a bacterial alkaline phosphatase (FLAG-BAP). $Rcr3^{pim}$ and $Rcr3^{lyc}$ were detected in the immunoprecipitate (IP) with anti-HA serum using different exposure times (t). (*C*) Binding of the fluorescent DCG-04 probe to the active site of $Rcr3^{pim}$ and $Rcr3^{lyc}$ after treatment with Gr-VAP1 and Avr2. Treatments with the cystatin, apoplastic fluids from agroinfiltrations with the empty binary expression vector (Empty Vector), and with buffer alone (Buffer) were included as controls.

binds to the active site of cysteine proteases (27). Pretreatment of $Rcr3^{pim}$ with E-64 in a subset of the samples reduced the binding of Gr-VAP1 to $Rcr3^{pim}$ (Fig. 2*A*), indicating that this interaction involves the active site of $Rcr3^{pim}$. To further examine the involvement of the active site of $Rcr3^{pim}$ in the interaction with Gr-VAP1, we used the fluorescent cysteine protease inhibitor DCG-04 for protease activity profiling on apoplastic fluids from agroinfiltrated *Nicotiana benthamiana* leaves either transiently expressing $Rcr3^{pim}$-His or $Rcr3^{lyc}$-His (Fig. 2*C*). Similar to Avr2, preincubating $Rcr3^{pim}$ with Gr-VAP1 strongly reduced the access of DCG-04 to the active site of $Rcr3^{pim}$. Remarkably, DCG-04 labeling of the $Rcr3^{lyc}$, which differs only in six amino acid residues from $Rcr3^{pim}$ (18), was reduced by Avr2 but not by Gr-VAP1. We therefore concluded that Gr-VAP1 specifically interacts with $Rcr3^{pim}$ and that this interaction perturbs the active site of $Rcr3^{pim}$.

Both Cf-2 and $Rcr3^{pim}$ Are Required for Defense-Related Hypersensitive Response and Resistance to *G. rostochiensis* in Tomato. The tomato cultivar MoneyMaker (*Cf-0*/$Rcr3^{lyc}$), a near-isogenic line of MoneyMaker specifically selected for *Cf-2* resistance [*Cf-2*/$Rcr3^{pim}$ (28)], and an EMS mutant of this near-isogenic line [*Cf-2*/*rcr3-3* (29)] were challenged with *G. rostochochiensis* pathotype Ro1-Mierenbos to study the effect of Cf-2 and $Rcr3^{pim}$ on susceptibility to nematodes. To assess the effect of $Rcr3^{pim}$ on the susceptibility of tomato plants to *G. rostochiensis* alone, we also included homozygous *Cf-0*/$Rcr3^{pim}$ and *Cf-0*/*rcr3-3* lines that were generated by crossing *Cf-0*/$Rcr3^{lyc}$ with *Cf-2*/$Rcr3^{pim}$ and *Cf-2*/*rcr3-3*, respectively. The presence of *Cf-2* and *Rcr3* transcripts in these tomato genotypes was checked with RT-PCR using gene-specific primers (Fig. 3*A*). Because the *Cf-2*/$Rcr3^{lyc}$ genotype shows a constitutive autoimmunity phenotype (18), it cannot be tested for susceptibility to nematodes. Compared with the susceptible reference genotype *Cf-0*/$Rcr3^{lyc}$ (30), the number of nematodes in *Cf-2*/$Rcr3^{pim}$ plants at 3 wk after inoculation was 68% lower (Fig. 3*B*). The nematode susceptibility of the genotypes *Cf-0*/*rcr3-3* and *Cf-2*/*rcr3-3* was similar to that of the *Cf-0*/$Rcr3^{lyc}$ reference plants. We concluded that the *C. fulvum* immune receptor Cf-2 also confers resistance to *G. rostochiensis* in tomato and that Cf-2–mediated resistance to *G. rostochiensis* requires $Rcr3^{pim}$. Interestingly, the number of nematodes in tomato plants that lack the *Cf-2* gene but harbor

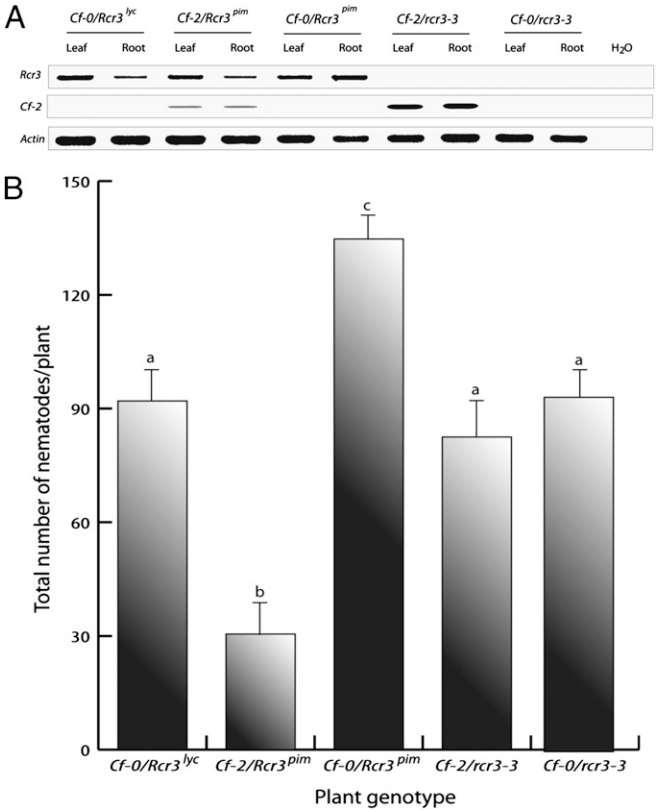

Fig. 3. Both Cf-2 and $Rcr3^{pim}$ are required for resistance to *G. rostochiensis* in tomato. (*A*) RT-PCR of the expression of *Cf-2*, *Rcr3*, and actin in nematode-infected roots of five tomato genotypes with (*Cf-2*) and without the *Cf-2* gene (*Cf-0*), either combined with the $Rcr3^{pim}$ allele of *S. pimpinellifolium*, the $Rrc3^{lyc}$ allele of *S. lycopersicum*, or the $Rcr3^{pim}$ null mutant allele *rcr3-3*. Reactions without template were included as controls (H_2O). (*B*) Mean number of nematodes per tomato plant per genotype at 3 wk after inoculation (bars indicate SD). Different characters indicate statistical differences as determined with a one-way ANOVA test (*P* values <0.001 with $n = 10$).

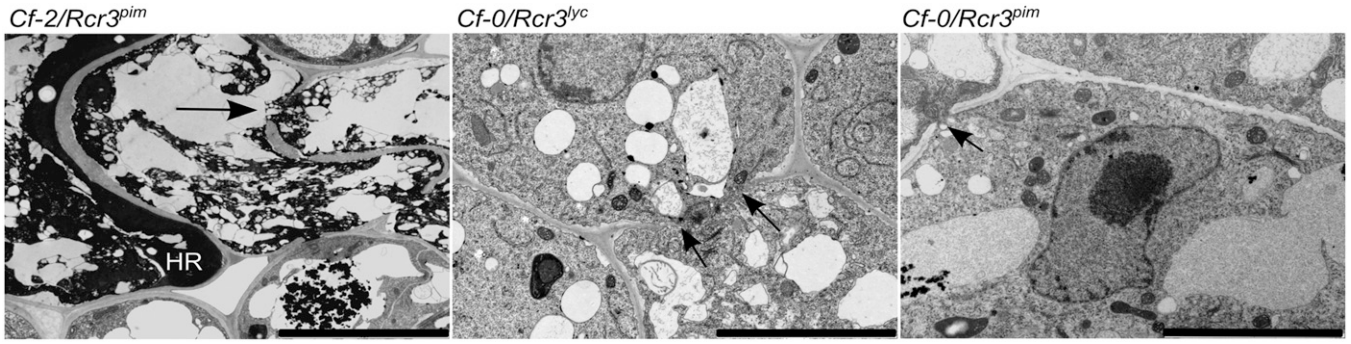

Fig. 4. Cf-2/Rcr3^{pim}-mediated nematode resistance involves a defense-related local cell death response in the infection site of *G. rostochiensis* in tomato. Infections with *G. rostochiensis* in the tomato genotype *Cf-2/Rcr3^{pim}*, but not in the genotypes *Cf-0/Rcr3^{lyc}* and *Cf-0/Rcr3^{pim}*, resulted in a local cell death associated with a hypersensitive response (HR) in the nematode-induced feeding structures in roots at 7 d after inoculation. Arrows in the transmission electron micrographs indicate the typical local cell wall dissolution and subsequent fusion of protoplasts of tomato root cells incorporated into the feeding structure of *G. rostochiensis*. (Scale bars, 5 μm.)

the functional *Rcr3^{pim}* allele (*Cf-0/Rcr3^{pim}*; Fig. 3*A*) was 40% higher than that in *Cf-0/Rcr3^{lyc}* and *Cf-0/rcr3-3* plants (Fig. 3*B*), demonstrating that Rcr3^{pim} from *S. pimpinellifolium* is most likely a virulence target of *G. rostochiensis*.

Cf-2–mediated resistance to *C. fulvum* in leaves of tomato involves a local programmed cell death, associated with a hypersensitive response, at the infection site of the fungus (31). To investigate whether Cf-2/Rcr3^{pim}–mediated nematode resistance also involves a local plant cell death at the infection site of the nematode, we analyzed the subcellular structure of nematode-infected roots of *Cf-0/Rcr3^{lyc}*, *Cf-0/Rcr3^{pim}*, and *Cf-2/Rcr3^{pim}* tomato plants with light and electron microscopy (Fig. 4 and Fig. S2). The nematode-infected roots of *Cf-2/Rcr3^{pim}* tomato plants revealed a specific cell death response in host cells inside most, but not all, of the infection sites of *G. rostochiensis.* From 7 d after invasion onward, a progressive local cell death was observed in cells directly surrounding the nematodes and in most of the nematode-induced feeding structures in *Cf-2/Rcr3^{pim}* plants. By contrast, the nematode infection sites in *Cf-0/Rcr3^{pim}* tomato plants, demonstrating increased susceptibility to *G. rostochiensis*, were structurally similar to those observed in the susceptible reference genotype *Cf-0/Rcr3^{lyc}* and showed no signs of cell death. The cell death response observed in nematode-infected *Cf-2/Rcr3^{pim}* plants resembles the response previously described for the nematode resistance genes *H1* and *Hero* (30, 32).

***G. rostochiensis* Effector Gr-VAP1 Triggers a Cf-2- and Rcr3^{pim}-Dependent Programmed Cell Death in Tomato.** To test whether the nematode effector Gr-VAP1 is able to trigger a Cf-2/Rcr3^{pim}-dependent programmed cell death, we transiently expressed *Gr-VAP1* with its native signal peptide for secretion in leaves of the tomato genotypes *Cf-2/Rcr3^{pim}*, *Cf-2/rcr3-3*, *Cf-0/Rcr3^{pim}*, and *Cf-0/Rcr3^{lyc}* (Fig. 5*A*). Just like *Avr2* of *C. fulvum*, transiently expressed *Gr-VAP1* only triggered a cell death response in leaves of *Cf-2/Rcr3^{pim}* plants. To further confirm that both Cf-2 and Rcr3^{pim} are required for this cell death response, *Gr-VAP1* was transiently coexpressed with and without *Rcr3^{pim}* in leaves of the tomato genotypes *Cf-2/rcr3-3* and *Cf-0/rcr3-3* that lack a functional *Rcr3* gene (Fig. 5*B*). Only the complementation of *Cf-2/rcr3-3* plants with *Rcr3^{pim}* resulted in a cell death response to Gr-VAP1. We therefore concluded that both Cf-2 and Rcr3^{pim} are required for the defense-related cell death response triggered by the nematode effector Gr-VAP1 in tomato.

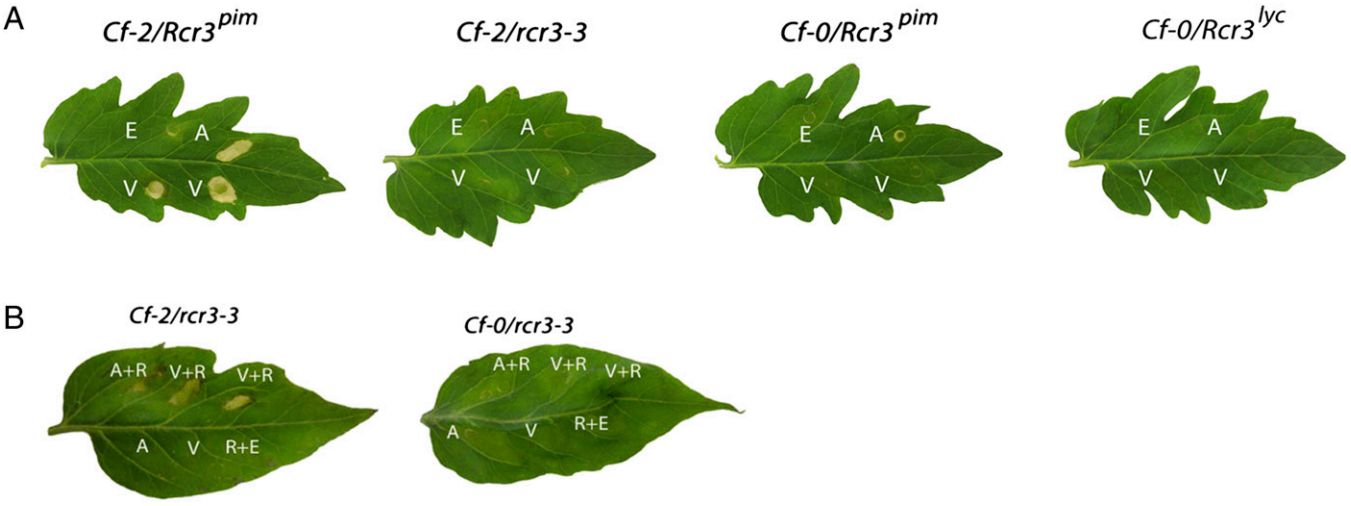

Fig. 5. Gr-VAP1 triggers a Cf-2- and Rcr3^{pim}-dependent defense-related cell death response in tomato. (*A*) *Agrobacterium tumefaciens*–mediated transient expression of *Gr-VAP1* (V), *Avr2* (A), or the corresponding empty binary expression vector (E) in infiltrated leaves of the tomato genotypes *Cf-2/Rcr3^{pim}*, *Cf-2/rcr3-3*, *Cf-0/Rcr3^{pim}*, and *Cf-0/Rcr3^{lyc}*. (*B*) Transient coexpression of *Gr-VAP1* (V) and *Avr2* (A) with the empty binary plant expression vector (E) or with *Rcr3^{pim}* (R) in leaves of the tomato genotypes *Cf-2/rcr3-3* and *Cf-0/rcr3-3*. Pictures were taken 5 d after infiltration.

Dual Cf-2–Mediated Resistance Requires a Common Virulence Target of Two Unrelated Pathogens. Our data demonstrate that *Cf-2* is a dual resistance gene that offers tomato plants protection against a leaf mold fungus and a root-parasitic nematode. The dual resistance specificity of Cf-2 results from perturbations in the apoplastic papain-like cysteine protease $Rcr3^{pim}$ induced by two structurally different effectors. Recently it has been shown that the oomycete plant-pathogen *Phytophthora infestans* secretes cystatin-like effectors that also interact with $Rcr3^{pim}$ (15, 33, 34). Although the perturbations of $Rcr3^{pim}$ induced by Gr-VAP1 of *G. rostochiensis* and by Avr2 of *C. fulvum* may be structurally different, both trigger a Cf-2–mediated programmed cell death and disease resistance. Surprisingly, the perturbations of $Rcr3^{pim}$ brought about by the effectors EPIC1 and EPIC2B of *P. infestans* do not trigger Cf-2–mediated defense responses (15), suggesting that they fall outside of the activation window of Cf-2. The constitutive autoimmune phenotype in *Cf-2/*$Rcr3^{lyc}$ plants (18), which is not observed in *Cf-2* plants lacking a functional *Rcr3* gene (i.e., *Cf-2/rcr3-3*), further suggests that association with either $Rcr3^{lyc}$ or Gr-VAP-1/Avr2-perturbed $Rcr3^{pim}$, but not the absence of Rcr3, activates Cf-2. The next step is to investigate whether the activation of Cf-2–mediated disease resistances indeed involves a physical association between this immune receptor and different effector–$Rcr3^{pim}$ complexes.

The dual specificity of Cf-2 via a common virulence target of a fungus and an invertebrate demonstrates the power of indirect recognition of pathogen-derived molecules for plants. By "guarding" critical host molecules targeted by multiple and unrelated pathogens, plants can efficiently expand the coverage of their innate immune system with a relatively small set of extracellular and cytoplasmic immune receptors. The recent finding that the *Mi-1.2* gene, originally identified as a root-knot nematode resistance gene, confers resistance to at least four different attackers by an unresolved mechanism suggests that multiple resistance specificities of immune receptors may contribute significantly to the resistance spectrum of plants (35–39). The relatively late discoveries of the multiple resistance specificities of *Cf-2* and *Mi-1.2*, which were both introgressed into cultivated tomato in the 1940s, further show how the paradigm of singular specificity of disease resistance genes (3) has dominated the screening methods of plant breeders. The notion that single immune receptors conferring multiple resistances to taxonomically unrelated pathogens may not be exceptional, gives breeders a strong incentive to identify and to use common virulence targets as leads to discover broad-specificity resistance genes.

Materials and Methods

Identification of Gr-VAP1 in Stylet Secretions. A fragment of 176 base pairs derived from the Gr-VAP1 transcript was identified in a cDNA-AFLP analysis of successive developmental stages of *G. rostochiensis* pathotype Ro1 Mierenbos (23). The transcript-derived fragment was extended using partially overlapping expressed sequence tags of *G. rostochiensis* in a nonredundant database (40). The 5′ and 3′ ends of the transcript-derived fragment were cloned by using rapid amplification of cDNA-ends (Invitrogen). The Gr-VAP1 transcripts were localized in freshly hatched preparasitic second-stage juveniles of *G. rostochiensis* pathotype Ro1 using whole-mount mRNA in situ hybrididization (26). Peptides matching the protein sequences of Gr-VAP1 and several cell wall-modifying proteins were detected with LC-MS/MS in collected stylet secretions of preparasitic second-stage juveniles of *G. rostochiensis* Ro1 (41). Details are given in *SI Materials and Methods*.

Yeast Two-Hybrid Screening. The Matchmaker System 3 (Clontech) was used to screen a tomato root cDNA library (*S. lycopersicum* cultivar CGR161) in yeast with Gr-VAP1 as bait (42). cDNA of *Gr-VAP1* without signal peptide for secretion was amplified with PCR using the primers Gr-VAP1-Y2HFw and Gr-VAP1-Y2HRv (Table S1) and subcloned into the EcoRI/BamHI site of pGBKT7-BD for expression in yeast (*Saccharomyces cerevisiae* strain AH109). Details are given in *SI Materials and Methods*.

Coimmunoprecipitation Experiments. Recombinantly produced Gr-VAP1, Avr2, and BAP with a FLAG tag were incubated with anti-FLAG serum labeled agarose beads. The beads were subsequently either incubated with recombinant $Rcr3^{pim}$-His-HA or $Rcr3^{lyc}$-His-HA in duplicates. In one sample of each duplicate set the active site of $Rcr3^{pim}$-His-HA or $Rcr3^{lyc}$-His-HA was blocked with the competitive cysteine protease inhibitor E64 first. The fractions of $Rcr3^{pim}$-His-HA and $Rcr3^{lyc}$-His-HA bound to Gr-VAP1, Avr2, and BAP on beads were visualized on Western blot with anti-HA serum. Details are given in *SI Materials and Methods*.

Fluorescent Protease Activity Profiling. $Rcr3^{pim}$ of *S. pimpinellifolium* and $Rcr3^{lyc}$ of *S. lycopersicum* were transiently overexpressed in the apoplastic fluids of *N. benthamiana* leaves after agroinfiltration. Apoplastic fluids were incubated with either Avr2, cystatin from chicken egg white, or Gr-VAP1. To label the remaining available active sites in $Rcr3^{pim}$ and $Rcr3^{lyc}$, the proteins were subsequently incubated with fluorescent DCG-04-TMR. Details are given in *SI Materials and Methods*.

Microscopy of Nematode Infections. In-vitro-germinated seedlings of three tomato genotypes *Cf-2/*$Rcr3^{pim}$, *Cf-0/*$Rcr3^{lyc}$, and *Cf-0/*$Rcr3^{pim}$ were challenged with infective second-stage juveniles of *G. rostochiensis* pathotype Ro1 Mierenbos. At 3, 7, 14, and 21 d after invasion, nematode-infected roots were collected, fixed, sectioned, and stained for transmission electron microscopy. Details are given in *SI Materials and Methods*.

Effector-Induced Plant Cell Death Response. *Gr-VAP1* and *Avr2* were transiently expressed in the apoplastic space of tomato mesophyll cells after agroinfiltration in leaves of tomato genotypes *Cf-2/*$Rcr3^{pim}$, *Cf-2/rcr3-3*, *Cf-0/*$Rcr3^{pim}$, and *Cf-0/*$Rcr3^{lyc}$. The development of a specific local cell death in the infiltrated leaf area from 3 to 7 d after infiltration was used as an indicator of a defense-related hypersensitive response. Details are given in *SI Materials and Methods*.

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