

# 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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Edited by David C. Baulcombe, University of Cambridge, Cambridge, United Kingdom, and approved May 10, 2012 (received for review February 19, 2012)

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<sup>pim</sup> protein of *S. pimpinellifolium*. Binding of the venom allergen-like effector protein Gr-VAP1 of *G. rostochiensis* to Rcr3<sup>pim</sup> perturbs the active site of this papain-like cysteine protease. In the absence of the Cf-2 receptor, Rcr3<sup>pim</sup> 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<sup>pim</sup> 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

**D**ominant 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–

Author contributions: J.L.L.-T., R.H.P.W., J.W.V.t.K., M.S., R.A.L.v.d.H., A.S., J.B., A.G., and G.S. designed research; J.L.L.-T., R.H.P.W., P.G., J.C.B., A.F.-T., J.H.G.C., A.H.P.A., H.A.O., J.W.V.t.K., L.B., M.S., and M.I. performed research; M.I. contributed new reagents/analytic tools; J.L.L.-T., R.H.P.W., P.G., A.F.-T., J.H.G.C., A.H.P.A., M.S., R.A.L.v.d.H., A.S., P.J.G.M.d.W., J.B., A.G., and G.S. analyzed data; and J.L.L.-T., R.H.P.W., M.S., R.A.L.v.d.H., A.S., P.J.G.M.d.W., J.B., A.G., and G.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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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This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1202867109/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1202867109/-DCSupplemental).

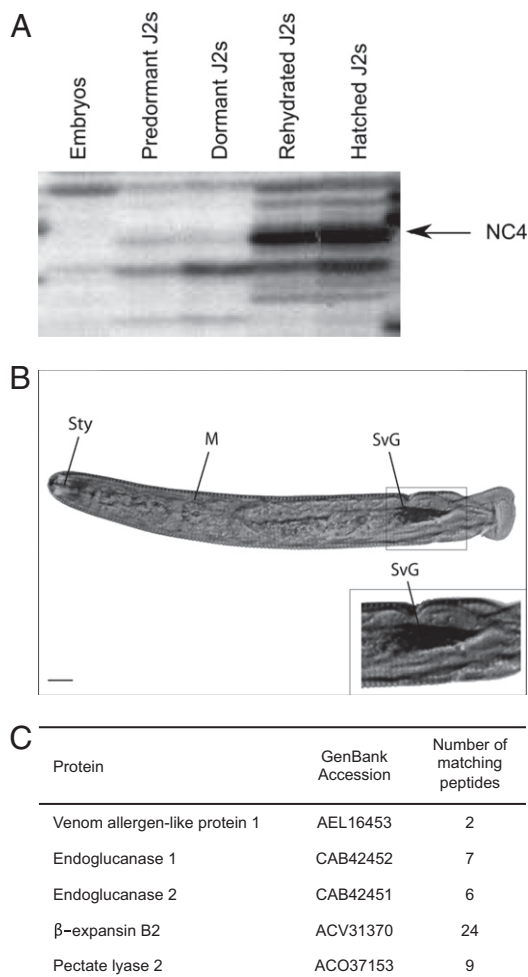
mediated resistance to *G. rostochiensis* in tomato plants. We found that *Cf-2*, which has been used for decades in tomato as a resistance gene against the leaf mold fungus *C. fulvum* (18), also confers resistance to the potato cyst nematode *G. rostochiensis*.

## 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. 1A). 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.

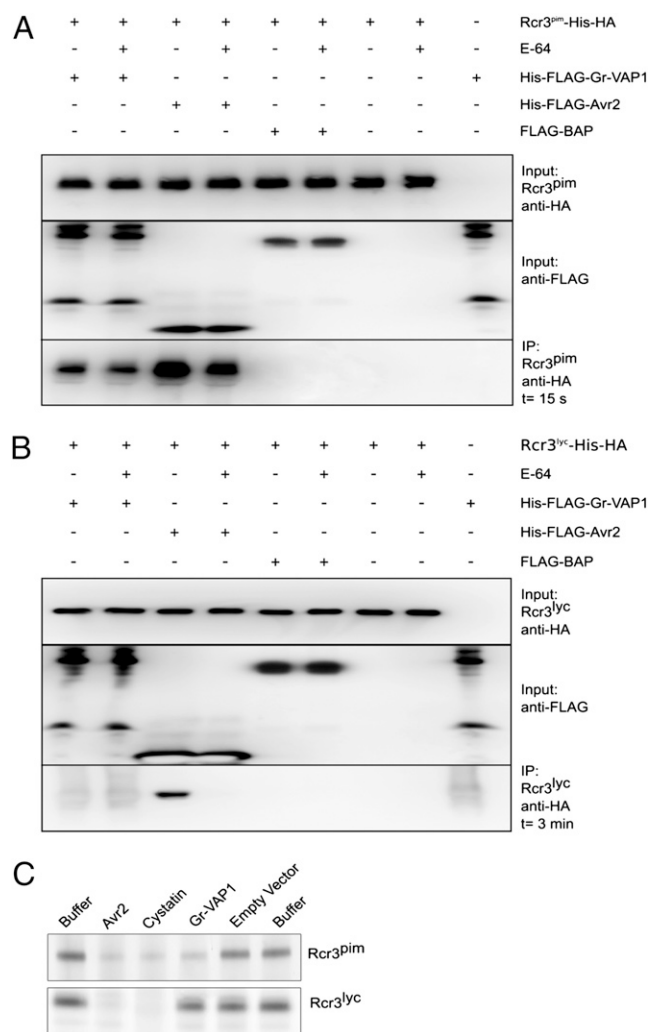
Effectors of plant-parasitic nematodes are produced in three single-celled esophageal glands that are connected to a hollow protrusible oral stylet. To investigate whether *Gr-VAP1* is expressed in the esophageal glands of *G. rostochiensis*, we used whole-mount in situ hybridization microscopy on infective 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. 1B). 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. 1C). 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.



**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, metacarpus). (Scale bar, 10  $\mu$ m.) (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*.

***G. rostochiensis* Effector Gr-VAP1 Interacts with the Apoplastic Cysteine Protease Rcr3<sup>pim</sup> of Tomato.** To identify host targets of Gr-VAP1, a tomato root cDNA library (*S. lycopersicum* 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. S14) 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. S14). 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. S18).

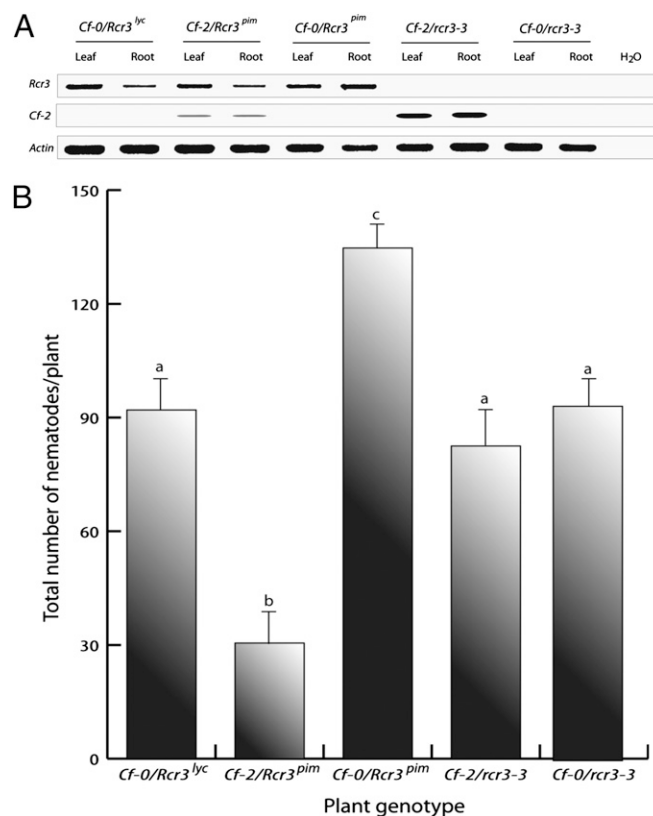
The Cf-2-mediated fungal resistance in tomato specifically requires binding of Avr2 to the protein encoded by the *S. pimpinellifolium* allele of Rcr3 [Rcr3<sup>pim</sup> (18, 19)]. We therefore examined whether affinity-tagged Gr-VAP1 also binds to recombinant Rcr3<sup>pim</sup> in an in vitro coimmunoprecipitation assay (Fig. 2A). Rcr3<sup>pim</sup>-His-HA, but not the affinity-tagged product of the Rcr3 allele of *S. lycopersicum* (Rcr3<sup>lyc</sup>-His-HA; Fig. 2B), was pulled down by His-FLAG-Gr-VAP1 on agarose beads. By contrast, affinity-tagged Avr2 (His-FLAG-Avr2) pulled down both Rcr3<sup>pim</sup>-His-HA (Fig. 2A) and Rcr3<sup>lyc</sup>-His-HA (Fig. 2B). Notably, the coimmunoprecipitation of Avr2 and Rcr3<sup>lyc</sup>-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<sup>lyc</sup>. A similarly tagged bacterial alkaline phosphatase of *Escherichia coli* (FLAG-BAP) did not pull down Rcr3<sup>pim</sup> or Rcr3<sup>lyc</sup> (Fig. 2A and B), demonstrating that the interaction between Gr-VAP1 and Rcr3<sup>pim</sup> 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<sup>pim</sup> of tomato. Coimmunoprecipitation of Rcr3<sup>pim</sup> (Rcr3<sup>pim</sup>-His-HA in A) and Rcr3<sup>lyc</sup> (Rcr3<sup>lyc</sup>-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<sup>pim</sup> and Rcr3<sup>lyc</sup> 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<sup>pim</sup> and Rcr3<sup>lyc</sup> 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.

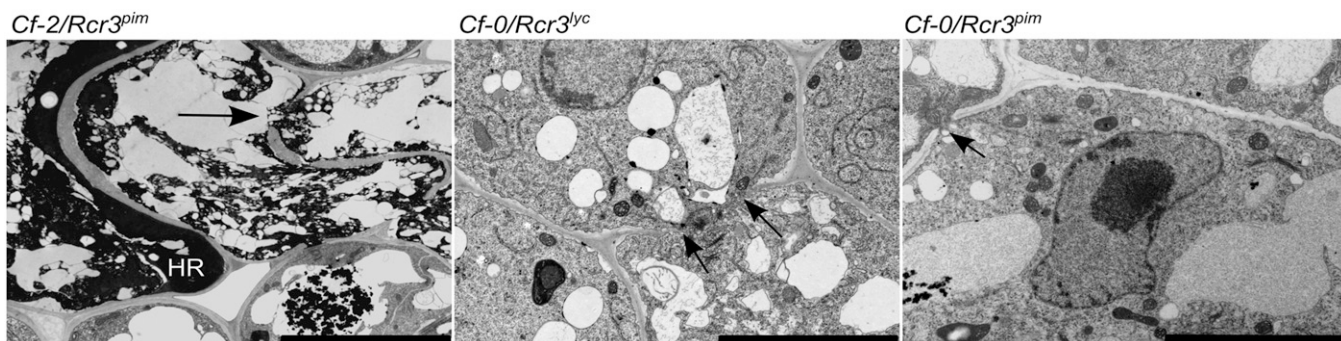
binds to the active site of cysteine proteases (27). Pretreatment of Rcr3<sup>pim</sup> with E-64 in a subset of the samples reduced the binding of Gr-VAP1 to Rcr3<sup>pim</sup> (Fig. 2A), indicating that this interaction involves the active site of Rcr3<sup>pim</sup>. To further examine the involvement of the active site of Rcr3<sup>pim</sup> 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<sup>pim</sup>-His or Rcr3<sup>lyc</sup>-His (Fig. 2C). Similar to Avr2, preincubating Rcr3<sup>pim</sup> with Gr-VAP1 strongly reduced the access of DCG-04 to the active site of Rcr3<sup>pim</sup>. Remarkably, DCG-04 labeling of the Rcr3<sup>lyc</sup>, which differs only in six amino acid residues from Rcr3<sup>pim</sup> (18), was reduced by Avr2 but not by Gr-VAP1. We therefore concluded that Gr-VAP1 specifically interacts with Rcr3<sup>pim</sup> and that this interaction perturbs the active site of Rcr3<sup>pim</sup>.

**Both Cf-2 and Rcr3<sup>pim</sup> Are Required for Defense-Related Hypersensitive Response and Resistance to *G. rostochiensis* in Tomato.** The tomato cultivar MoneyMaker (*Cf-0/Rcr3<sup>lyc</sup>*), a near-isogenic line of MoneyMaker specifically selected for *Cf-2* resistance [*Cf-2/Rcr3<sup>pim</sup>* (28)], and an EMS mutant of this near-isogenic line [*Cf-2/rcr3-3* (29)] were challenged with *G. rostochiensis* pathotype Ro1-Mierenbos to study the effect of *Cf-2* and Rcr3<sup>pim</sup> on susceptibility to nematodes. To assess the effect of Rcr3<sup>pim</sup> on the susceptibility of tomato plants to *G. rostochiensis* alone, we also included homozygous *Cf-0/Rcr3<sup>pim</sup>* and *Cf-0/rcr3-3* lines that were generated by crossing *Cf-0/Rcr3<sup>lyc</sup>* with *Cf-2/Rcr3<sup>pim</sup>* 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. 3A). Because the *Cf-2/Rcr3<sup>lyc</sup>* genotype shows a constitutive autoimmunity phenotype (18), it cannot be tested for susceptibility to nematodes. Compared with the susceptible reference genotype *Cf-0/Rcr3<sup>lyc</sup>* (30), the number of nematodes in *Cf-2/Rcr3<sup>pim</sup>* plants at 3 wk after inoculation was 68% lower (Fig. 3B). The nematode susceptibility of the genotypes *Cf-0/rcr3-3* and *Cf-2/rcr3-3* was similar to that of the *Cf-0/Rcr3<sup>lyc</sup>* 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<sup>pim</sup>. Interestingly, the number of nematodes in tomato plants that lack the *Cf-2* gene but harbor



**Fig. 3.** Both *Cf-2* and Rcr3<sup>pim</sup> 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<sup>pim</sup>* allele of *S. pimpinellifolium*, the *Rcr3<sup>lyc</sup>* allele of *S. lycopersicum*, or the *Rcr3<sup>pim</sup>* null mutant allele *rcr3-3*. Reactions without template were included as controls (H<sub>2</sub>O). (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).





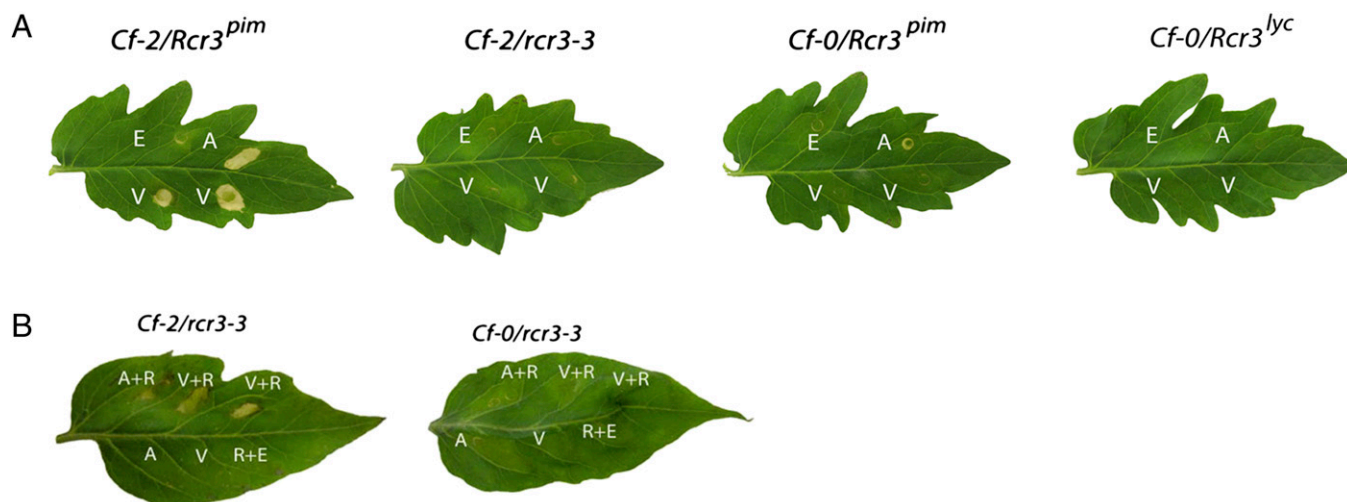
**Fig. 4.** Cf-2/Rcr3<sup>pim</sup>-mediated nematode resistance involves a defense-related local cell death response in the infection site of *G. rostochiensis* in tomato. Infections with *G. rostochiensis* in the tomato genotype Cf-2/Rcr3<sup>pim</sup>, but not in the genotypes Cf-0/Rcr3<sup>lyc</sup> and Cf-0/Rcr3<sup>pim</sup>, 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*<sup>pim</sup> allele (Cf-0/Rcr3<sup>pim</sup>; Fig. 3A) was 40% higher than that in Cf-0/Rcr3<sup>lyc</sup> and Cf-0/rcr3-3 plants (Fig. 3B), demonstrating that *Rcr3*<sup>pim</sup> 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<sup>pim</sup>-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<sup>lyc</sup>, Cf-0/Rcr3<sup>pim</sup>, and Cf-2/Rcr3<sup>pim</sup> tomato plants with light and electron microscopy (Fig. 4 and Fig. S2). The nematode-infected roots of Cf-2/Rcr3<sup>pim</sup> 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<sup>pim</sup> plants. By contrast, the nematode infection sites in Cf-0/Rcr3<sup>pim</sup> tomato plants, demonstrating increased susceptibility to *G. rostochiensis*, were structurally similar to those observed in the susceptible reference genotype Cf-0/Rcr3<sup>lyc</sup> and showed no signs of cell death.

The cell death response observed in nematode-infected Cf-2/Rcr3<sup>pim</sup> 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<sup>pim</sup>-Dependent Programmed Cell Death in Tomato.** To test whether the nematode effector Gr-VAP1 is able to trigger a Cf-2/Rcr3<sup>pim</sup>-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<sup>pim</sup>, Cf-2/rcr3-3, Cf-0/Rcr3<sup>pim</sup>, and Cf-0/Rcr3<sup>lyc</sup> (Fig. 5A). Just like *Avr2* of *C. fulvum*, transiently expressed *Gr-VAP1* only triggered a cell death response in leaves of Cf-2/Rcr3<sup>pim</sup> plants. To further confirm that both Cf-2 and Rcr3<sup>pim</sup> are required for this cell death response, *Gr-VAP1* was transiently coexpressed with and without *Rcr3*<sup>pim</sup> in leaves of the tomato genotypes Cf-2/rcr3-3 and Cf-0/rcr3-3 that lack a functional *Rcr3* gene (Fig. 5B). Only the complementation of Cf-2/rcr3-3 plants with *Rcr3*<sup>pim</sup> resulted in a cell death response to *Gr-VAP1*. We therefore concluded that both Cf-2 and Rcr3<sup>pim</sup> 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<sup>pim</sup>-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<sup>pim</sup>, Cf-2/rcr3-3, Cf-0/Rcr3<sup>pim</sup>, and Cf-0/Rcr3<sup>lyc</sup>. (B) Transient coexpression of *Gr-VAP1* (V) and *Avr2* (A) with the empty binary plant expression vector (E) or with *Rcr3*<sup>pim</sup> (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<sup>pim</sup> 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<sup>pim</sup> (15, 33, 34). Although the perturbations of Rcr3<sup>pim</sup> 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<sup>pim</sup> 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<sup>lyc</sup>* 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<sup>lyc</sup> or Gr-VAP1/Avr2-perturbed Rcr3<sup>pim</sup>, 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<sup>pim</sup> 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 parasitic second-stage juveniles of *G. rostochiensis* pathotype Ro1 using whole-mount mRNA in situ hybridization (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 parasitic 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<sup>pim</sup>-His-HA or Rcr3<sup>lyc</sup>-His-HA in duplicates. In one sample of each duplicate set the active site of Rcr3<sup>pim</sup>-His-HA or Rcr3<sup>lyc</sup>-His-HA was blocked with the competitive cysteine protease inhibitor E64 first. The fractions of Rcr3<sup>pim</sup>-His-HA and Rcr3<sup>lyc</sup>-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<sup>pim</sup> of *S. pimpinellifolium* and Rcr3<sup>lyc</sup> 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<sup>pim</sup> and Rcr3<sup>lyc</sup>, 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<sup>pim</sup>*, *Cf-0/Rcr3<sup>lyc</sup>*, and *Cf-0/Rcr3<sup>pim</sup>* 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<sup>pim</sup>*, *Cf-2/rcr3-3*, *Cf-0/Rcr3<sup>pim</sup>*, and *Cf-0/Rcr3<sup>lyc</sup>*. 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*.

**ACKNOWLEDGMENTS.** We thank Bart Thomma and Ton Bisseling for their critical comments on the manuscript. Funding was provided by the European Commission's Sixth Framework Programme project BIOEXPLOIT (CT FOOD-2005-513959, to J.L.L.-T., J.W.V.t.K., P.J.G.M.d.W., J.B., A.G., and G.S.), European Commission's COST Action 872 (to A.G., G.S., M.S., and L.B.), a VIDI grant from the Netherlands Organization of Scientific Research (to G.S.), the Deutscher Akademischer Austausch Dienst (M.I.), and the Higher Education Commission of Pakistan (M.I.).

- Dangl JL, Jones JDG (2001) Plant pathogens and integrated defence responses to infection. *Nature* 411:826–833.
- Dodds PN, Rathjen JP (2010) Plant immunity: towards an integrated view of plant-pathogen interactions. *Nat Rev Genet* 11:539–548.
- Flor HH (1971) Current status of the gene-for-gene concept. *Annu Rev Phytopathol* 9: 275–296.
- Thomma BPHJ, Nürnberger T, Joosten MHAI (2011) Of PAMPs and effectors: The blurred PTI-ETI dichotomy. *Plant Cell* 23:4–15.
- Scofield SR, et al. (1996) Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato. *Science* 274:2063–2065.
- Tang X, et al. (1996) Initiation of plant disease resistance by physical interaction of AvrPto and Pto kinase. *Science* 274:2060–2063.
- Jia Y, McAdams SA, Bryan GT, Hershey HP, Valent B (2000) Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. *EMBO J* 19:4004–4014.
- Deslandes L, 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.
- Dodds PN, 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.
- Chen Y, Liu Z, Halterman DA (2012) Molecular determinants of resistance activation and suppression by *Phytophthora infestans* effector IPI-O. *PLoS Pathog* 8:e1002595.
- Krasileva KV, Dahlbeck D, Staskiewicz BJ (2010) Activation of an *Arabidopsis* resistance protein is specified by the in planta association of its leucine-rich repeat domain with the cognate oomycete effector. *Plant Cell* 22:2444–2458.
- Van der Biezen EA, Jones JDG (1998) Plant disease-resistance proteins and the gene-for-gene concept. *Trends Biochem Sci* 23:454–456.
- Jones JDG, Dangl JL (2006) The plant immune system. *Nature* 444:323–329.
- Mukhtar MS, et al.; European Union Effectoromics Consortium (2011) Independently evolved virulence effectors converge onto hubs in a plant immune system network. *Science* 333:596–601.
- Song J, et al. (2009) Apoplastic effectors secreted by two unrelated eukaryotic plant pathogens target the tomato defense protease Rcr3. *Proc Natl Acad Sci USA* 106: 1654–1659.

16. Collier SM, Moffett P (2009) NB-LRRs work a "bait and switch" on pathogens. *Trends Plant Sci* 14:521–529.
17. Maekawa T, Kufer TA, Schulze-Lefert P (2011) NLR functions in plant and animal immune systems: So far and yet so close. *Nat Immunol* 12:817–826.
18. Krüger J, et al. (2002) A tomato cysteine protease required for Cf-2-dependent disease resistance and suppression of autonecrosis. *Science* 296:744–747.
19. Rooney HCE, et al. (2005) *Cladosporium* Avr2 inhibits tomato Rcr3 protease required for Cf-2-dependent disease resistance. *Science* 308:1783–1786.
20. Shabab M, et al. (2008) Fungal effector protein AVR2 targets diversifying defense-related cysteine proteases of tomato. *Plant Cell* 20:1169–1183.
21. van Esse HP, et al. (2008) The *Cladosporium fulvum* virulence protein Avr2 inhibits host proteases required for basal defense. *Plant Cell* 20:1948–1963.
22. Cantacessi C, et al. (2009) A portrait of the "SCP/TAPS" proteins of eukaryotes—developing a framework for fundamental research and biotechnological outcomes. *Biotechnol Adv* 27:376–388.
23. Qin L, et al. (2000) An efficient cDNA-AFLP-based strategy for the identification of putative pathogenicity factors from the potato cyst nematode *Globodera rostochiensis*. *Mol Plant Microbe Interact* 13:830–836.
24. Popeijus H, et al. (2000) Degradation of plant cell walls by a nematode. *Nature* 406:36–37.
25. Qin L, et al. (2004) Plant degradation: A nematode expansin acting on plants. *Nature* 427:30.
26. Smant G, et al. (1998) Endogenous cellulases in animals: Isolation of  $\beta$ -1, 4-endoglucanase genes from two species of plant-parasitic cyst nematodes. *Proc Natl Acad Sci USA* 95:4906–4911.
27. Hanada K, Tamai M, Yamagishi M (1978) Isolation and characterization of E 64, a new thiol protease inhibitor. *Agric Biol Chem* 42:523–528.
28. Tigchelaar EC (1984) Collections of isogenic tomato stocks. *Rep Tomato Genet Coop* 34:55–57.
29. Dixon MS, Golstein C, Thomas CM, van Der Biezen EA, Jones JDG (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.
30. Sobczak M, et al. (2005) Characterization of susceptibility and resistance responses to potato cyst nematode (*Globodera* spp.) infection of tomato lines in the absence and presence of the broad-spectrum nematode resistance *Hero* gene. *Mol Plant Microbe Interact* 18:158–168.
31. Hammond-Kosack KE, Silverman P, Raskin I, Jones JDG (1996) Race-specific elicitors of *Cladosporium fulvum* induce changes in cell morphology and the synthesis of ethylene and salicylic acid in tomato plants carrying the corresponding Cf disease resistance gene. *Plant Physiol* 110:1381–1394.
32. Rice SL, Leadbeater BSC, Stone AR (1985) Changes in cell structure in roots of resistant potatoes parasitized by potato cyst-nematodes. I. Potatoes with resistance gene *H1* derived from *Solanum tuberosum* ssp. *andigena*. *Physiol Mol Plant Pathol* 27:219–234.
33. Tian M, et al. (2007) A *Phytophthora infestans* cystatin-like protein targets a novel tomato papain-like apoplastic protease. *Plant Physiol* 143:364–377.
34. Kaschani F, et al. (2010) An effector-targeted protease contributes to defense against *Phytophthora infestans* and is under diversifying selection in natural hosts. *Plant Physiol* 154:1794–1804.
35. Casteel CL, Walling LL, Paine TD (2006) Behavior and biology of the tomato psyllid, *Bactericera cockerelli*, in response to the *Mi-1.2* gene. *Entomol Exp Appl* 121:67–72.
36. Vos P, et al. (1998) The tomato *Mi-1* gene confers resistance to both root-knot nematodes and potato aphids. *Nat Biotechnol* 16:1365–1369.
37. Milligan SB, et al. (1998) The root knot nematode resistance gene *Mi* from tomato is a member of the leucine zipper, nucleotide binding, leucine-rich repeat family of plant genes. *Plant Cell* 10:1307–1319.
38. Seifi A, et al. (2011) Linked, if not the same, *Mi-1* homologues confer resistance to tomato powdery mildew and root-knot nematodes. *Mol Plant Microbe Interact* 24:441–450.
39. Rossi M, et al. (1998) The nematode resistance gene *Mi* of tomato confers resistance against the potato aphid. *Proc Natl Acad Sci USA* 95:9750–9754.
40. Popeijus M, et al. (2000) Analysis of genes expressed in second stage juveniles of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida* using the expressed sequence tag approach. *Nematology* 2:567–574.
41. Smant G, et al. (1997) Potato root diffusate-induced secretion of soluble, basic proteins originating from the subventral esophageal glands of potato cyst nematodes. *Phytopathology* 87:839–845.
42. Rehman S, et al. (2009) A secreted SPRY domain-containing protein (SPRYSEC) from the plant-parasitic nematode *Globodera rostochiensis* interacts with a CC-NB-LRR protein from a susceptible tomato. *Mol Plant Microbe Interact* 22:330–340.