

# Recognition of the *Hyaloperonospora parasitica* effector ATR13 triggers resistance against oomycete, bacterial, and viral pathogens

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Phytopathogenic oomycetes cause some of the most devastating diseases affecting agricultural crops. *Hyaloperonospora parasitica* is a native oomycete pathogen of *Arabidopsis* and is related to other oomycete phytopathogens that include several species of *Phytophthora*, including the causal agent of potato late blight. Recently, four oomycete effector genes have been isolated, and several oomycete genomes have been sequenced. We have developed an efficient and genetically amenable system to test putative effector genes using the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000. The *H. parasitica* effector protein ATR13 was delivered via *P. syringae* by fusing the ATR13 gene with the *avrRpm1* type three secretion signal peptide, a bacterial sequence that allows transfer of proteins into the host cell through the bacterial type III secretion system. We also inserted ATR13 into the genome of the turnip mosaic virus, a single-stranded RNA virus. Our results show that delivery of ATR13 via the bacterial or viral pathogen triggers defense responses in plants containing the cognate resistance protein RPP13<sup>Nd</sup>, which restricts proliferation of both pathogens. Hence, recognition of ATR13 by RPP13 initiates defense responses that are effective against oomycete, bacterial and viral pathogens, pointing to a common defense mechanism. We have characterized regions of the RPP13<sup>Nd</sup> resistance protein that are essential for effector recognition and/or downstream signaling, using transient coexpression in *Nicotiana benthamiana*.

plant disease resistance | effector-triggered plant immunity | plant pathogenesis

**H***yaloperonospora parasitica* is a native oomycete pathogen of *Arabidopsis* (1). This plant-pathogen interaction became established as a laboratory model in the 1990s and led to the identification of many *Arabidopsis* resistance genes (R-genes) (1). However, cloning *H. parasitica*'s effector genes has been much more difficult because of its obligate biotrophic lifestyle and lack of genetic tractability. Recently, four oomycete effector genes have been isolated, and this coincides with the sequencing of several oomycete genomes (including *H. parasitica*) and has allowed prediction of a large number of effector genes that share signal peptide sequences with the four known effectors (2). The *H. parasitica* effector *Arabidopsis thaliana* recognized 13 (ATR13) is recognized by the *A. thaliana* R-gene Resistance to *Peronospora parasitica*13 (RPP13) (3). ATR13 contains two predicted signal sequences at its N terminus. The first sequence is believed to allow secretion of the protein across the oomycete plasma membrane; the second sequence contains an RxLR motif, which has been shown to target *Phytophthora infestans* effector proteins across the host cell plasma membrane (4). The RxLx motif is also conserved in the human malaria parasite *Plasmodium falciparum* and is responsible for vacuolar export of proteins (4). Once inside the plant cell, ATR13 is detected by the cognate R-protein RPP13, a NBS-LRR protein of the coiled-coil (CC) class (3). Recognition leads to induction of defense pathways and is visible as an HR (hypersensitive cell death response). Allen *et al.* (3) showed by biolistic transient expression in *Arabidopsis* that the RPP13 gene product from ecotype

Niederzenz (RPP13<sup>Nd</sup>) recognizes ATR13 from *H. parasitica* strains Emco5, Maks9, Aswa1, and Goco1, but not from strains Emoy2 and Hind4.

We have developed an efficient and genetically amenable system to test ATR13-RPP13 recognition using the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*). *Pst* is a Gram-negative bacterium that delivers effector proteins into host cells via a type III secretion system (TTSS). We delivered ATR13 alleles via *Pst* by fusing the ATR13 gene with the AvrRpm1 signal peptide, a bacterial sequence that allows transfer of proteins into the host cell through the TTSS (5, 6). Our results show that delivery of ATR13<sup>Emco5</sup>, but not ATR13<sup>Emoy2</sup>, via the bacterial pathogen triggers defense responses in the plant expressing the cognate resistance protein RPP13<sup>Nd</sup>. This leads to restriction of bacterial proliferation and thus validates our delivery system. The system can now be used to test other predicted effector proteins from *H. parasitica* for their ability to be recognized by a wide range of diverse ecotypes of *A. thaliana*.

We also inserted ATR13 into the genome of the turnip mosaic virus, a single-stranded RNA virus. Again, expression of ATR13<sup>Emco5</sup> (but not ATR13<sup>Emoy2</sup>) by the viral pathogen triggers defense responses in *A. thaliana* containing RPP13<sup>Nd</sup> and stops viral proliferation. A hypersensitive cell death response (HR) is also triggered when RPP13<sup>Nd</sup> and ATR13<sup>Emco5</sup> are coexpressed in *Nicotiana benthamiana* by *Agrobacterium* infiltration, indicating that *N. benthamiana* contains the signaling components necessary to trigger HR. This has allowed us to characterize regions of the RPP13<sup>Nd</sup> R-protein that are essential for effector recognition and/or downstream signaling.

## Results

**Construction of a Surrogate Bacterial TTSS to Deliver *H. parasitica* Effector Proteins into Plant Cells.** *Pst* causes disease on *A. thaliana*, a pathosystem that has been well characterized. To deliver putative *H. parasitica* (*Hp*) effectors via *Pst* DC3000, we used the *Pseudomonas avrRpm1* promoter plus secretion-translocation signal of AvrRpm1 reported in refs. 5 and 7). The promoter region plus the *Pseudomonas syringae* type III secretion signal peptide (*PsSP*) were cloned into the wide host range vector pVSP61 that can replicate in *Pst* (Fig. 1A). The addition of the Gateway destination cassette allows rapid cloning of putative effector genes. *Pseudomonas* constructs were preinduced in

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The authors declare no conflict of interest.

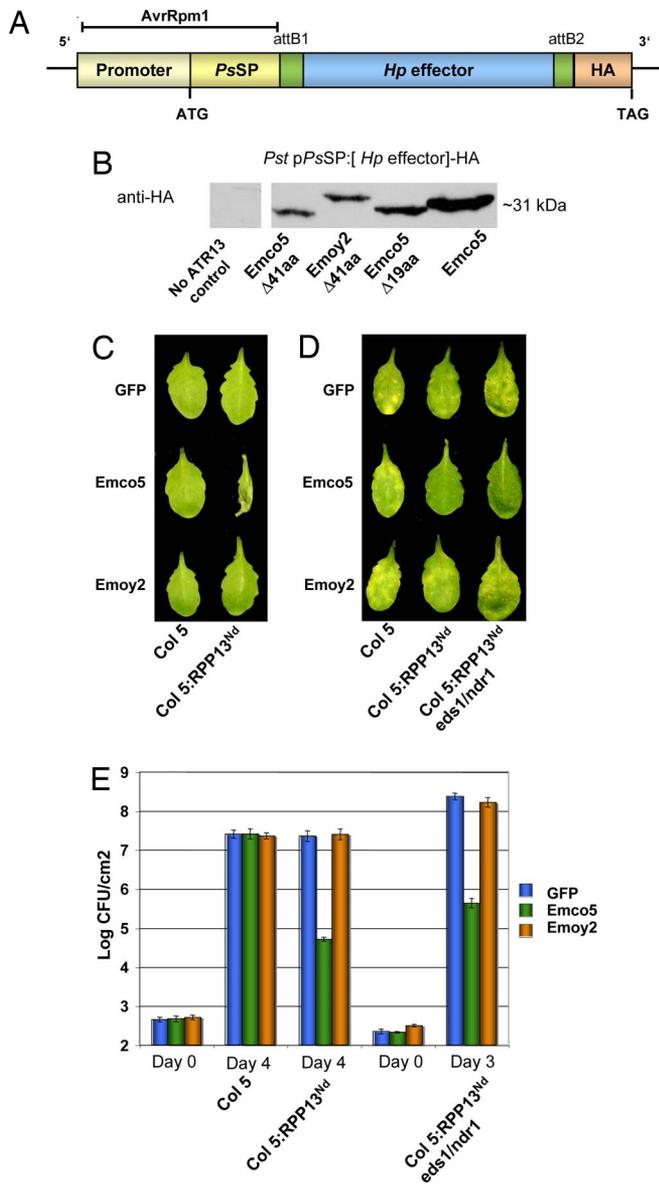
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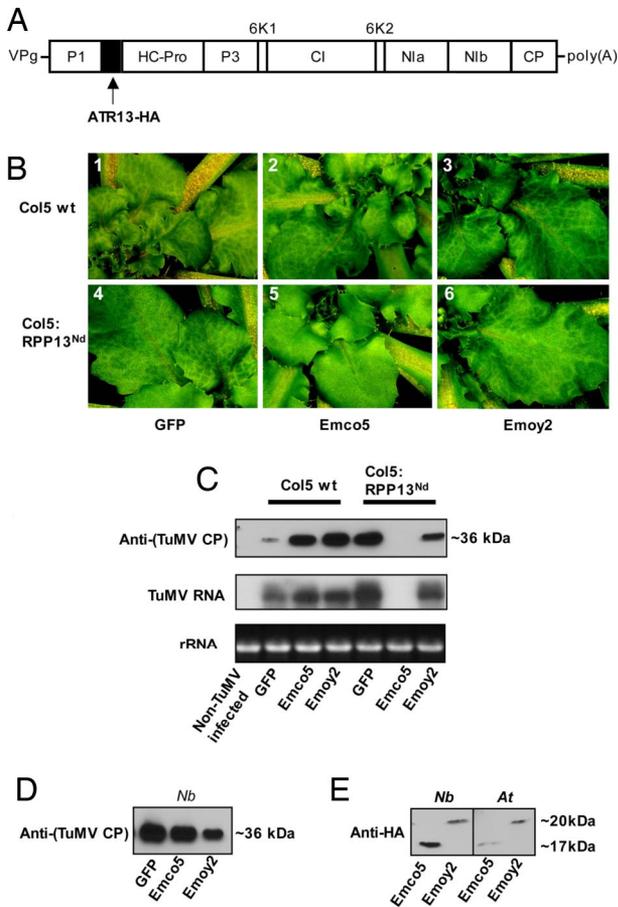


**Fig. 1.** Surrogate *Pst* DC3000 bacterial TTSS to deliver *H. parasitica* effector protein ATR13 to detect RPP13 bacterial resistance. (A) Expression vector pVSP\_PsSPdes for delivery of putative effector proteins via the TTSS of *Pst* DC3000. Expression vectors are gateway-compatible to enable easy effector cloning and epitope tagging. (B) Immunoblot analysis to detect effector expression in *Pst* DC3000 after preinduction in *Pseudomonas*-promoter induction media. HA epitope-tagged *Hp* effector constructs with the ATR13<sup>Emco5</sup> full-length ( $\approx 32$  kDa), ATR13<sup>Emco5- $\Delta$ 19aa</sup> ( $\approx 31$  kDa), ATR13<sup>Emco5- $\Delta$ 41aa</sup> ( $\approx 28$  kDa), and the nonrecognized allele ATR13<sup>Emoy2</sup>. (C) Leaves were inoculated with high-density suspensions of *Pst* DC3000 ( $5 \times 10^7$  CFU/ml). Delivery of  $\Delta 41$ ATR13<sup>Emco5</sup> triggered an HR within 24 hpi in Col5 plants expressing RPP13<sup>Nd</sup>, but not in Col5 WT plants. Delivery of GFP or  $\Delta 41$ ATR13<sup>Emoy2</sup> does not induce an HR. (D) Leaves were inoculated with low-density suspensions of *Pst* DC3000 ( $1 \times 10^4$  CFU/ml). Chlorotic disease symptoms do not develop for DC3000 delivering ATR13<sup>Emco5</sup> on Col-5 RPP13<sup>Nd</sup> and Col-5 (*ndr1/eds1*) RPP13<sup>Nd</sup>. (E) Bacterial growth levels were assessed 3 and 4 days after inoculation. Delivery of  $\Delta 41$ ATR13<sup>Emco5</sup> triggered RPP13-specific resistance, as evident from the absence of chlorotic areas (D) and decreased bacterial growth (E). RPP13<sup>Nd</sup> signaling is independent of *EDS1* and *NDR1* because homozygous double mutants still exhibit RPP13-specific resistance to *Pst* DC3000 delivering  $\Delta 41$ ATR13<sup>Emco5</sup>. The nonrecognized allele  $\Delta 41$ ATR13<sup>Emoy2</sup> does not activate RPP13 resistance. Error bars represent standard deviation for three replicated samples. Growth assay experiments were carried out three times with similar results.

*Pseudomonas*-promoter induction media (8), and HA epitope tagged chimeric effector expression was confirmed by immunoblot detection of the predicted chimeric proteins (Fig. 1B).

**ATR13-Specific Activation of RPP13 Resistance Is Effective Against a Bacterial Pathogen.** We tested *Hp* effector activation of RPP13 resistance by effector delivery through the *Pst*TTSS of DC3000. We compared alleles of ATR13 from the two *Hp* strains, one (Emco5) that activates isolate specific resistance on RPP13<sup>Nd</sup> and one that does not (Emoy2) (9). The first 19 aa constitute a putative *Hp* signal peptide and cleavage site (3). In addition to full-length ATR13, we made a  $\Delta 19$  (no *Hp* signal peptide) version of ATR13<sup>Emco5</sup> and ATR13<sup>Emoy2</sup>. Also  $\Delta 41$  (no *Hp* signal peptide or RxLR motif) versions were made to evaluate the role of this RxLR motif. Although Allen *et al.* (3) reported that a full-length ATR13<sup>Emco5</sup> construct with *Hp* signal peptide delivered biolistically was active, our full-length ATR13<sup>Emco5</sup> construct delivered from *Pst* was not active (data not shown). We detected recognition for both  $\Delta 19$  (no signal peptide) and  $\Delta 41$  (no signal peptide or RXLR motif). Detailed disease assays were carried out by using the  $\Delta 41$  version of ATR13. At high inoculation density, *Pst* delivering ATR13<sup>Emco5</sup> triggered an HR after 24 h on Col-5:RPP13<sup>Nd</sup> but not on Col-5 WT plants (Fig. 1C). Disease assays were performed 3 or 4 days after low-density inoculation. For *Pst* delivering ATR13<sup>Emco5</sup> on Col-5:RPP13<sup>Nd</sup> and Col-5(*ndr1/eds1*):RPP13<sup>Nd</sup>, there were no chlorotic disease symptoms (Fig. 1D), which was confirmed by *in planta* bacterial growth assays (Fig. 1E). Bacterial growth was reduced 1,000-fold on Col-5:RPP13<sup>Nd</sup> for strains delivering  $\Delta 41$ ATR13<sup>Emco5</sup>, confirming that RPP13 is effective against a bacterial pathogen. Growth was also reduced 1,000-fold on Col-5:RPP13<sup>Nd</sup> plants carrying the *ndr1/eds1* mutations, confirming that the defense response triggered by RPP13 is *NDR1*- and *EDS1*-independent (Fig. 1D and E). The nonactive allele, ATR13<sup>Emoy2</sup>, also was unable to activate RPP13<sup>Nd</sup> resistance against the bacterial pathogen. For our *AvrRpm1*-ATR13 effectors, we observe no enhanced virulence for *Pst* on Col-5. Resistance to *Pst* strains delivering the control effectors *AvrRpt2* and *AvrRps4* was abolished in the double mutant (data not shown), because these signaling pathways require *NDR1* and *EDS1*, respectively (10, 11). We conclude that delivery of the oomycete effector protein ATR13 via the TTSS of *Pst* triggers a resistance response in *Arabidopsis* containing the *R*-gene RPP13 consistent with the *Hp* isolate-specific resistance response previously reported (9). This resistance is specific to interactions between cognate effector-*R*-gene pairs, and is functional against the bacterial pathogen *Pst* DC3000.

**$\Delta 41$ ATR13<sup>Emco5</sup> Activates Resistance to a Viral Pathogen in Plants Expressing RPP13<sup>Nd</sup>.** Turnip mosaic virus (TuMV) is able to systemically infect *A. thaliana* (12, 13). The virus belongs to the class of single-stranded (+) RNA potyviruses, with nonoverlapping ORFs (Fig. 2A). This allows insertion of genes into the viral genome, which are subsequently translated into a polyprotein by the host cell and cleaved into individual proteins by viral proteases. We inserted GFP,  $\Delta 41$ ATR13-HA<sup>Emco5</sup> and  $\Delta 41$ ATR13-HA<sup>Emoy2</sup> between the *PI* and *HcPro* genes of the TuMV virus. Challenge of WT Col-5 with viral particles led to a systemic infection with visible mottling symptoms on young rosette leaves (Fig. 2B). TuMV-GFP-infected plants exhibited GFP fluorescence under a UV light, which corresponded to mottled areas. This confirms that the symptoms were due to viral spread (data not shown). WT Col5 plants with mottling symptoms contained high levels of coat protein and viral RNA (Fig. 2C). TuMV containing GFP and  $\Delta 41$ ATR13-HA<sup>Emoy2</sup> replicated to similar levels on Col-5 plants expressing RPP13<sup>Nd</sup>, but virus expressing ATR13<sup>Emco5</sup> was unable to replicate. No mottling symptoms were observed on leaves, and coat protein and viral



**Fig. 2.** Delivery of *ATR13<sup>Emco5</sup>* within the turnip mosaic virus (TuMV) genome activates RPP13 resistance against TuMV systemic infection of *A. thaliana*. (A) TuMV genome. The transgene insertion site (*GFP*, *ATR13<sup>Emco5</sup>*, or *ATR13<sup>Emoy2</sup>*) is indicated by an arrow. Upon translation, viral proteases cleave the polyprotein into separate proteins. RPP13<sup>Nd</sup> recognizes ATR13 from strain Emco5, but not strain Emoy2, when the gene is delivered within the TuMV genome, leading to resistance against the viral pathogen. (B) Leaves were inoculated with infectious viral particles containing *GFP*,  $\Delta 41ATR13^{Emco5}$ , or  $\Delta 41ATR13^{Emoy2}$  fused to the HA epitope sequence as described in *Materials and Methods*. Leaves were inspected for symptoms 8 days pi (after infiltration). Diseased plants showed mottling symptoms on young leaves near the center of the rosette (1–4, 6). Expression of  $\Delta 41ATR13^{Emco5}$  from within the viral genome triggered resistance in Col5 plants expressing RPP13<sup>Nd</sup> (5) but not in Col5 WT plants (2), as evident from the absence of mottling symptoms. Resistance is not induced on delivery of *GFP* or  $\Delta 41ATR13^{Emoy2}$ , with leaves showing clear mottling (4, 6). Contrast and brightness of images was altered in Adobe Photoshop. (C) By using immunoblot analysis and Northern blotting infected plants (1), Col5 WT plants (2) Col-5 RPP13<sup>Nd</sup> (3) Col-5 (*ndr1eds1*) RPP13<sup>Nd</sup> were tested for levels of viral coat protein and viral RNA 8 days pi. Coat protein (CP) and viral genome were absent in plants expressing RPP13<sup>Nd</sup> challenged with TuMV containing  $\Delta 41ATR13^{Emco5}$ . Blots were probed with anti-CP antibody and <sup>32</sup>P-labeled coat protein DNA, respectively. Ethidium bromide-stained 25S rRNA in the RNA gel is shown as a loading control. A minimum of three independent experiments were carried out for each TuMV strain. (D) Leaf discs were taken for protein extraction from the youngest leaf of TuMV-infected *N. benthamiana* (*Nb*) immediately before processing tissue for *Arabidopsis* infection inoculum. Immunoblot analysis with anticoot protein (CP) detects similar levels for all TuMV strains. (E) Immunoblot analysis of TuMV infected *N. benthamiana* (*Nb*) and *A. thaliana* (*At*) detects ATR13<sup>Emco5</sup>-HA and ATR13<sup>Emoy2</sup>-HA that are processed from the TuMV polypeptide. Protein was extracted from infected leaf discs, and blots were probed with anti-HA antibody.

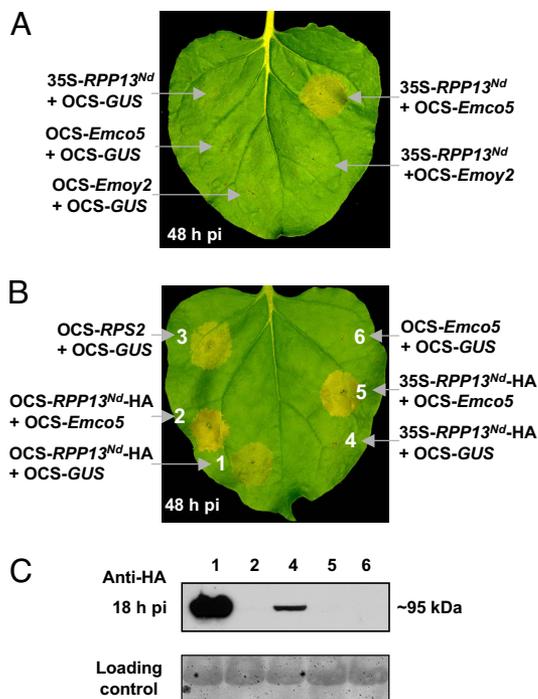
RNA were absent (Fig. 2 B and C). Hence, RPP13<sup>Nd</sup> is able to induce a defense response in the presence of ATR13<sup>Emco5</sup> that is effective against the viral pathogen TuMV. This defense

response is independent of *NDRI* and *EDS1*, because TuMV containing *ATR13<sup>Emco5</sup>* cannot replicate on Col5 *ndr1eds1* mutants expressing RPP13<sup>Nd</sup> (data not shown). TuMV containing  $\Delta 19ATR13^{Emco5}$  also triggered resistance (data not shown). We confirmed that viral particles for all three strains were present at comparable levels in *N. benthamiana* before harvest of inoculum used to infect *A. thaliana* (Fig. 2D). To confirm that ATR13-HA protein was processed from the TuMV polyprotein in infected *N. benthamiana* and *Arabidopsis* leaves, we analyzed protein expression by immunoblot analysis (Fig. 2E).  $\Delta 41ATR13\text{-HA}^{Emco5}$  ( $\approx 17$  kDa) and  $\Delta 41ATR13\text{-HA}^{Emoy2}$  ( $\approx 20$  kDa) were detected in both TuMV-infected *N. benthamiana* and *Arabidopsis* and migrated close to their predicted sizes.

**Coexpression of Cognate RPP13 and ATR13 Proteins Induces a Cell Death Response in *N. benthamiana*.** Transient coexpression of R-proteins and their corresponding effector proteins in *N. benthamiana* has been used as a powerful tool to study R-gene-effector gene recognition (14). To determine whether coexpression of RPP13<sup>Nd</sup> and ATR13<sup>Emco5</sup> could trigger a specific cell death response, we transferred each gene into a gateway compatible *Agrobacterium* binary vector where transient expression is driven by either the 35S promoter or the very strong OCS promoter (15). The resulting plasmids were conjugated into *Agrobacterium tumefaciens* C58C1. A C58C1 strain carrying 35S-RPP13<sup>Nd</sup>-HA was coinfiltrated with C58C1 strains carrying either a *GUS*-control or one of the *ATR13* allele binary constructs (Fig. 3A). RPP13<sup>Nd</sup>-HA and  $\Delta 41ATR13^{Emco5}$  together induced a clear HR within 22 hours after inoculation (hpi). No HR was detected for coinoculations of 35S-RPP13<sup>Nd</sup>-HA with *GUS* control or the nonrecognized allele,  $\Delta 41ATR13^{Emoy2}$ . The coinoculation of  $\Delta 41ATR13^{Emco5}$  or  $\Delta 41ATR13^{Emoy2}$  with *GUS* control was also negative.

A second very strong promoter construct OCS-RPP13<sup>Nd</sup>-HA triggered a  $\Delta 41ATR13^{Emco5}$ -specific HR at 16 hpi, whereas the 35S-RPP13<sup>Nd</sup>-HA triggered an HR at 22 hpi (data not shown). By 48 hpi, the OCS-RPP13<sup>Nd</sup>-HA triggered an  $\Delta 41ATR13^{Emco5}$ -independent HR. This autoactivated HR was not observed for lower expressing 35S-RPP13<sup>Nd</sup>-HA construct. Immunoblot analysis to detect RPP13 expression at 18 hpi confirms the stronger OCS promoter construct expression (Fig. 3C). In the case of the 35S-RPP13<sup>Nd</sup>-HA, it appears that the coexpression of the RPP13 construct with  $\Delta 41ATR13^{Emco5}$  destabilizes the RPP13 protein even before a visible HR is observed.

**Characterization of RPP13 Domains Required for ATR13-Specific Induction of the Cell Death Response.** To define regions of the RPP13<sup>Nd</sup> gene that are necessary for induction of cell death in the *N. benthamiana* transient expression assay, we constructed chimeras between the active RPP13<sup>Nd</sup> (Nd) and the inactive RPP13<sup>Col</sup> (Col) alleles in a native RPP13 promoter *Agrobacterium* binary vector. We used restriction sites conserved between the two genes to swap 5' and 3' ends of the two alleles (Fig. 4A). As expected, *Agrobacterium* coexpression of the WT RPP13<sup>Nd</sup> with  $\Delta 41ATR13^{Emco5}$  induced an HR at 24 hpi (Fig. 4B), whereas coexpression with *GUS*-control triggered no HR (data not shown). Coexpression of RPP13<sup>Col</sup> with  $\Delta 41ATR13^{Emco5}$  induced no HR (Fig. 4B). Only the RPP13 chimera proteins Col-BstXI-Nd and Nd-MslI-Col gave HR at 24 hpi (Fig. 4B). All other chimeras failed to show any responses. The results indicate that amino acids between positions 382–729 of RPP13<sup>Nd</sup> are required for ATR13<sup>Emco5</sup> recognition and cell death induction. This corresponds to the most variable region between these two alleles. Immunoblot analysis with anti-HA for detection of the C-terminal HA epitope tag of all RPP13 constructs revealed that all constructs that have RPP13<sup>Nd</sup> sequences at the C terminus yield the predicted 95-kDa protein, whereas all constructs with the RPP13<sup>Col</sup> sequences at the C terminus were not detectable

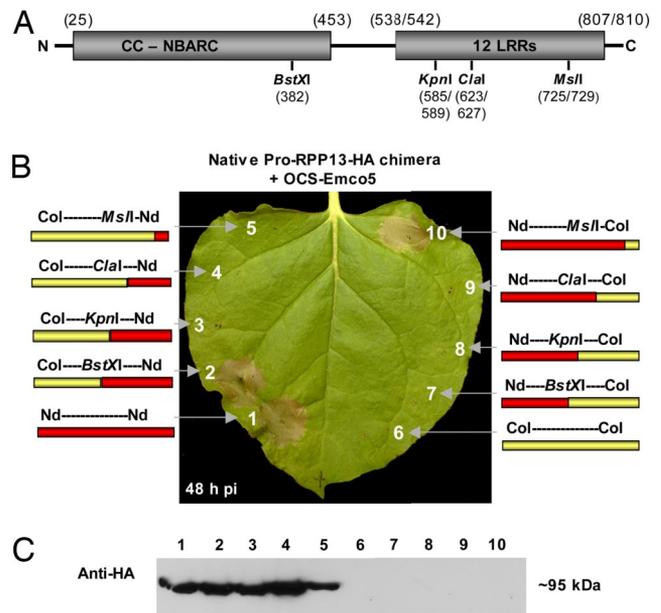


**Fig. 3.** *Agrobacterium*-mediated coexpression of *ATR13*<sup>Emco5</sup> and *RPP13*<sup>Nd</sup> in *N. benthamiana* induces an effector-specific hypersensitive cell death response (HR). *A. tumefaciens* strain (C58C1) was used for transient coexpression, and *N. benthamiana* leaves were used where coinfiltrated constructs were mixed at a final OD<sub>600</sub> of 0.3 for each. (A) *tumefaciens* delivers the various constructs from three different gateway-compatible binary plasmids, pMD1-35Sdes with the 35S promoter and from pE1776des or pE1776-T7des with the stronger OCS promoter. (A) Coexpression of 35S-*RPP13*<sup>Nd</sup>-HA with OCS- $\Delta$ 41*ATR13*<sup>Emco5</sup> induced an effector-specific HR within 22 hpi. Coexpression of 35S-*RPP13*<sup>Nd</sup>-HA with either the nonrecognized *ATR13* allele OCS- $\Delta$ 41*ATR13*<sup>Emoy2</sup> or the OCS-*GUS* control did not induce an HR. Leaf was photographed at 48 hpi. (B) Coexpression of OCS-*RPP13*<sup>Nd</sup>-HA with OCS- $\Delta$ 41*ATR13*<sup>Emco5</sup> induced an effector-specific (HR) within 18 hpi (data not shown); however, by 48 hpi, coexpression of OCS-*RPP13*<sup>Nd</sup>-HA with the OCS-*GUS* control induced an effector-independent HR. Coexpression of 35S-*RPP13*<sup>Nd</sup>-HA and OCS- $\Delta$ 41*ATR13*<sup>Emco5</sup> gave the same response as in A, and the OCS-*RPS2* construct served as a positive HR control. (C) Immunoblot analysis of coexpression constructs at 18 hpi detects elevated levels of RPP13-HA for the strong OCS promoter construct, however, no RPP13 was detected when the effector OCS- $\Delta$ 41*ATR13*<sup>Emco5</sup> was coexpressed. Loading control (Ponceau S stain of Western blot membrane after probing with anti HA)

even though Nd-*MslI*-*Col* was able to give HR. This suggests that the constructs with the *RPP13*<sup>Col</sup> sequences at the C terminus may have been expressed but the HA epitope was not detectable (Fig. 4C). The sequence of the epitope region was reconfirmed for all *Agrobacterium* ex-conjugates.

## Discussion

The genomes of *H. parasitica*, *P. infestans*, *Phytophthora ramorum*, *Phytophthora sojae*, and *Phytophthora capsici* have recently been sequenced (<http://genome.jgi-psf.org> and <http://phytophthora.vbi.vt.edu>), enabling us to predict putative effector proteins by computational analysis. In *Hp* alone, 149 candidate effector proteins were predicted based on the presence of signal peptide and RxLR motifs (2). To date, genetic analysis of oomycete effector proteins has been hampered by the difficulty (or inability) to culture these organisms *in vitro* and to genetically manipulate strains. To circumvent this problem, we tested whether we could individually deliver oomycete effector proteins into resistant *Arabidopsis* lines via the TTSS of the phytopathogenic bacterium *Pst*DC3000 and trigger a disease-resistance response. We constructed a broad host



**Fig. 4.** The LRR domain of *RPP13*<sup>Nd</sup> is required for induction of hypersensitive cell death response when *ATR13*<sup>Emco5</sup> is coexpressed with chimeric *RPP13* constructs between the active Niederenz allele and the inactive Columbia allele. All *Agrobacterium*-transient expression constructs were inoculated at OD<sub>600</sub> 0.3. (A) Diagram of the *RPP13* protein. Amino acid positions are indicated by numbers. Where the amino acid positions differ between the *RPP13*<sup>Nd</sup> and *RPP13*<sup>Col</sup> alleles, both are indicated in the format *RPP13*<sup>Nd/Col</sup>. *KpnI* cuts within the third LRR, *Clal* cuts between the fourth and fifth LRRs, and *MslI* cuts between the eighth and ninth LRRs (9). (B) OCS-*ATR13*<sup>Emco5</sup> is coexpressed with chimeric native promoter *RPP13* constructs in *N. benthamiana* leaves at a final OD<sub>600</sub> of 0.3 for each strain. Strains carried the binary plasmid pMD1 nP13des containing the indicated chimeric constructs of *RPP13*<sup>Nd</sup> and *RPP13*<sup>Col</sup> (format: N terminus–restriction site–C terminus). All chimeras were coexpressed with OCS- $\Delta$ 41*ATR13*<sup>Emco5</sup>. Coexpression of *RPP13* constructs Nd-Nd, Col-*BstXI*-Nd, and Nd-*MslI*-Col with *ATR13*<sup>Emco5</sup> induced an HR by 24 hpi. All other constructs failed to induce an HR. The image was taken 48 hpi. (C) Immunoblot analysis with anti-HA for detection of the C-terminal HA epitope tag of all *RPP13* constructs revealed that all constructs that have *RPP13*<sup>Nd</sup> at the C terminus yield the predicted 95-kDa protein, whereas all constructs with the *RPP13*<sup>Col</sup> at the C terminus were not detectable even though Nd-*MslI*-Col was able to give a cell death response.

range vector that contained the first 89 aa of the bacterial *AvrRpm1* effector protein (which encodes a signal for type III secretion), a C-terminal HA epitope tag sequence, and the Gateway *ccdB* cassette, allowing for rapid cloning of candidate effector genes into our expression vector. The resulting plasmid was then introduced into *Pst* and inoculated onto *Arabidopsis* plants and scored for disease resistance.

Plants have developed the capacity to recognize pathogens from diverse classes and induce defenses, which inhibit pathogen growth and multiplication. To date, the majority of identified plant R proteins fall into the category of proteins containing NBS-LRR motifs. These proteins have been shown to confer resistance to all major classes of pathogens, including viruses, bacteria, fungi, oomycetes, nematodes, and insects. In this study, we demonstrate that the resistance response triggered by activation of the *Arabidopsis* R-gene *RPP13* is effective not only against an oomycete but also against bacterial and viral pathogens. The resistance response was specific to the cognate effector and R-protein pair.

Transient coexpression of effector proteins with their cognate R-proteins in *N. benthamiana* has served as a powerful tool to investigate the specificity of R-gene recognition, protein localization, and protein–protein interactions (16). When we coex-

pressed RPP13<sup>Nd</sup> with ATR13<sup>Emco5</sup>, we observed a rapid cell death response in *N. benthamiana* (Fig. 3A). This response was specific to the interaction of cognate effector and R-protein, because coexpression with GUS or ATR13<sup>Emoy2</sup> failed to induce an HR. When expressed at very high levels, RPP13 alone did induce a delayed HR (Fig. 3B), indicating that the protein is kept in an inactive state, which can be activated if overexpressed.

The RPP13 allele from *Arabidopsis* ecotype Columbia is unable to recognize ATR13<sup>Emco5</sup> (3, 17–19). To identify essential regions for ATR13 recognition and signaling within the RPP13<sup>Nd</sup> protein, we created chimeras between the active RPP13<sup>Nd</sup> allele and the inactive RPP13<sup>Col</sup> allele (Fig. 4B). Our results show that the C-terminal LRR region between amino acids 382 and 725 of RPP13<sup>Nd</sup> is required for induction of cell death upon coexpression with ATR13<sup>Emco5</sup> (Fig. 4B) and is correspondingly the region of highest variability between the two alleles. LRR domains are commonly involved in protein–protein interactions and ligand binding (20) and would therefore be expected to mediate binding to the effector protein. Indeed, genetic analysis of the flax L protein, a TIR-NBS-LRR, has suggested that the LRR is critical for determining the specificity of effector recognition (21), supported by a recent report of direct interactions of this R-protein with its effector in a yeast-two-hybrid assay (22). However, other NBS-LRR proteins, such as the tobacco N protein and *Arabidopsis* R-proteins RPM1 and RPS5, have been shown to interact with their effectors or other signaling molecules through the N-terminal TIR or CC domains (23–25).

In brief, we have shown that ATR13 recognition by its cognate RPP13 allele confers resistance to pathogens from different kingdoms that employ various infection strategies. The mechanism of this resistance has yet to be determined, but a forward genetic mutagenesis screen of Col5:RPP13<sup>Nd</sup> seed may shed light on the pathway involved in RPP13-mediated disease resistance. Additionally, the region of RPP13 implicated in ATR13 recognition has been narrowed down to the amino acid residues between 382 and 725 of the LRR. Focusing on this region, a positive selection analysis will be performed to identify critical residues involved in the ATR13 recognition event, followed by site-directed mutagenesis of these residues to determine functionality and possibly discover novel specificities.

## Materials and Methods

**Strains and Growth.** *Escherichia coli* strains DH5 $\alpha$ , Top10, and DB3.1 and *A. tumefaciens* strains C58C1 and GV2260 were grown on Luria–Bertani agar containing the appropriate antibiotics, at 37°C and 28°C respectively. *Pst* was grown on NYGA agar at 28°C. *N. benthamiana* plants were grown at 24°C, 16-h light/8-h dark cycle. *A. thaliana* plants were grown at 22°C, 8-h light/16-h dark cycle.

**Plasmid Constructs.** For *Hp* effector delivery via the TTSS of *Pst* DC3000, a destination vector was constructed, by using the broad host-range vector pVSP61 as a backbone. The promoter (201 bp) and signal peptide leader sequence (267 bp) of AvrRpm1 (5, 26) were amplified by PCR with primers 5'-agtcgaattcGGAGGCTGCAGTATTCGG-3' and 5'-aatgggatccGATATCCTCG-GTTGCACCATCAGTTTTCT-3'. This EcoRI–AvrRpm1–EcoRV–BamHI fragment was digested with EcoRI and BamHI and inserted into pVSP61. An HA epitope plus stop codon tag annealed from two primers (5'-GATCCTATCCGTACGAGC-TACCAGACTACGCATGAA-3', and 5'-AGCTTTCATGCGTAGTCTGGTACGTCG-TACGGATAG-3') was inserted at the BamHI and HindIII sites. This intermediate plasmid was redigested with BamHI, filled in with Klenow, and religated so that when the destination cassette is cloned in later, the HA epitope is maintained as a translational fusion onto the C terminus of various pENTR clones. This intermediate plasmid was cut at the introduced EcoRV site for cloning a EcoRV fragment containing the ccdB cassette reading frame A (Invitrogen) between the AvrRpm1 TTSS leader sequence and the HA epitope plus stop codon sequence to make the *P. syringae* TTSS signal peptide (PsSP) destination vector pVSP PsSPdes.

Alleles of ATR13 from *H. parasitica* isolates Emco5 and Emoy2 were amplified from spore cDNA by PCR and cloned into pENTR by using the Directional

TOPO Cloning kit (Invitrogen). The following primers were used: forward  $\Delta$ 19 (no signal peptide) 5'-CACCAATCTGCTCCACGCCCATGCGCT-3',  $\Delta$ 41 (no RXLR motif) 5'-CACCGCAGCCAGCGAAGTATTTGG-3'; reverse: ATR13<sup>Emco5</sup> 5'-CTGTCTGCAAGAGCATCCCGA-3', ATR13<sup>Emoy2</sup> 5'-CTGACTGGCAACG-GCAGTCTG-3'. The pENTR ATR13 constructs were transferred to pVSP PsSPdes by performing an LR recombination reaction (Invitrogen). For ATR13 delivery by TuMV,  $\Delta$ 19 ATR13<sup>Emco5</sup>,  $\Delta$ 41 ATR13<sup>Emco5</sup>, and  $\Delta$ 41 ATR13<sup>Emoy2</sup> fragments were amplified from plasmid DNA with primers adding a 5' NcoI site and a 3' HA epitope sequence, followed by a KpnI site. The PCR product was cloned into vector p5PKTuMCS, inserting ATR13 between the viral P1 and HC-Pro genes. This plasmid was digested with SmaI and KpnI, and the fragment containing ATR13 was cloned into p35StuMV. The resulting p35StuMVATR13 plasmid was digested with NcoI and ApaI, and the fragment containing ATR13 was ligated into the binary vector pCBTuMV.

For ATR13 delivery by *Agrobacterium*-mediated transient expression, the binary vector pE1776 with a MAS promoter plus triple chimera OCS UAS and one MAS UAS (15) was first modified with a T7 epitope plus start codon tag inserted at the XhoI and SpeI sites. This intermediate plasmid was redigested with SpeI and filled in with Klenow for cloning an EcoRV fragment containing the ccdB cassette reading frame B to make pE1776-T7des. ATR13 Emco5 $\Delta$ 19+stop and Emoy2 $\Delta$ 19+stop constructs were PCR amplified with the same forward  $\Delta$ 19 primer and the Emco5 and Emoy2 reverse primers, with an additional (5'-CTA-3') stop codon added, were cloned directionally in pENTR. The pENTR ATR13 constructs were transferred to pE1776-T7des by performing an LR recombination reaction (Invitrogen).

For RPP13 delivery by *Agrobacterium*-mediated transient expression, three binary vectors with different strength promoters were used. For the 35S promoter, pMD1 (27) was first modified with a linker annealed from two primers (5'-TCGAGACTAGTTATCCCTACGACGTACCAGACTACGCATTGCA-AGCTCCACCGCTGAGAGGGCTACTGTAGG-3') and (5'-TCGACCTACAGT-TAGCCCTCAGCGGGTGGAGCTTGAATGCGTAGTCTGGTACGTCGTAGGGA-TAAGTAGTC-3') and inserted at the XhoI site. This intermediate plasmid was redigested with SmaI for cloning an EcoRV fragment containing the ccdB cassette reading frame B to make pMD1–35Sdes. For the strong OCS promoter, an EcoRV fragment containing the ccdB cassette reading frame A was cloned into T4 filled-in SacI and KpnI sites of pE1776 to make pE1776des. For RPP13 native promoter, the 35S promoter of pMD1–35Sdes vector was replaced with a HindIII and XbaI native promoter fragment for RPP13 generated with primers: forward (5'-AAGCTTCGTGATATCGCAATCTGT-3'), reverse (5'-TCTAGACTTCTCAATATCGATTGA-3') to make pMD1-nP13des. RPP13<sup>Nd</sup> and RPP13<sup>Col</sup> alleles were PCR amplified from genomic DNA from *Arabidopsis* ecotypes Niederzenz and Columbia, and cloned into pENTR by using primers: common forward (5'-CACCATGGTAGATCGCATCCGGAGT-TCGT-3'); reverse with a SpeI site before the stop codon: RPP13<sup>Nd</sup> (5'-CTAACTAGTAACGCTCGCAATCGGTTTG-3') and RPP13<sup>Col</sup> (5'-CTAACTAGT-GCAGTAGATTGACCAAACGCT-3'). The pENTR RPP13<sup>Nd</sup> and pENTR RPP13<sup>Col</sup> constructs were further modified by adding an HA epitope plus stop codon at the SpeI site. The pENTR RPP13 constructs were transferred to both pE1776-T7des and pMD1–35Sdes by performing an LR recombination reaction.

For pENTR RPP13 Nd and Col chimera constructs, the common BstXI, KpnI, ClaI, and MspI sites of the pENTR RPP13<sup>Nd</sup> and pENTR RPP13<sup>Col</sup> were used with the unique AscI pENTR vector site to generate the various combinations. The pENTR RPP13 chimera constructs were transferred to pMD1 nP13des by performing an LR recombination reaction.

**Bacterial Disease Assays.** All *A. thaliana* bacterial disease assays were performed as described (16).

**TuMV Infection.** *A. tumefaciens* strain GV2260 with binary TuMV constructs were first used to infect *N. benthamiana* and subsequent infectious TuMV extracts from *N. benthamiana* were used to infect *A. thaliana* plants as described in ref. 13. Mottling of young *A. thaliana* leaves was observed after 6–8 days. Viability of viral particles from infected *A. thaliana* were also tested by re-infecting *N. benthamiana*. Infected leaves displayed comparable numbers of distinct lesions 5–6 days pi (data not shown).

**Agrobacterium-Mediated Transient Expression.** *A. tumefaciens* strain C58C1 carrying the binary vector with the gene of interest were cultured, preinduced, and infiltrated into leaves of *N. benthamiana* as described (5).

**Northern Blot Analysis.** Total RNA was extracted from individual plants infected with TuMV by using the RNeasy Plant Total RNA kit (Qiagen) according

to the manufacturer's instructions. Northern blot analysis was performed as described (28). All probes were prepared by PCR, by using the following primers: coat protein: CP for 5'-TGACAGACGAGCAAAGCAG-3'; CP rev 5'-ATGTTCCGATTAACGTCTC-3' (from pCBTuMVGFP plasmid) tub for 5'-GTGAACTCCATCTCGTCCAT-3'; tub rev 5'-CCTGATAAATTCTTTGG-3' (from cDNA).

**Plant and Bacterial Immunoblot Analysis.** *N. benthamiana* transiently expressed proteins were extracted and immunoblot analysis performed as described in ref. 5. *Pst* cultures were washed and cultured for 3 h in the *Pseudomonas* effector promoter induction media with sucrose (8) before protein extraction and immunoblot analysis. Protein bolts were probed with either Anti-TuMV-CP: rabbit polyclonal antibody PVAS34 or Anti-HA: mouse monoclonal antibody (Covance) and secondary HRP-anti-rabbit or HRP-anti-mouse (Bio-Rad).

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**Note Added in Proof.** Sohn *et al.* (29) have also shown that ATR13 and ATR1 can be delivered to plant cells via the *P. syringae* TTSS and can also promote disease susceptibility.

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