The Plant Journal (2006) **48**, 165–176 doi: 10.1111/j.1365-313X.2006.02866.x

The C-terminal half of *Phytophthora infestans* RXLR effector AVR3a is sufficient to trigger R3a-mediated hypersensitivity and suppress INF1-induced cell death in *Nicotiana benthamiana*

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Summary

The RXLR cytoplasmic effector AVR3a of *Phytophthora infestans* confers avirulence on potato plants carrying the *R3a* gene. Two alleles of *Avr3a* encode secreted proteins that differ in only three amino acid residues, two of which are in the mature protein. Avirulent isolates carry the *Avr3a* allele, which encodes AVR3a^{KI} (containing amino acids C^{19}, K^{80} and I^{103}), whereas virulent isolates express only the virulence allele *avr3a*, encoding AVR3a^{EM} (S^{19}, E^{80} and M^{103}). Only the AVR3a^{KI} protein is recognized inside the plant cytoplasm where it triggers R3a-mediated hypersensitivity. Similar to other oomycete avirulence proteins, AVR3a^{KI} carries a signal peptide followed by a conserved motif centered on the consensus RXLR sequence that is functionally similar to a host cell-targeting signal of malaria parasites. The interaction between *Avr3a* and *R3a* can be reconstructed by their transient co-expression in *Nicotiana benthamiana*. We exploited the *N. benthamiana* experimental system to further characterize the *Avr3a–R3a* interaction. R3a activation by AVR3a^{KI} is dependent on the ubiquitin ligase-associated protein SGT1 and heat-shock protein HSP90. The AVR3a^{KI} and AVR3a^{EM} proteins are equally stable *in planta*, suggesting that the difference in R3a-mediated death cannot be attributed to AVR3a^{EM} protein instability. AVR3a^{KI} is able to suppress cell death induced by the elicitin INF1 of *P. infestans*, suggesting a possible virulence function for this protein. Structure–function experiments indicated that the 75-amino acid C-terminal half of AVR3a^{KI}, which excludes the RXLR region, is sufficient for avirulence and suppression functions, consistent with the view that the N-terminal region of AVR3a^{KI} and other RXLR effectors is involved in secretion and targeting but is not required for effector activity. We also found that both polymorphic amino acids, K^{80} and I^{103}, of mature AVR3a contribute to the effector functions.

Keywords: oomycetes, pathogen, effector, hypersensitive response, virulence.

Introduction

Plant pathogens secrete effector proteins to re-program the defense circuitry of their host cells, thereby enabling parasitic colonization. Although effectors primarily function as virulence factors, specific effector molecules can be recognized by plant disease resistance (R) proteins, resulting in the activation of innate immunity embodied in a variety of defense responses, such as the hypersensitive response (HR; Dangl and Jones, 2001; Staskawicz *et al.*, 1995). In such cases, the effector is termed an avirulence (AVR) protein, and the interaction typically follows the gene-for-gene model, which postulates that the concurrent expression of matching pairs of pathogen *Avr* genes and plant *R* genes results in resistance (Dangl and Jones, 2001; Staskawicz *et al.*, 1995). In its simplest biochemical illustration, the gene-for-gene model assumes direct interaction between the *Avr* and *R* gene products. However, recent studies suggest that several

gene-for-gene interactions follow a more complex basis of recognition, in which perception by R proteins is indirect and involves at least a third component (Dangl and Jones, 2001; Martin *et al.*, 2003). According to the 'guard hypothesis' (Van der Biezen and Jones, 1998), this component is a virulence target (VT) that is recognized by the AVR effector in both susceptible and resistant plants. The R protein thus acts as a 'guard' that monitors alterations of the VT mediated by the AVR effector. Evidence supporting indirect recognition models is accumulating with the characterization of VT proteins and the biochemical perturbations caused by AVR effectors in several gene-for-gene interactions (Innes, 2004; Schneider, 2002). One significant implication of indirect recognition models, such as the guard hypothesis, is that the identification of AVR proteins not only provides insight into their roles as triggers of plant innate immunity but also contributes to our understanding of virulence and pathogenicity mechanisms (Alfano and Collmer, 2004; Espinoza and Alfano, 2004).

Oomycetes, such as *Phytophthora*, form a unique branch of eukaryotic plant pathogens (Sogin and Silberman, 1998). The most notable oomycete is *Phytophthora infestans*, the Irish potato famine pathogen. *P. infestans* causes late blight, a devastating and re-emerging disease of potato and tomato (Birch and Whisson, 2001; Kamoun and Smart, 2005). This pathogen establishes intimate associations with plants and requires living host cells to complete its infection cycle, a process known as biotrophy (Kamoun and Smart, 2005). *P. infestans* secretes two classes of effectors that target distinct sites in the host plant (Kamoun, 2006). Some effectors, termed apoplastic effectors, are secreted into the plant extracellular space where they interact with extracellular targets and surface receptors. In contrast, cytoplasmic effectors are translocated inside the plant cell, presumably through specialized structures such as infection vesicles and haustoria that invaginate living host cells. A comprehensive knowledge of the structure and function of these various classes of *P. infestans* effectors and the perturbations they cause in plants is essential for understanding the molecular basis of late blight disease (Kamoun, 2006).

Four oomycete *Avr* genes, *ATR1*^{NdWsB} and *ATR13* from the downy mildew *Hyaloperonospora parasitica*, *Avr1b-1* from the soybean pathogen *Phytophthora sojae*, and *Avr3a* from *P. infestans*, have been cloned recently (Allen *et al.*, 2004; Armstrong *et al.*, 2005; Rehmany *et al.*, 2005; Shan *et al.*, 2004). *Avr3a* was identified using an association genetics approach that aimed at linking polymorphic candidate effector genes with avirulence on potato *R* genes (Armstrong *et al.*, 2005; Bos *et al.*, 2003). *Avr3a* encodes at least two polymorphic secreted proteins of 147 amino acids that differ in only three residues, two of which are in the mature protein (Armstrong *et al.*, 2005). *P. infestans* isolates that are avirulent on *R3a* potato carry the avirulence allele *Avr3a*, which encodes AVR3a^{KI} (containing amino acids C^{19}, K^{80} and I^{103}), whereas virulent isolates carry only the virulence allele *avr3a*, encoding AVR3a^{EM} (S^{19}, E^{80} and M^{103}). Biolistic and agro-infiltration co-expression assays showed that *R3a*-mediated cell death was specifically induced by the *Avr3a* allele, confirming interaction between the gene pair (Armstrong *et al.*, 2005). All examined *P. infestans* isolates carry a functional *Avr3a* and/or *avr3a* allele, and no apparent null mutants were detected, suggesting that this gene plays an important function in the pathogen. Remarkably, *P. infestans Avr3a* and *H. parasitica ATR1*^{NdWsB} reside in syntenic regions, suggesting an ancestral effector locus in these oomycete pathogens (Armstrong *et al.*, 2005).

Similar to the great majority of R proteins that target oomycete pathogens, R3a belongs to the intracellular class of NBS-LRR (nucleotide binding site and leucine-rich repeat) domain proteins, suggesting that recognition of AVR3a^{KI} occurs inside the plant cytoplasm (Huang *et al.*, 2005). AVR3a^{KI} and the three other oomycete AVR proteins carry a signal peptide followed by a conserved motif centered on the consensus RXLR (Rehmany *et al.*, 2005). When directly expressed *in planta*, AVR3a^{KI}, ATR1^{NdWsB} and ATR13 did not require the signal peptide sequence to trigger the HR, and are therefore recognized inside the plant cytoplasm (Allen *et al.*, 2004; Armstrong *et al.*, 2005; Birch *et al.*, 2006; Rehmany *et al.*, 2005). How these effectors are translocated inside host cells is unknown but the RXLR sequence might be implicated (Birch *et al.*, 2006; Rehmany *et al.*, 2005). This motif is similar to a host cell-targeting signal that is required for translocation of proteins from malaria parasites (*Plasmodium* species) into the cytoplasm of host red blood cells (Hiller *et al.*, 2004; Marti *et al.*, 2004), leading to the hypothesis that the RXLR motif also functions as a signal that mediates trafficking into host cells (Birch *et al.*, 2006; Rehmany *et al.*, 2005). Recently, the AVR3a^{KI} RXLR leader sequence was shown to mediate export of the green fluorescent protein (GFP) from the *Plasmodium falciparum* parasite to the host erythrocyte (Bhattacharjee *et al.*, 2006). This suggests that plant and animal eukaryotic pathogens share similar secretory signals for effector delivery into host cells (Bhattacharjee *et al.*, 2006). Bioinformatic analyses indicate that the RXLR motif is frequent among secreted proteins of *P. infestans*, *P. sojae* and *Phytophthora ramorum*, with at least 100 proteins identified in each genome (Bhattacharjee *et al.*, 2006). This raises the possibility that *Phytophthora* spp. deliver a complex set of RXLR effectors to the host cytoplasm, resulting in intricate re-programming of plant defenses (Birch *et al.*, 2006; Kamoun, 2006).

We previously showed that the interaction between *Avr3a* and *R3a* can be reconstructed by concurrent agro-infiltration in *Nicotiana benthamiana* (Armstrong *et al.*, 2005). In this study, we exploited the *N. benthamiana* experimental system to further characterize the structure and function of *Avr3a*. Most notably, we found that, in addition to triggering R3a-dependent HR, AVR3a^{KI} is able to suppress the cell

death induced by the elicitin INF1 of *P. infestans* (Kamoun *et al.*, 1998). Also, the 75-amino acid C-terminal half of AVR3a^{KI}, which excludes the RXLR region, was sufficient for avirulence and cell death suppression functions, consistent with the view that the N-terminal region of AVR3a^{KI} and other RXLR effectors is involved in secretion and targeting but is not required for effector activity.

Results

R3a but not its three paralogs specifically recognizes AVR3a^{KI}

Infiltration of *N. benthamiana* leaves with mixtures of *Agrobacterium tumefaciens* strains expressing R3a and the AVR3a proteins results in a rapid cell death response to AVR3a^{KI} but not AVR3a^{EM} (Armstrong *et al.*, 2005). We took advantage of this assay to further characterize this gene-for-gene interaction. *R3a* belongs to a complex late blight *R* gene locus on chromosome 11 of *Solanum tuberosum* (*R3* locus) that comprises four cloned paralogs: I2GA-SH23-1 (*R3-1*), I2GA-SH23-2 (*R3a*), I2GA-SH23-3 (*R3-3*) and I2GA-SH194-2 (*R3-4*; Huang *et al.*, 2005). To determine whether any of these R3a paralogs can recognize AVR3a, we co-expressed them with the two *Avr3a* alleles by agro-infiltration in *N. benthamiana*. No macroscopic symptoms were observed for any of the combinations involving *R3-1*, *R3-3* and *R3-4* (Figure 1), whereas panels infiltrated with the positive control combination of R3a and AVR3a^{KI} showed a confluent necrosis starting at 3–4 days post-infiltration (dpi). These results indicate that AVR3a^{KI} is specifically recognized by *R3a* among the examined NBS-LRR genes of the *R3* locus.

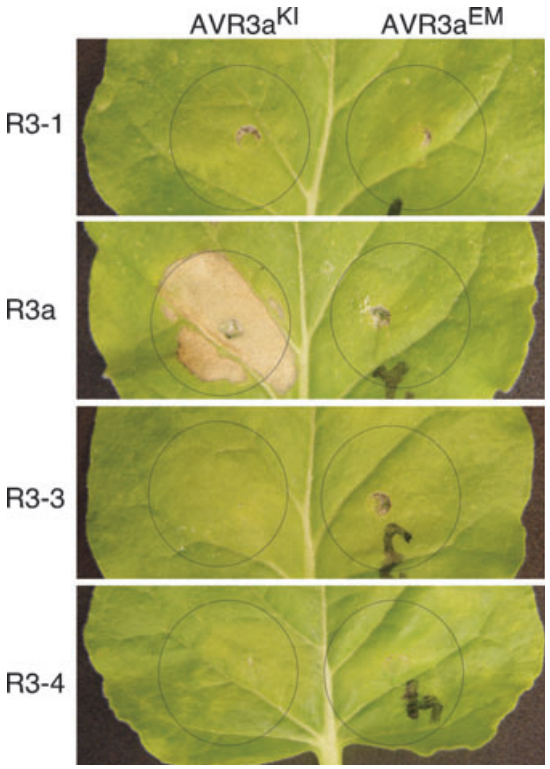

Figure 1. *R3a,* but not its three paralogs, specifically recognizes AVR3a^{KI}. Leaves of *Nicotiana benthamiana* were infiltrated with *Agrobacterium tumefaciens* strains to co-express the three *R3a* paralogs (*R3-1, R3-3* and *R3-4*) with AVR3a^{KI} and AVR3a^{EM}. The R3a and AVR3a^{KI} combination was the only one to induce the hypersensitive response (HR) starting from 3 days post-infiltration (dpi). Photographs were taken at 8 dpi.

The AVR3a^{KI} and AVR3a^{EM} proteins are equally stable in planta

To determine whether the difference in activity between the two *Avr3a* alleles was due to differences in protein stability, we constructed fusions between the FLAG epitope tag and the mature portions of the two AVR3a proteins resulting in the constructs pGR106-FLAG-AVR3a^{KI} and pGR106-FLAG-AVR3a^{EM}. *A. tumefaciens* strains carrying the two constructs were used to infiltrate *N. benthamiana* leaves, and protein extracts were collected 4 days after infiltration and used in Western blot hybridizations with FLAG antisera. As shown in Figure 2(a), a strongly reacting band of about 20 kDa, consistent with the expected size of the recombinant AVR3a, was detected in both treatments. Considering that no differences in intensity were observed between the AVR3a^{KI} and AVR3a^{EM} proteins, we conclude that the two proteins are equally stable *in planta*, and that the difference in R3a-mediated HR cannot be attributed to AVR3a^{EM} protein instability.

AVR3a^{EM} triggers weak R3a-specific responses

In some gene-for-gene interactions, weak responses to the virulent allele may be observed, especially in overexpression assays (Joosten *et al.*, 1997; Westerink *et al.*, 2004). To investigate whether the virulent *avr3a* allele triggers weak R3a-mediated responses, we performed co-agro-infiltration experiments in *N. benthamiana* followed by fluorescent light microscopy for visualization of autofluorescence of phenolic compounds associated with cell death. As expected, no macroscopic cell death was observed at infiltration sites expressing R3a and AVR3a^{EM} (Figure 3d). However, localized autofluorescence was noted, suggesting that AVR3a^{EM} triggers weak R3a responses (Figure 3e). On the other hand, sites infiltrated with the R3a and AVR3a^{KI} combination showed confluent macroscopic cell death and extensive autofluorescence starting at around 3–4 dpi (Figure 3b,c). The induction of a weak response by AVR3a^{EM} was observed in two separate experiments and was dependent on R3a. No significant responses were observed when we expressed AVR3a or R3a alone (Figure S1).

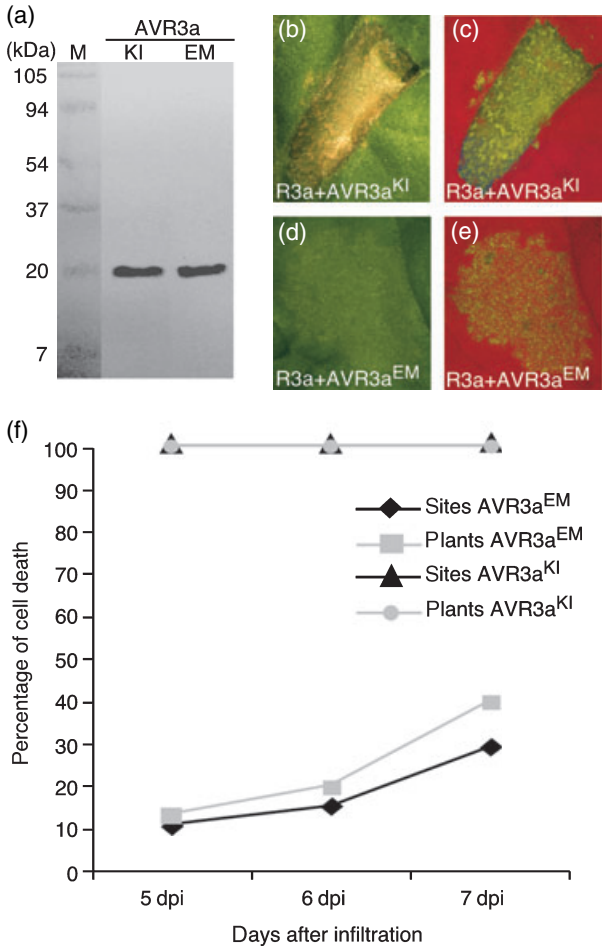

Figure 2. AVR3a^{KI} and AVR3a^{EM} proteins are equally stable *in planta*, and R3a weakly recognizes AVR3a^{EM}.
(a) *In planta* expression of FLAG-AVR3a. FLAG Western blot analysis was performed on total protein extracts of leaves of *Nicotiana benthamiana* following agro-infiltration with constructs expressing FLAG-AVR3a. An approximately 20 kDa protein band representing recombinant AVR3a was present in total extracts of plant tissues expressing either AVR3a^{KI} or AVR3a^{EM}. Molecular weight markers (M) are indicated on the left.
(b–e) Symptoms at *N. benthamiana* agro-infiltration sites expressing the R3a and AVR3a combinations. Symptoms induced by the R3a and AVR3a^{KI} combination (b) and the R3a and AVR3a^{EM} combination (d) were analyzed under a dissecting microscope. Accumulation of autofluorescent phenolic compounds associated with cell death induced by the R3a and AVR3a^{KI} combination (c) and the R3a and AVR3a^{EM} combination (e) were visualized under ultraviolet (UV) light (480/40 nm excitation filter; 510 nm barrier). Photographs were taken at 6 days post-infiltration. Note the localized autofluorescence foci associated with the R3a and AVR3a^{EM} combination.
(f) Percentages of plants and infiltration sites showing induction of macroscopic cell death upon expression of the R3a and AVR3a^{EM} combination in *N. benthamiana* plants. Circles and squares indicate the percentages of plants out of a total of 15 that show macroscopic symptoms of cell death induced by the R3a and AVR3a^{KI} or R3a and AVR3a^{EM} combinations, respectively. Triangles and diamonds indicate the percentages of infiltration sites out of a total of 45 that show macroscopic symptoms of cell death induced by the R3a and AVR3a^{KI} or R3a and AVR3a^{EM} combinations, respectively.

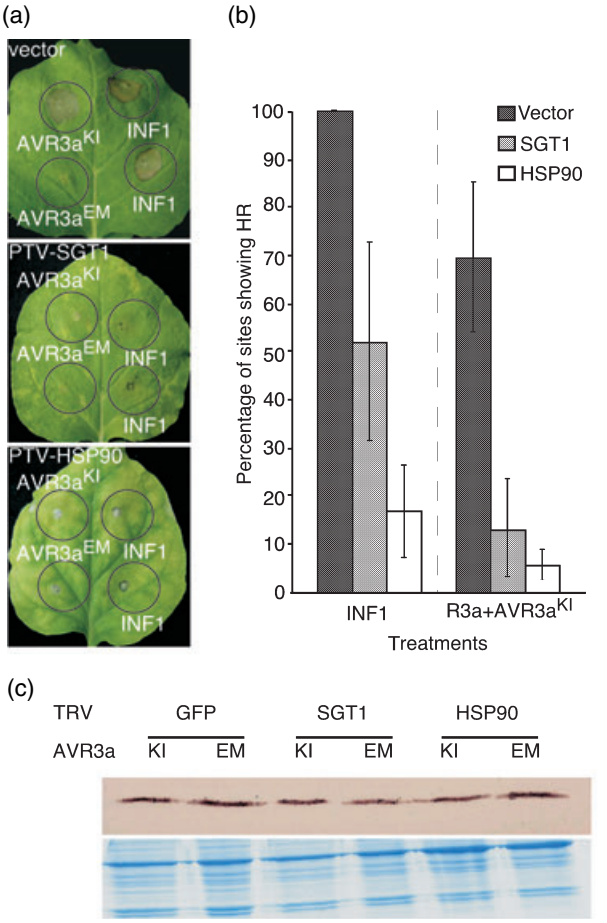

Figure 3. The R3a-mediated hypersensitive response (HR) requires the plant signaling components SGT1 and HSP90.
Control (PTV00 or PTV-GFP), SGT1- and HSP90-silenced *Nicotiana benthamiana* plants were challenged by agro-infiltration of *Agrobacterium tumefaciens* expressing the R3a and AVR3a^{KI} combination or INF1 elicitin. INF1 and the R3a and AVR3a^{KI} combination were expressed in silenced plants in parallel. Control silenced plants showed symptoms of the hypersensitive response (HR) induced by the R3a and AVR3a^{KI} combination around 4 days post-infiltration (dpi) and by INF1 from 2–3 dpi.
(a) Leaves of *N. benthamiana* vector control (PTV-GFP), SGT1- and HSP90-silenced plants at 5 dpi with the AVR3a and INF1 constructs.
(b) Percentages of infiltration sites showing the HR induced by INF1 or the R3a and AVR3a^{KI} combination in vector control, SGT1- and HSP90-silenced plants. Infiltration sites were scored at 4 dpi. The percentages of sites were based on three independent experiments. Error bars indicate the standard error.
(c) Accumulation of FLAG-AVR3a in control, SGT1- and HSP90-silenced *N. benthaminana* leaves. An approximately 20 kDa protein band representing recombinant AVR3a was detected in total extracts of plant tissues expressing either AVR3a^{KI} or AVR3a^{EM}. The bottom panel shows a Coomassie stained gel as a loading control.

To further explore this finding, we conducted a similar agro-infiltration experiment using a larger set of 15 plants. Upon co-expression of R3a and AVR3a^{EM}, up to 28% of the total number of infiltration sites in six of the 15 plants showed non-confluent macroscopic cell death starting from 5 dpi (Figure 3f). In contrast, co-expression of R3a and AVR3a^{KI} resulted in confluent cell death in 100% of the infiltration sites starting from 3 to 4 dpi. These results

confirm that AVR3a^{EM} is weakly recognized by R3a when expressed in *N. benthamiana*.

R3a signaling is dependent on SGT1 and HSP90

The ubiquitin ligase-associated protein SGT1 and heat-shock protein HSP90 are required for the activation of resistance mediated by several NBS-LRR R proteins (Liu *et al.*, 2004; Muskett and Parker, 2003; Takahashi *et al.*, 2003). We tested whether the HR mediated by R3a requires these signaling components using virus-induced gene silencing (VIGS) followed by agro-infiltration assays (Huitema *et al.*, 2004). For this purpose, we infiltrated *N. benthamiana* plants (five-leaf stage) with mixtures of *A. tumefaciens* strains carrying combinations of pBINTRA6 (TRV RNA1) and either pTV-GFP/pTV00 (vector controls), pTV-SGT1 or pTV-HSP90 (TRV RNA2). Three weeks after TRV infection, plants silenced with pTV-SGT1 and pTV-HSP90 showed stunted phenotypes indicative of silencing of the SGT1 and HSP90 plant genes (Figure S2). We also confirmed silencing of SGT1 and HSP90 by RT-PCR and Western blotting (Figure S2). Silenced plants were infiltrated with a mixture of *A. tumefaciens* strains containing pGR106-AVR3a^{KI} or pGR106-AVR3a^{EM} and a strain expressing *R3a*. On pTV vector control-treated plants, the R3a and AVR3a^{KI} combination caused visible HR cell death at 4 dpi in about 70% of the total number of infiltration sites based on three independently replicated experiments (Figure 3a,b). In contrast, on SGT1- and HSP90-silenced plants, cell death was only observed in about 13% and 6% of the infiltration sites, respectively. As expected, no necrotic responses were observed upon co-expression of R3a and AVR3a^{EM} on all plants. As a positive control and to further check for the effectiveness of gene silencing, we infiltrated in parallel with *A. tumefaciens* carrying p35S-INF1, as both SGT1 and HSP90 are required for HR induction by INF1 elicitin (Kanzaki *et al.*, 2003; Peart *et al.*, 2002). INF1 induced the HR in 100% of the infiltration sites on control plants but only in about 50% and 17% of the sites in SGT1- and HSP90-silenced plants, respectively (Figure 3a,b). These findings indicate that, in *N. benthamiana*, R3a triggers the HR through SGT1- and HSP90-mediated signaling pathways similar to other NBS-LRR proteins.

AVR3a^{KI} and AVR3a^{EM} are stable in SGT1- and HSP90-silenced plants

To determine whether the loss of R3a-mediated cell death in SGT1- and HSP90-silenced plants was the result of AVR3a protein instability, we expressed FLAG-AVR3a^{KI} or FLAG-AVR3a^{EM} in the silenced plants and examined AVR3a accumulation by Western blot. An expected 20 kDa band, representing FLAG-AVR3a^{KI} or FLAG-AVR3a^{EM}, was detected in all examined plants (Figure 3c). No consistent differences in protein levels were detected in SGT1- and HSP90-silenced plants compared to the control plants. This suggests that the loss of R3a response in the silenced plants is not the result of reduced AVR3a stability.

AVR3a^{KI} suppresses the HR induced by INF1 elicitin

To identify virulence-related functions of the AVR3a proteins, we tested whether they can suppress cell death induced by the *P. infestans* effectors INF1 elicitin (Kamoun *et al.*, 2003), CRN2 (Torto *et al.*, 2003) and the Nep1-like protein (NLP) PiNPP1.1 (Kanneganti *et al.*, 2006). First, we infiltrated *A. tumefaciens* strains carrying pGR106-AVR3a^{KI} (Δ23–147), pGR106-AVR3a^{EM} (Δ23–147) or pGR106-ΔGFP (vector control) in *N. benthamiana* to express the candidate suppressors. After 1 day, infiltration sites were challenged by infiltration of *A. tumefaciens* strains carrying p35S-INF1, p35S-CRN2 or p35-PiNPP1.1 (Kamoun *et al.*, 2003; Kanneganti *et al.*, 2006; Torto *et al.*, 2003), and cell death symptoms were observed and scored 2–5 days later. Interestingly, AVR3a^{KI} impaired the HR induced by INF1, whereas the AVR3a^{EM} and the vector construct did not (Figure 4a,b). In contrast, both AVR3a^{KI} and AVR3a^{EM} did not interfere with the cell death induced by CRN2 and PiNPP1.1 (data not shown).

To independently confirm INF1 suppression, we performed systemic expression of AVR3a in *N. benthamiana*. Plantlets were inoculated with *A. tumefaciens* strains carrying pGR106-FLAG-AVR3a^{KI} and pGR106-FLAG-AVR3a^{EM}. The recombinant *Potato virus X* (PVX) was allowed to replicate and spread throughout the plant resulting in mosaic symptoms in the upper leaves. Then, we challenged the systemically infected leaves expressing FLAG-AVR3a with recombinant INF1 protein expressed in *Escherichia coli* (Figure S3). As expected, AVR3a^{KI} suppressed INF1 hypersensitivity (Figure S3). These experiments confirm the suppression activity of AVR3a^{KI} and indicate that suppression can take place in a bioassay that does not involve delivery by *A. tumefaciens*.

AVR3a^{KI} does not require the RXLR motif for perception by R3a

When directly expressed *in planta* by agro-infiltration or particle bombardment, AVR3a^{KI} did not require a signal peptide sequence to trigger R3a-mediated HR, indicating that it is recognized inside the plant cytoplasm (Armstrong *et al.*, 2005). Interestingly, a construct carrying the full ORF of AVR3a^{KI}, including the signal peptide, did not trigger cell death in the biolistic assay (Armstrong *et al.*, 2005). We assayed a similar full-length AVR3a^{KI} construct cloned in the binary PVX vector, pGR106-SP-AVR3a^{KI}, by agro-infiltration together with the *R3a* construct and found that it triggered R3a-dependent HR in *N. benthamiana* (Figure 5). One likely

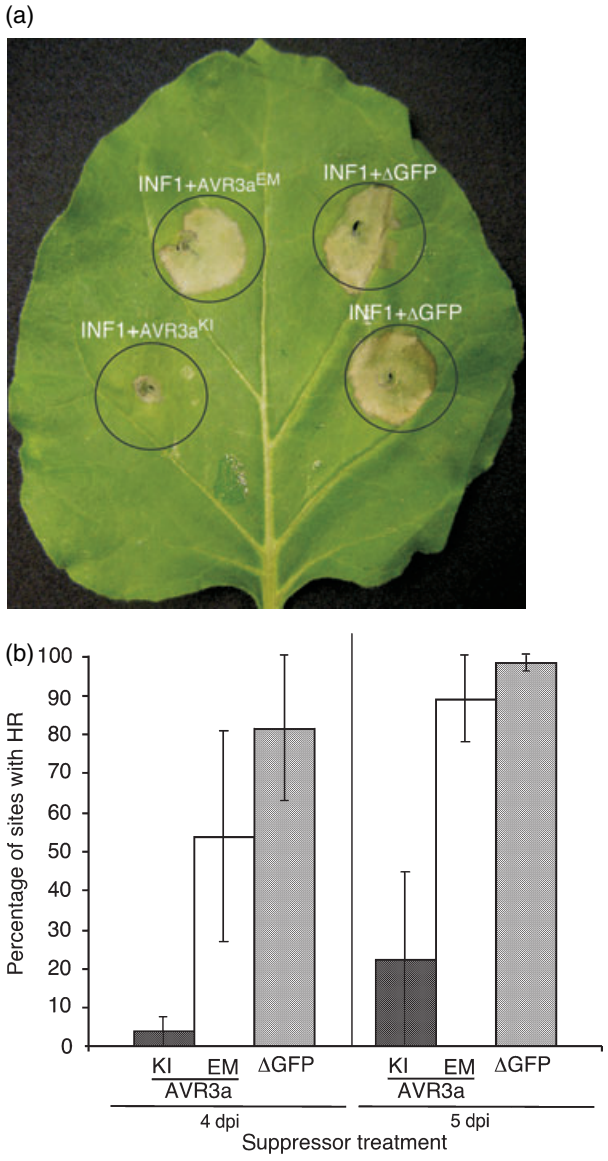

Figure 4. AVR3a suppresses the hypersensitive response (HR) induced by *Phytophthora infestans* INF1 elicitin.
Agro-infiltration sites expressing either AVR3a^{KI}, AVR3a^{EM} or ΔGFP (vector control) were challenged with *Agrobacterium tumefaciens* expressing the INF1 elicitin in *Nicotiana benthamiana*. The INF1-induced hypersensitive response (HR) was observed from 2 to 3 days post-infiltration (dpi) in infiltration sites expressing ΔGFP or AVR3a^{EM} but was suppressed in the AVR3a^{KI} sites.
(a) Symptoms of infiltration sites co-expressing AVR3a or ΔGFP with INF1. The photograph was taken at 6 dpi.
(b) Percentages of infiltration sites showing HR upon co-expression of AVR3a or ΔGFP with INF1 at 4 and 5 dpi. AVR3a and ΔGFP were expressed side-by-side in the examined leaves. The percentages were based on three independent experiments with 6–18 infiltrations per experiment. Error bars indicate the standard error.

explanation for this difference is that agro-infiltration results in significantly higher expression than biolistic delivery, resulting in mis-targeting of some AVR3a^{KI} from the endoplasmic reticulum into the cytoplasm. However, an alternative possibility is that some of the secreted AVR3a^{KI} proteins re-enter plant cells, possibly through the RXLR leader sequence. To test whether the RXLR motif is required for cell death induction by the full-length AVR3a^{KI}, we mutated this sequence to AXAA. Agro-infiltration of the mutated AVR3a^{KI} with *R3a* in *N. benthamiana* resulted in a confluent HR at 3–4 dpi that was similar to the response triggered by the wild-type AVR3a^{KI} (Figure 5). This shows that the RXLR motif of AVR3a^{KI} is not required for recognition by R3a. This experiment is, however, inconclusive with respect to the potential contribution of the RXLR motif for translocation inside plant cells.

The 75-amino-acid C-terminal half of AVR3a^{KI} is sufficient for activation of R3a-mediated cell death

To delineate the functional domain of AVR3a^{KI}, we assayed three N-terminal and C-terminal deletion mutants for activation of R3a cell death by agro-infiltration in *N. benthamiana*. These experiments indicated that the 75-amino-acid C-terminal region of AVR3a^{KI} is sufficient for triggering R3a-mediated cell death (Figure 5). This 75-amino-acid C-terminal region of AVR3a^{KI} excludes the RXLR leader sequence but includes the two polymorphic amino acids K^{80} and I^{103}. Deletion of 16 C-terminal amino acids within this region completely abolished the recognition by R3a.

The 75-amino-acid C-terminal half of AVR3a^{KI} is sufficient for suppression of INF1-induced cell death

We used agro-infiltration to assay the AVR3a^{KI} mutant constructs for suppression of INF1-induced cell death. Both full-length AVR3a^{KI} constructs (RXLR and AXAA mutant) were altered in their ability to suppress INF1-induced cell death compared with the construct with no signal peptide (Figure 5). Also, these two full-length AVR3a^{KI} constructs lost the ability to suppress INF1-induced cell death. Apparently, the presence of the signal peptide interfered with the suppressor activity of the AVR3a^{KI} effector.

We also tested the AVR3a^{KI} deletion constructs for suppressor activity. As with the R3a activation activity, the 75-amino-acid C-terminal region was sufficient for suppression of INF1-induced cell death (Figure 5). However, the suppression ability of the construct expressing the 75-amino-acid region (Δ73-147) was reduced compared to the mature AVR3a^{KI} protein (Δ23-147) or the Δ60-147 deletion (Figure 5). Similarly to the R3a assay, deletion of 16 C-terminal amino acids abolished suppressor activity. Altogether, our data demonstrate that the 75-amino-acid C-terminal half of AVR3a^{KI}, including the two polymorphic amino acids K^{80} and I^{103}, is sufficient for both the suppression of INF1-induced cell death and perception by R3a.

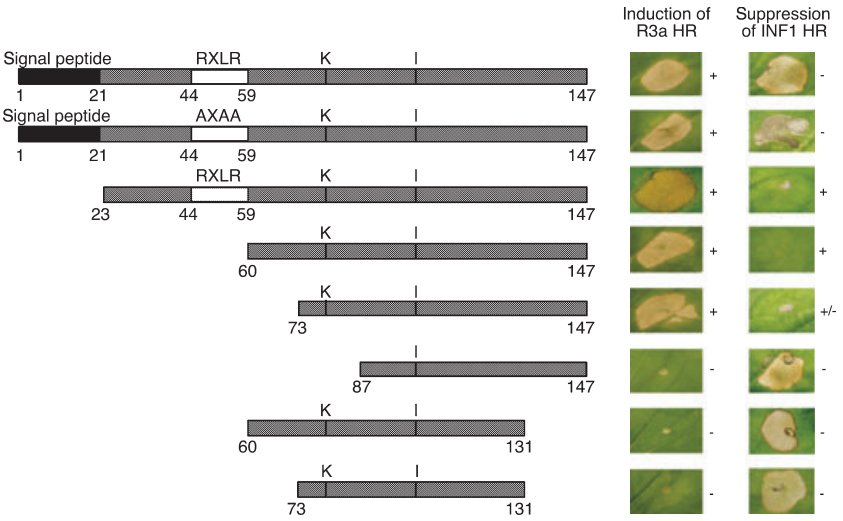

Figure 5. The C-terminal region of AVR3a is sufficient for effector functions.
Various AVR3a^{KI} (deletion) mutants were co-expressed with R3a or INF1 by agro-infiltration in *Nicotiana benthamiana* to determine the AVR3a domains required for induction of the R3a-mediated hypersensitive response (HR) and suppression of INF1-induced hypersensitivity. A schematic view of the various mutant and deletion constructs is shown on the left. Symptoms of infiltration sites co-expressing the AVR3a constructs with R3a or INF1 are shown on the right. Plus and minus signs indicate the presence and absence of effector activity, respectively.

Both K^{80} and I^{103} of AVR3a^{KI} are important for recognition by R3a and suppression of INF1-induced cell death

To evaluate the involvement of the two polymorphic amino acid sites (K^{80} and I^{103}) in AVR3a^{KI} effector activities, we generated AVR3a mutants containing all four combinations of the two residues. Corresponding pGR106-AVR3a (Δ23–147) constructs were used in agro-infiltrations of *N. benthamiana* to express the mature AVR3a proteins in combination with R3a or INF1 (Figure 6a). AVR3a^{KM} induced R3a-mediated hypersensitivity at levels similar to AVR3a^{KI} (Figure 6b). In contrast, AVR3a^{EI} induced an R3a response at lower levels but was consistently more effective than AVR3a^{EM} in several side-by-side infiltrations (Figure 6b). This indicates that both K^{80} and I^{103} contribute to perception by R3a, with K^{80} having a critical effect.

We also performed INF1 suppression experiments with the four AVR3a forms using the co-infiltration assay described above. Both AVR3a^{KM} and AVR3a^{EI} showed reduced ability to suppress INF1-induced cell death compared to AVR3a^{KI}, but were significantly more effective than AVR3a^{EM} (Figure 6c). This suggests that K^{80} and I^{103} contribute to suppression of INF1-induced cell death.

Discussion

The recent cloning of race-specific oomycete *Avr* genes has generated new opportunities for studying the mechanisms of host specificity and pathogenicity in these economically important pathogens (Allen *et al.*, 2004; Armstrong *et al.*, 2005; Rehmany *et al.*, 2005; Shan *et al.*, 2004). AVR3a^{KI} and the other oomycete AVR proteins belong to the RXLR family of cytoplasmic effectors, which are thought to be delivered inside host cells where they contribute to virulence by reprogramming plant defense responses (Kamoun, 2006). In this paper, we follow up on the cloning of *Avr3a* and its cognate *Solanum demissum R* gene *R3a* (Armstrong *et al.*, 2005; Huang *et al.*, 2005) to further characterize this gene-pair interaction. In particular, we took advantage of the experimental capacity to reconstruct the perception of AVR3a^{KI} by R3a using concurrent agro-infiltration in *N. benthamiana* (Armstrong *et al.*, 2005) to alleviate some of the limitations of the *Solanum* system and address a number of outstanding issues about this gene-for-gene interaction. Our main findings consist of insights into R3a-mediated defense signaling and specificity of the *Avr3a–R3a* interaction, as well as evidence that AVR3a^{KI} suppresses INF1-induced cell death. We also showed that the C-terminal half of AVR3a^{KI} is sufficient for effector functions. Finally, we found that both amino acids that are polymorphic between mature AVR3a^{KI} and AVR3a^{EM} contribute to recognition by R3a and suppression of INF1 hypersensitivity.

The *R3a* gene occurs in a complex chromosome 11 NBS-LRR gene cluster that carries multiple resistance specificities to *P. infestans* (Huang *et al.*, 2005). Among the four cloned NBS-LRR genes of this cluster, *R3a* is the only one that recognizes AVR3a^{KI}. This is consistent with the finding that, in transgenic potatoes, these *R3a* paralogs do not exhibit resistance against *P. infestans* isolates expressing AVR3a^{KI} although they are constitutively expressed (Huang *et al.*, 2005). In *N. benthamiana*, R3a perception of AVR3a^{KI} requires the plant signaling components SGT1 and HSP90 (Figure 3). These proteins are believed to function together as (co)chaperones to regulate plant defense signaling of

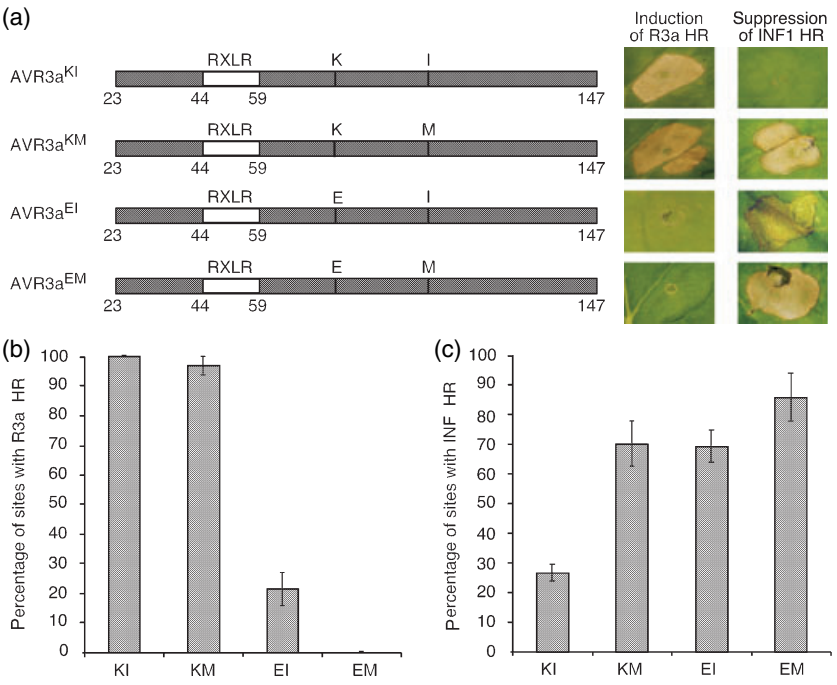

Figure 6. Amino acids K^{80} and I^{103} of $AVR3a^{KI}$ are important for recognition by R3a and suppression of INF1-induced cell death.
AVR3a mutants containing the four combinations of amino acids at the K^{80} and I^{103} polymorphic positions were co-expressed with R3a or INF1 by agro-infiltration in *Nicotiana benthamiana*.
(a) A schematic view of the various mutant constructs is shown on the left. Symptoms of infiltration sites expressing the various AVR3a and R3a combinations or AVR3a and INF1 combinations are shown on the right.
(b) Percentages of infiltration sites with R3a-mediated hypersensitive response (HR) based on four independent experiments scored at 4–8 days post-infiltration (dpi). Error bars indicate standard errors.
(c) Percentages of infiltration sites with INF1-induced HR based on four independent experiments scored at 5–8 dpi. Error bars indicate standard errors.

multiple R proteins (Shirasu and Schulze-Lefert, 2003). In this model, the conformation and kinetics of large multi-protein complexes (signalosomes) are regulated by the activity of SGT1 and HSP90 either through stabilization or recruitment of co-factors into the complex (Liu *et al.*, 2004; Takahashi *et al.*, 2003). It therefore appears that, upon activation by $AVR3a^{KI}$, R3a may associate with such signalosome complexes to regulate defense response signaling.

In transient overexpression assays in *N. benthamiana*, R3a weakly recognizes $AVR3a^{EM}$, resulting in limited and delayed necrotic symptoms compared to $AVR3a^{KI}$. Similar weak recognition of virulent alleles has been reported in some but not all gene-for-gene interactions. For instance, virulent isoforms of Avr4 and Avr4E of the fungus *Cladosporium fulvum* can trigger cell death when co-expressed with their cognate *Cf-4* locus *R* genes, *Hcr9-4D* and *Hcr9-4E*, respectively (Joosten *et al.*, 1997; Westerink *et al.*, 2004). The Avr4 isoforms were unstable in tomato apoplast, suggesting that reduced stability enables evasion of recognition and resistance mediated by *Hcr9-4D* (Joosten *et al.*, 1997). In contrast, Avr4E and its virulent isoform $avr4E^{LT}$ were both stable in tomato apoplast (Westerink *et al.*, 2004). In the present study, we showed that the inability of $AVR3a^{EM}$ to trigger strong R3a-dependent hypersensitivity is not due to lower protein stability *in planta* as the two AVR3a proteins exhibited comparable levels of stability when expressed in *N. benthamiana* by agro-infiltration (Figure 2a). Therefore, the amino acid substitutions in $AVR3a^{EM}$ are likely to directly impact the biochemical activity of this protein and reduce its ability to elicit the R3a response. The AVR3a polymorphism could either affect the binding of AVR3a to R3a in the case of a direct interaction, or alter binding to the host target(s) that may be guarded by R3a. At present, the interaction mechanism of AVR3a recognition by R3a remains to be elucidated.

In an effort to assign virulence-related functions to AVR3a, we discovered that $AVR3a^{KI}$ suppresses the hypersensitive cell death induced by the major *P. infestans* elicitin INF1. In recent years, suppression of innate immunity has emerged as a widespread function for plant pathogen effectors, particularly the type III secretion system (TTSS) effectors of bacterial phytopathogens (Espinoza and Alfano, 2004). Several TTSS effectors suppress the HR elicited by various AVR proteins, explaining in some cases earlier observations of epistatic interactions among *Avr* genes (Abramovitch *et al.*, 2003; Jamir *et al.*, 2004; Kim *et al.*, 2005a; Tsiamis *et al.*, 2000). Apparently, a given strain of a bacterial pathogen delivers a specific repertoire of TTSS effectors, which, depending on the effector combination, may result in the masking of particular avirulence phenotypes (Espinoza and Alfano, 2004). Other TTSS effectors contribute to virulence by suppressing basal defenses induced by conserved pathogen epitopes, known as pathogen-associated molecular patterns (PAMPs; Hauck *et al.*, 2003; Kim *et al.*, 2005b). Consequently, $AVR3a^{KI}$ suppression of INF1-elicited HR could be a genuine, biologically relevant, virulence function. *P. infestans* transformants silenced for INF1 production induced disease lesions in *N. benthamiana*, suggesting that INF1 functions as an avirulence factor that conditions resistance in this species (Kamoun *et al.*, 1998). Interestingly, 88069, the *P. infestans* isolate used in that study, does not express the $AVR3a^{KI}$ allele (Armstrong *et al.*,

2005). It is possible that *P. infestans* strains that produce both AVR3a^{KI} and INF1 are altered in their ability to infect *Nicotiana* spp. For example, suppression of INF1-induced cell death by AVR3a^{KI} could explain the occurrence of *P. infestans* isolates that produce normal levels of INF1 but still colonize *N. benthamiana* (Kamoun, 2001; Restrepo *et al.*, 2005). On the other hand, INF1-activated signaling pathways could overlap with the pathways elicited by other, as yet uncharacterized effectors of *P. infestans*. AVR3a^{KI} could then generally suppress the HR induced by effectors other than INF1 by targeting common signaling components. The extent to which AVR3a^{KI} can suppress cell death triggered by other oomycete RXLR effectors is still under investigation.

AVR3a^{KI} suppresses the cell death induced by INF1 but not by the PiNPP1 and CRN2 effectors of *P. infestans*. This is perhaps not surprising in light of our recent findings that PiNNP1 and CRN2 elicit distinct and antagonistic cell death signaling pathways compared to INF1 (Kanneganti *et al.*, 2006). It also points to a degree of specificity in the suppression activity of AVR3a^{KI}.

Although mutations, such as deletions, premature stop codons and frameshifts, have been frequently reported for the virulence alleles of fungal *Avr* genes (Lauge and De Wit, 1998; Van't Slot and Knogge, 2002), null *avr3a* alleles have not been identified while intact *avr3a* ORFs have been detected among more than 70 isolates of *P. infestans* (Armstrong *et al.*, 2005 and unpublished data). The preponderance of the *avr3a* allele in pathogen populations, despite the selective pressure imposed by *R3a*, implies that AVR3a^{EM} possesses a biological function that contributes to *P. infestans* fitness. Unlike AVR3a^{KI}, AVR3a^{EM} did not suppress INF1-induced cell death, suggesting a different virulence-related activity that remains to be identified.

Deletion analysis revealed that the effector domain of AVR3a^{KI} is limited to its C-terminal half. When AVR3a^{KI} is directly expressed inside plant cells, its N-terminal region, which includes the signal peptide and RXLR motif, is not required for activation of R3a-dependent hypersensitivity and suppression of INF1-induced cell death. These results are consistent with the emerging view that RXLR effectors have two distinct domains: (i) the signal peptide and RXLR motif that target the mature protein to the host cytoplasm, and (ii) the C-terminal region that encodes the effector domain (Kamoun, 2006). Further support for this model is provided by the higher levels of polymorphisms, particularly non-synonymous substitutions, in the C-terminal regions of RXLR effectors. For example, the two out of the three polymorphic residues between the two *Avr3a* alleles, amino acids 80 and 103, are located in the C-terminal effector domain. Also, the C-terminal region of *H. parasitica* ATR1 and ATR13 exhibits higher levels of non-synonymous polymorphisms than the N-terminal region, suggesting that the effector activity is localized to the C-terminal domain (Allen *et al.*, 2004; Rehmany *et al.*, 2005).

In planta expression of cytoplasmic effectors, such as *H. parasitica* ATR1 and ATR13, with a signal peptide does not result in HR induction, in contrast to cytoplasmic expression (Allen *et al.*, 2004; Rehmany *et al.*, 2005). One reasonable explanation is that the majority of the expressed proteins are secreted, resulting in the reduced activity. Here, we observed that the full-length AVR3a^{KI} protein did not result in consistent suppression of INF1 hypersensitivity compared to the truncated constructs, possibly reflecting lower levels of AVR3a^{KI} accumulation inside plant cells (Figure 5). Interestingly, the full-length AVR3a^{KI} protein triggered R3a-mediated hypersensitivity in agro-infiltration assays in *N. benthamiana* although a similar construct did not in biolistic assays in potato (Armstrong *et al.*, 2005). This difference could be explained by the higher expression levels obtained by agro-infiltration with a PVX vector-derived construct, which may result in (i) mis-targeting of the protein to the cytoplasm, or (ii) secretion of AVR3a^{KI} followed by re-entry of the protein inside the plant cell. We favor the first explanation considering that it is well established that incorrectly folded proteins can be translocated back from the endoplasmic reticulum (ER) into the cytosol of plants through retrograde transport (Brandizzi *et al.*, 2003; Di Cola *et al.*, 2001). Thus, it is likely that overexpression of the full-length AVR3a^{KI} results in mis-targeting of some of the protein from the ER to the cytosol, resulting in R3a activation. Attempts to exploit the full-length AVR3a^{KI} construct to evaluate the role of the RXLR motif in translocation inside host cells were inconclusive, as an RXLR to AXAA mutant retained the ability to elicit an R3a response. However, this experiment complements the deletion analyses and confirms that the RXLR motif is not required for effector activity.

Site-specific mutation analysis of the two polymorphic residues of mature AVR3a^{KI} showed that both K^{80} and I^{103} contribute to R3a elicitation and suppression of HR, although to different degrees. The K^{80} residue was critical for triggering R3a-mediated HR regardless of the polymorphism at position 103. A similar observation was reported for the PVX coat protein, which triggers resistance mediated by the potato gene *Rx* (Bendahmane *et al.*, 1995). Two polymorphic key residues contributed to the avirulence function, with a lysine having the largest contribution (Bendahmane *et al.*, 1995). It should be noted, however, that the two single-residue mutants AVR3a^{KM} and AVR3a^{EI} retained a higher level of activity compared with the virulent allele AVR3a^{EM}. It is therefore tempting to speculate that mutations of both K^{80} and I^{103} were required for the evolution of a virulent allele. Future experiments involving transformation of *P. infestans* with the single-residue mutants will help to assess their ability to confer avirulence on *R3a* potato.

Experimental procedures

Microbial strains and growth conditions

Agrobacterium tumefaciens strains GV3101, LBA4404 and AGL0 (Hellens *et al.*, 2000) were used in molecular cloning experiments and were routinely cultured at 28°C in Luria–Bertani (LB) media using appropriate antibiotics (Sambrook and Russell, 2001). All bacterial DNA transformations were conducted by electroporation using standard protocols (Sambrook and Russell, 2001).

Plasmid constructs

All primers and plasmids used in this study are described in Tables S1 and S2, respectively. New constructs include pTRV-GFP that carries part of the *gfp* gene. We amplified the *gfp* fragment using GFP-F and GFP-R. The amplified fragments were digested with *Xma*I and *Bam*HI and ligated into the pTV00 vector to yield PTV-GFP. The *Avr3a* derivatives were cloned in the binary PVX vector pGR106 (Lu *et al.*, 2003) using the oligonucleotide combinations indicated in Supplementary Table S1. *Avr3a* sequences were amplified by PCR and cloned into *Cla*I- and *Not*I-digested pGR106. *Avr3a* mutant constructs were generated by overlap-extension PCR using the strategy described by Kamoun *et al.* (1999). Fragments for generating the pFLAG-ATS-AVR3a constructs were amplified using the oligonucleotides AVR3a_FLAG-F and Avr3a_-FLAG-R, and were digested with the *Eco*RI and *Kpn*I restriction enzymes for cloning into the pFLAG-ATS vector (Sigma, St Louis, MO, USA). Subsequently, fragments for generating the pGR106-FLAG-AVR3a constructs were amplified from pFLAG-ATS-AVR3a plasmid DNA using the oligonucleotides PVX-FLAG-F and Avr3a_147-R and ligated into *Cla*I- and *Not*I-digested pGR106. The pGR106-ΔGFP construct was generated by cloning DNA that had been PCR-amplified with oligonucleotides GFP-F and GFP-R into *Cla*I- and *Not*I-digested pGR106.

Agro-infiltration and PVX agro-infection assays

Recombinant *A. tumefaciens* strains were grown as described previously (Van der Hoorn *et al.*, 2000), except that culturing steps were performed in LB media supplemented with 50 μg/ml kanamycin. Agro-infiltration experiments were performed on 4–6-week-old *N. benthamiana* plants. Plants were grown and maintained throughout the experiments in a greenhouse with an ambient temperature of 22–25°C and high light intensity. In the experiments corresponding to Figures 1–4 we used the pGR106-AVR3a^{KI} (Δ23–147) and pGR106-AVR3a^{EM} (Δ23–147) constructs to express the AVR3a mature proteins. AVR3a constructs were delivered using *A. tumefaciens* strain LBA4404 (Figures 1, 2b–f, 3a,b, 4 and S1) or GV3101 (Figures 2a, 3c, 5, 6, S2 and S3). In side-by-side infiltrations, the two *A. tumefaciens* strains produced similar results in experiments involving AVR3a. However, it should be noted that LBA4404 (pGR106-AVR3a^{EM} Δ23-147) consistently gave a higher frequency of cell-death induction than GV3101 (pGR106-AVR3a^{EM} Δ23-147).

Transient co-expression of *R3a* and *Avr3a* were performed as follows. *A. tumefaciens* strains carrying the respective constructs were mixed in a 2:1 ratio in MMA induction buffer (1 l MMA: 5 g MS salts, 1.95 g MES, 20 g sucrose, 200 μM acetosyringone, pH 5.6) to a final OD_{600} of 0.4.

Similar procedures were used for the suppression assays, except that, for expression of *Avr3a* or Δ*gfp*, the final OD_{600} of recombinant *A. tumefaciens* strains was 0.3 in induction buffer. The infiltration sites were challenged 1 day after infiltration with recombinant *A. tumefaciens* carrying p35-INF1, p35S-CRN2 or p35S-PiNPP1.1 at a final OD_{600} of 0.3 in induction buffer. All suspensions were incubated for 1–3 h prior to infiltration. Symptom development was monitored from 3–8 days after infiltration.

Western blot detection of recombinant AVR3a protein was performed as follows. *N. benthamiana* leaves were infiltrated with strain GV3101 expressing pGR106-FLAG-AVR3a^{KI} or pGR106-FLAG-AVR3a^{EM} in combination with a strain containing pJL3-p19 (obtained from J. Lindbo, Ohio State University, OARDC, Wooster, OH, USA), a binary vector that expresses the suppressor of post-transcriptional gene silencing p19 of *Tomato bushy stunt virus* (TBSV; Voinnet *et al.*, 2003). Strains expressing AVR3a and p19 were mixed in induction buffer in a ratio of 2:1 (final OD_{600} of 0.6). For Western blot detection of recombinant AVR3a proteins in SGT1- and HSP90-silenced plants, infiltrations were performed in the same manner but in the absence of p19.

For PVX agro-infection of *N. benthamiana* with the pGR106-FLAG-AVR3a constructs, 2-week-old plantlets were 'toothpick-inoculated' with the corresponding *A. tumefaciens* strains. Three weeks after inoculation, plants became systemically infected with the recombinant virus and displayed virus mosaic symptoms. Leaves with mosaic symptoms were infiltrated with recombinant wild-type INF1 protein or the inactive mutant INF1S1, produced from *E. coli* carrying plasmids pFB53-INF1 and pFB52-INF1S1 respectively (Kamoun *et al.*, 1997).

TRV-induced gene silencing

We performed gene silencing as described previously (Huitema *et al.*, 2004).

Agrobacterium tumefaciens suspensions expressing the binary TRV RNA1 construct, pBINTRA6, and the TRV-RNA2 vector, PTV00, PTV-GFP, PTV-HSP90 or PTV-SGT1 were mixed in a 2:1 ratio (RNA1:RNA2) in induction buffer (final OD_{600} of 0.6).

Western blotting

Leaf tissue was harvested at 4 dpi, and protein extracts were prepared as described by Moffett *et al.* (2002). The presence of recombinant FLAG-AVR3a was determined by SDS–PAGE and Western blotting as described by Tian *et al.* (2004). Monoclonal FLAG M2 antibodies were obtained from Sigma. Polyclonal HSP90 antibodies were obtained from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA, USA).

RT-PCR analysis

Total RNA from control (GFP), SGT1- and HSP90-silenced *N. benthamiana* leaves was isolated using the Trizol reagent (Gibco-BRL, Bethesda, MD, USA). RT-PCR was performed on equal amounts of RNA using the OneStep RT-PCR kit (Qiagen, Valencia, CA, USA). Primers used to amplify SGT1 that annealed outside the VIGS target region were SGT1-F-RT: 5′-GAAGTGATGTCCACCAAAATTG-3′ and SGT1-R-RT: 5′-CCCATTTCTTCAGCTCCATGCC-3′. Primers for amplification of tubulin were tubulin-F: 5′-ATCGCATCCGAAAG-CTTGCAG-3′ and tubulin-R: 5′-ACATCAACATTCAGAGCTCCATC-3′.

Acknowledgements

We thank I. Malcuit and D. Baulcombe for providing PVX and TRV vectors, Sanwen Huang for the R3a constructs, John Lindbo for

providing the pJL3-p19 construct, and Diane Kinney for technical assistance. This work was supported by NSF Plant Genome Research Program grant DBI-0211659. Salaries and research support were provided, in part, by state and federal funds appropriated to the Ohio Agricultural Research and Development Center, Ohio State University.

Supplementary Material

The following supplementary material is available for this article online:

Figure S1. R3a and AVR3a agro-infiltration controls.

Figure S2. Virus-induced gene silencing (VIGS) controls.

Figure S3. Suppression of INF1-induced cell death in *Nicotiana benthamiana* plants systemically infected with recombinant *Potato virus X* (PVX) expressing AVR3a.

Table S1 Primers used for cloning

Table S2 Bacterial strains and plasmids used in this study

This material is available as part of the online article from http://www.blackwell-synergy.com

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