Relocalization of Late Blight Resistance Protein R3a to Endosomal Compartments Is Associated with Effector Recognition and Required for the Immune Response

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An important objective of plant–pathogen interactions research is to determine where resistance proteins detect pathogen effectors to mount an immune response. Many nucleotide binding–Leu-rich repeat (NB-LRR) resistance proteins accumulate in the plant nucleus following effector recognition, where they initiate the hypersensitive response (HR). Here, we show that potato (Solanum tuberosum) resistance protein R3a relocates from the cytoplasm to endosomal compartments only when coexpressed with recognized Phytophthora infestans effector form AVR3a\textsuperscript{KI} and not unrecognized form AVR3a\textsuperscript{EM}. Moreover, AVR3a\textsuperscript{KI}, but not AVR3a\textsuperscript{EM}, is also relocalized to endosomes in the presence of R3a. Both R3a and AVR3a\textsuperscript{KI} colocalized in close physical proximity at endosomes in planta. Treatment with brefeldin A (BFA) or wortmannin, inhibitors of the endocytic cycle, attenuated both the relocalization of R3a to endosomes and the R3a-mediated HR. No such effect of these inhibitors was observed on HRs triggered by the gene-for-gene pairs Rx1/PVX-CP and Stoi/IpiO1. An R3a(D501V) autoactive MHD mutant, which triggered HR in the absence of AVR3a\textsuperscript{KI}, failed to localize to endosomes. Moreover, BFA and wortmannin did not alter cell death triggered by this mutant. We conclude that effector recognition and consequent HR signaling by NB-LRR resistance protein R3a require its relocalization to vesicles in the endocytic pathway.

INTRODUCTION

Plants have an efficient two-layer receptor system that detects potential pathogens and activates defense responses to prevent disease. Plasma membrane–localized pattern recognition receptors detect highly conserved microbial molecules known as microbe-associated molecular patterns (Zipfel, 2008). The consequent pattern-triggered immunity is suppressed by adapted pathogens, which deploy virulence factors known as effectors (Chisholm et al., 2006; Jones and Dangl, 2006). In recent years, our knowledge, especially of prokaryotic effectors, has demonstrated how microbes can use different strategies to manipulate host processes, creating a suitable environment for infection (Block et al., 2008).

In the second layer of the inducible immune system, effectors are perceived by direct or indirect interaction with intracellular nucleotide binding–Leu-rich repeat (NB-LRR) resistance (R) receptors. NB-LRR genes are one of the largest gene families in plants. Approximately 150 such genes have been described in Arabidopsis thaliana (Meyers et al., 2003) and more than 430 in potato (Solanum tuberosum) (Jupe et al., 2012). Members of the NB-LRR family can be divided into TIR genes carrying an N-terminal domain with similarity to the Drosophila melanogaster Toll and human interleukin-1 receptor and those with a predicted coiled coil (CC) structure at the N terminus. Following perception of effectors (then termed avirulence, or AVR, proteins) R proteins activate effector-triggered immunity, which often includes a localized programmed cell death. Indirect recognition involves the R protein monitoring (or guarding) the biochemical state of a host protein that is targeted by an effector. Bacterial effectors are often recognized indirectly. For example, Pseudomonas syringae effectors AvrR and AvrRPM1 mediate phosphorylation of the host RPM1–INTERACTING PROTEIN 4, RIN4, which is detected by the host immune receptor RESISTANCE TO PSEUDOMONAS SYRINGAE PV. MACULICOLA 1, RPM1 (Chung et al., 2011), whereas RESISTANCE TO PSEUDOMONAS SYRINGAE 2, RPS2, perceives the proteolytic cleavage of RIN4 by P. syringae effector AvrRpt2 (Belkhadir et al., 2004). In contrast with most bacterial effectors, recognition of filamentous pathogen AVR proteins by NB-LRR proteins is often direct (Jia et al., 2000; Dodds et al., 2006; Krasileva et al., 2010).

NB-LRR proteins in unchallenged plant cells have several subcellular localizations. Upon activation, resistance proteins often relocalize to the nucleus where defense responses are initiated (Caplan et al., 2008; Eitas and Dangl, 2010). The potato CC-NB-LRR protein Rx1, which confers extreme resistance to Potato virus X, is activated in the cytoplasm but both nuclear and cytoplasmic pools of Rx1 are required for full function, a partitioning which is regulated by Ran GTase-activating protein 2 (Slootweg et al., 2010; Tameling et al., 2010). The nuclear localization of the tobacco (Nicotiana tabacum) TIR resistance protein 2
protein N, which specifically recognizes the Tobacco mosaic virus p50 helicase domain, is essential for initiating defenses (Burch-Smith et al., 2007). Another example is the Arabidopsis RESISTANCE TO PSEUDOMONAS SYRINGAE 4 protein RPS4, for which accumulation in the nucleus is necessary to trigger immunity in the presence of the P. syringae effector AvrRps4 (Wirthmueller et al., 2007). Recent findings of Gao et al. (2011), however, argue against a universal rule regarding nuclear re-localization of NB-LRR proteins for defense induction. Nuclear localization of RPM1 protein is not required for efficient disease resistance upon activation caused by AvrB and AvrRpm1. Inactive and active RPM1 resides in the plant plasma membrane, an observation that is further supported by experiments using an autoactive RPM1 mutant (Gao et al., 2011). More recently, a number of NB-LRR resistance proteins have been shown to be constitutively localized to a variety of endomembranes, although the mechanistic basis for these localizations in terms of effector recognition and hypersensitive response (HR) signaling is unknown (Takemoto et al., 2012).

One of the best studied oomycete effectors is AVR3a from Phytophthora infestans. AVR3a occurs as two forms differing in only two amino acids: Avr3a<sup>EBDM103</sup> (Avr3a<sup>EM</sup>) and Avr3a<sup>KB01103</sup> (Avr3a<sup>KI</sup>). The potato resistance protein R3a, a member of the CC-NB-LRR class, perceives Avr3a<sup>EM</sup>, whereas Avr3a<sup>KI</sup> evades resistance protein and AVR3a<sup>KI</sup> colocalize at endosomes and are in close proximity in planta. Our data demonstrate that a resistance protein and its cognate avirulence protein can be relocalized to a component of the endocytic cycle, which acts as a site for subsequent activation of the immune response.

RESULTS

R3a Is Relocalized to Vesicles in the Presence of Recognized Effectors

To investigate the localization of R3a upon activation, we fused yellow fluorescent protein (YFP) N-terminally to R3a and transiently expressed this construct in Nicotiana benthamiana in the presence or absence of P. infestans effectors. Fluorescence indicated a cytoplasmic localization of YFP-R3a in the absence of effector proteins (Figure 1A; see Supplemental Figure 1A online). R3a was excluded from the nucleus as fluorescence in the nucleoplasm was not detected. The cytoplasmic localization of R3a remained unaffected after coexpression with AVR3a<sup>EM</sup>, P. infestans avirulence effector AVR2 (Gilroy et al., 2011b), and Pex147-2, an AVR3a paralog (Armstrong et al., 2005) (Figure 1A), none of which trigger R3a-dependent HR (see Supplemental Figure 1B online). However, a dramatic relocalization of R3a from the cytoplasm to rapidly moving vesicle-like structures of various sizes occurred in the presence of AVR3a<sup>EM</sup> and Pex147-3 (Figure 1A), both of which are recognized by R3a (see Supplemental Figure 1B online; Armstrong et al., 2005; Oh et al., 2009). Immunoblot analyses (Figure 1B) confirm the stability of YFP-R3a fusion protein in the presence or absence of AVR3a, indicating that intact resistance protein fusion undergoes the observed relocalization. Interestingly, relocalization does not represent a symptom of dying cells caused by the onset of the HR, as YFP fused N-terminally to R3a effectively prevents the development of the HR in the presence of effectors known to cause R3a-mediated cell death (see Supplemental Figure 1B online).

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To confirm that AVR3a<sup>EM</sup> causes a relocalization of YFP-R3a and to investigate the timing of this event, we coexpressed YFP-R3a with dexamethasone-inducible forms of AVR3a<sup>EM</sup> and AVR3a<sup>KI</sup>. Following dexamethasone treatment, YFP-R3a alone, or coexpressed with dex:AVR3a<sup>EM</sup>, remained cytoplasmic. However, when coexpressed with dex:AVR3a<sup>KI</sup>, relocalization of YFP-R3a was observed from cytoplasm to vesicles from 2 h post-treatment (hpt) with dexamethasone (Figure 2A). The visible symptoms of HR, following expression of untagged R3a with dex:AVR3a<sup>KI</sup>, were not detected at 4 hpt but were clearly visible by 24 hpt with dexamethasone (Figures 2B and 2C). However, weak HR symptoms were first detected in some R3a/dex:AVR3a<sup>KI</sup> coexpression sites by 8 hpt with dexamethasone (examples shown in Figure 2C). This suggests that relocalization of YFP-R3a occurs prior to the initiation of cell death.

R3a Is Relocalized to Late Endosomes

To identify the sites of R3a relocalization in the presence of AVR3a<sup>EM</sup>, we conducted a series of colocalization experiments with diverse subcellular markers. Supplemental Figure 2 online shows that fluorescent protein (FP)-R3a fusions do not relocalize to chloroplasts, nuclei, or peroxisomes. The vesicle-like structures exhibit a characteristic motility, which, together with their different sizes, prompted us to investigate components of the endocytic cycle. Using coexpression with a Golgi marker, we demonstrated that monomeric red fluorescent protein (mRFP)-R3a is not relocalized to these structures (see Supplemental Figure 2 online). We used two additional subcellular markers, Ara6 and Ara7, which belong to the RAB GTPase family. Ara6 and Ara7 are key regulators of endosomal trafficking, endocytosis,
and vacuolar transport (Kotzer et al., 2004; Richter et al., 2009; Ebine et al., 2011). AVR3aK-mediated relocation of green fluorescent protein (GFP)-R3a partially coincided with the marker fluorescence of both Ara6-mRFP and mRFP-Ara7, indicating that, potentially, sorting endosomes are involved in the recognition process (Figure 3). Ara6-mRFP and mRFP-Ara7 have distinct but overlapping localization patterns (Ebine et al., 2011) and label a variety of endosomal compartments. We thus tried the specific prevacuolar compartment (PVC) marker PS1–cyan fluorescent protein (CFP) (Saint-Jean et al., 2010). We observed colocalization of mRFP-R3a with the PVC marker when coexpressed with AVR3aK, demonstrating that late endosomes are a site of R3a relocation (Figure 3).

We extended our investigations with the endocytic tracer FM4-64, which labels early endosomes within a few minutes (Dettmer et al., 2006), followed by labeling of PVCs that constitute a site where endocytic and vacuolar trafficking merge (Tse et al., 2004). Confocal microscopy showed colocalization of FM4-64 with relocalized GFP-R3a (Figure 3), which could be observed from 10 up to 30 min after FM4-64 infiltration, supporting the observation that late endosomes are sites at which activated R3a is localized. Interestingly, not all endosomes labeled by FM4-64 were sites of GFP-R3a relocation (Figure 3), indicating specificity in the endosomes to which R3a is associated.

In addition, we coexpressed YFP-R3a with dex:AVR3aK and PS1–CFP. At 2 hpt with dexamethasone, the earliest point at which relocation of YFP-R3a was observed, we saw colocalization between YFP-R3a and PS1–CFP fluorescence, indicating the presence of R3a at late endosomes (see Supplemental Figure 3 online). As with the FM4-64 tracer above, not all endosomes associated with the PS1–CFP marker were sites for relocation of YFP-R3a, supporting specificity to certain endosomes in the relocation process.

AVR3aK, but Not AVR3aEM, Is Relocalized to Endosomes Prior to R3a-Mediated HR

The results shown in Figure 1 suggest that relocation of R3a is associated with effector recognition, as unrecognized AVR3aEM and Pex147-2 do not provoke this response. Nevertheless, N-terminal FP tagging of wild-type R3a prevents effector-mediated cell death. To test whether relocation of R3a is required for R3a-mediated cell death, we fused R3a C-terminally to GFP. However, the R3a-YFP fusion also did not trigger HR when coexpressed with AVR3aK (see Supplemental Figure 4 online). As we had noted previously (Bos et al., 2010) that N-terminal FP tagging of AVR3aK faithfully yielded R3a-dependent HR, we thus extended our analyses to the localization of recognized and unrecognized effector forms in the presence of untagged R3a.

We first performed colocalization studies following transient coexpression of AVR3aK and R3a, both containing N-terminal fluorescent tags, in N. benthamiana. It was shown previously that AVR3aK, overexpressed alone, is present in the cytoplasm and the nucleoplasm and does not localize to vesicles (Boe et al., 2010). After coexpression of a GFP-AVR3aK fusion with mRFP-R3a, we observed the expected relocation of the mRFP fluorescence to vesicles. In addition, however, we observed relocation of GFP-AVR3aK fluorescence to the same subcellular compartments (Figure 4A), indicating that recognition is associated with close proximity between R3a and AVR3aK.

GFP-AVR3aK and GFP-AVR3aEM were each coexpressed with untagged, functional R3a and with the PVC marker PS1–CFP. Agrobacterium tumefaciens expressing R3a was infiltrated into inoculation sites 24 h after infiltration of Agrobacterium strains expressing GFP-AVR3a alleles and PS1–CFP marker. Confocal images taken 24 h postinoculation of untagged R3a revealed that, whereas GFP-AVR3aK clearly colocalized with the PS1–CFP marker, no such colocalization was seen between GFP-AVR3aEM and PS1–CFP (Figure 4B; see Supplemental Figure 5 online). Moreover, in the absence of R3a, AVR3aK did not colocalize with the PS1 marker, indicating that its association with late endosomes is specific to coexpression with R3a (see Supplemental Figure 5 online). As seen for relocation of FP–R3a in Figure 3, GFP-AVR3aK was relocated in the presence of untagged R3a to endosomes containing the endocytic tracer FM4-64 (Figure 4C). Critically, R3a-mediated HR was not

Figure 1. R3a Relocalizes to Vesicles in the Presence of Recognized Effectors.

(A) Confocal laser scanning microscopy following transient expression (by agroinfiltration) in N. benthamiana of YFP-R3a alone or transiently coexpressed with either AVR3aEM, AVR3aK, AVR2, Pex147-2, or Pex147-3 as indicated in the panels at 2 d after inoculation. Experiments were repeated at least five times. Bar = 25 μm.
(B) Immunoblot probed with α-GFP following transient expression of YFP-R3a alone or coexpressed with AVR3aEM or AVR3aK in N. benthamiana at 2 d after inoculation. Protein sizes are indicated (in kilodaltons), and protein loading is shown by Ponceau stain (PS).
Figure 2. Relocalization of R3a Occurs before Development of HR Symptoms.

(A) Confocal laser scanning microscopy following transient expression (by agroinfiltration) in N. benthamiana of YFP-R3a alone or in the presence of conditionally coexpressed Avr3aEM and Avr3aKI at the indicated time points after dexamethasone treatment at 2 d after inoculation. Bar = 20 μm.
inhibited in these assays, and macroscopic cell death was clearly visible by 48 h postinoculation of untagged R3a and only in the presence of GFP-AVR3aKI (Figure 4D). This indicates that relocalization of both R3a and AVR3aKI to endosomes occurs prior to HR symptoms.

To further investigate the close proximity between R3a and AVR3aKI, we used bimolecular fluorescence complementation (BiFC), or split-YFP, to analyze and localize protein–protein interactions in plant cells (Walter et al., 2004; Bos et al., 2010). We fused N and C terminus–encoding portions of YFP to the N terminus of R3a, AVR3aKI, and to the unrecognized effector form AVR3aEM. Constructs expressing fusions to complementary YFP halves were transiently expressed in N. benthamiana. As shown in Figure 5A, fluorescence was undetectable following coexpression of YN-R3a with YC-AVR3aEM. Constructs expressing fusions to complementary YFP halves were transiently expressed in N. benthamiana. As shown in Figure 5A, fluorescence was undetectable following coexpression of YN-R3a with YC-AVR3aEM. By contrast, YN-R3a/YC-AVR3aKI coexpression yielded strong fluorescence signals in a pattern reminiscent of relocalization to the endosomal vesicles, indicating close physical proximity between R3a and AVR3aKI sufficient to reconstitute YFP fluorescence. Immunoblot analyses confirmed the stability of all expressed fusion proteins in planta (Figure 5B).

To confirm the identity of vesicles observed in these experiments, YN-R3a, YC-AVR3aKI, and the PS1-CFP marker were coexpressed and revealed colocalization of reconstituted YFP fluorescence with PS1-CFP at endosomal compartments (Figure 5C). These results support relocalization of both R3a and AVR3aKI into close proximity at compartments within the endocytic pathway.

Direct Interaction between R3a and AVR3a Is Not Detectable Using Yeast Two-Hybrid or Coimmunoprecipitation Analyses

The close proximity observed between Avr3aKI and R3a in the colocalization and split-YFP experiment detailed above prompted us to investigate potential direct recognition of AVR3a by R3a. We
Figure 4. Avr3aKI, but Not Avr3aEM, Relocalizes to Late Endosomes in the Presence of Untagged R3a Prior to HR Development.

(A) Confocal laser scanning microscopy following transient coexpression by agroinfiltration in N. benthamiana of GFP-AVR3aKI (left picture) and mRFP-R3a (right picture), with overlay (middle picture) indicating colocalization of GFP and mRFP fluorescence at 2 d after inoculation.
conducted a series of yeast two-hybrid (Y2H) experiments using different R3a bait clones with three different AVR3a prey constructs: AVR3aEM, AVR3aKI, and AVR3aKI\_D\_Y147 (described in Bos et al., 2010). The AVR3aKI\_D\_Y147 mutant construct is unable to interact with the target protein in potato, CMPG1, but is nevertheless still recognized by R3a (Bos et al., 2010). No direct protein–protein interactions were observed between wild-type R3a with any AVR3a forms (see Supplemental Figure 6 online), since no colonies grew on triple-dropout medium (-L,-W,-Ade) and β-galactosidase activity was not detected. Moreover, no significant reporter gene activity was observed after coexpressing R3a subfragments with the AVR3a forms (see Supplemental Figure 6 online), indicating that, at least in the yeast system, AVR3a does not interact directly with R3a. This observation was not caused by fusion protein instability since all expressed R3a fusion proteins (see Supplemental Figure 6 online), and AVR3a fusion proteins (Bos et al., 2010) were detectable in yeast by immunoblot.

Although Y2H analysis is a facile, powerful, and high-throughput approach to analyze protein–protein interactions, it is prone to false-negative results, meaning that true interactions are sometimes not detected. Since yeast cytoplasmic and nucleoplasmic conditions do not mimic the plant cell environment in which effector recognition occurs naturally, misfolding of the fusion protein, steric hindrance, or nonspecific protein aggregation could prevent an R3a–AVR3a interaction, despite the proteins being stably expressed (see Supplemental Figure 6 online). To further investigate whether R3a interacts directly with AVR3a, we conducted in planta coexpression and coimmunoprecipitation studies.

Figure 4. (continued).

(B) GFP-Avr3a\_KI or GFP-Avr3a\_EM (left picture, as indicated), each coexpressed with PVC marker PS1-CFP (right picture) and untagged R3a, with overlay (middle picture), indicating colocalization of GFP and CFP fluorescence only in the case of GFP-Avr3a\_KI. (C) GFP-Avr3a\_KI (left picture) coexpressed with untagged R3a. Localization of FM4-64 (right picture) was examined 20 min after its infiltration. Overlay (middle picture) indicates colocalization of GFP and FM4-64 fluorescence. For experiments in (B) and (C), agroinfiltration to express untagged R3a was performed 24 h after agroinfiltration to express GFP-Avr3a (and PS1-CFP, as indicated). Images in (B) and (C) were taken 2 d after inoculation of GFP-Avr3a\_KI (1 d after inoculation for R3a). Bars = 10 µm.

(D) Transient expression of untagged R3a and GFP-Avr3a\_KI alone or coexpressed (agroinfiltration to express R3a was 24 h after agroinfiltration to express GFP-Avr3a). Photos of HRs were taken at indicated time points (h p.i., hours post inoculation) and are representative of multiple independent assays. Circles indicate the infiltrated area on the leaf panel.
**Figure 6.** Wortmannin and BFA Attenuate R3a Relocalization.

(A) Confocal laser scanning microscopy following transient coexpression in *N. benthamiana* by agroinfiltration of YFP-R3a with AVR3aKI in the absence (-wortmannin, -BFA, left pictures) and 30 min after infiltration of 33 µM Wortmannin or 20 min after infiltration of 20 µg/mL BFA (right picture). Bar = 25 (top images) and 20 (bottom images) µm. Pictures were taken 2 d after agroinfiltration, and experiments were repeated three times.

(B) Immunoblot probed with α-GFP following transient coexpression of YFP-R3a with AVR3aKI (left picture) and transient coexpression of GFP-Avr3a with mRFP-R3a (right picture) in *N. benthamiana* by agroinfiltration at 2 d after inoculation to show stability of R3a and Avr3a after application of 20 µg/mL BFA and 30 µM wortmannin for 2 h. Protein sizes are indicated (in kilodaltons), and protein loading is shown by Ponceau S (PS).
We transiently coexpressed GFP-Avr3a fusions and FLAG-R3a fusions in *N. benthamiana* via agroinfiltration and immunoprecipitated GFP-Avr3a using magnetic GFP-Trap beads. Despite a detectable enrichment of GFP-Avr3a (see Supplemental Figure 7 online), we could not coimmunoprecipitate FLAG-R3a, indicating that a direct interaction between Avr3a and R3a possibly does not occur, that the interaction is too transient, or that it is potentially disrupted during the total protein extraction process. Unfortunately, it was not possible to conduct reciprocal coimmunoprecipitation experiments due to nonspecific binding of AVR3a to all beads tested.

### Inhibitors of the Endocytic Cycle Attenuate R3a Relocalization to Late Endosomes

To investigate the contribution of late endosomes to either recognition of AVR3a and/or the signaling of R3a-dependent HR, we monitored the influence of two inhibitors of the endocytic cycle, brefeldin A (BFA) and wortmannin, on the relocalization of R3a in the presence of AVR3aKI. BFA is reported to inhibit the activation of ARF-GTPase that is involved in multiple trafficking pathways (Chardin and McCormick, 1999; Nielsen et al., 2008; Robinson et al., 2008). Golgi-localized proteins are redistributed to the endoplasmic reticulum (ER) upon BFA treatment. Furthermore, BFA causes aggregation of the trans-Golgi network and multivesicular endosomes (PVCs in plants) into BFA bodies (Uemura et al., 2004; Jaillais et al., 2008; Ebine et al., 2011). Wortmannin suppresses the formation of PVCs by inhibiting phosphatidylinositol 3-kinase and affects the later steps of clathrin-coated vesicle formation that eventually inhibits endocytosis (Emans et al., 2002; Ebine et al., 2011; Ito et al., 2011). Additionally, wortmannin causes late endosome/multivesicular endosome (PVC) deformation (Ebine et al., 2011).

The application of both compounds had a dramatic effect on FP-R3a relocalization. Only 20 to 30 min after treatment, both wortmannin and BFA (Figure 6A) caused a significant increase in cytoplasmic fluorescence compared with untreated samples. The cytoplasmic fluorescence in the presence of both BFA and wortmannin is not due to degradation of FP-R3a caused by mislocalization, as immunoblot analyses confirmed the stability of FP-R3a in planta following these treatments (Figure 6B). Due to the high cytoplasmic background of FP-AVR3a, it was not possible to perform reciprocal experiments to study the effects of the inhibitors on (preventing or reducing) FP-AVR3a relocalization in the presence of untagged R3a. However, we confirmed that GFP-AVR3a also remained stable in planta in the presence of mRFP-R3a following treatments with BFA and wortmannin (Figure 6B).

### The R3a(D501V) Autoactive Mutant Is Cytoplasmic

As some mutations in the NB-ARC domain in general, and in the Met-His-Asp (MHD) motif in particular, are known to cause autoactivation of resistance proteins (Tameling et al., 2006; Gao et al., 2011; Williams et al., 2011), we further investigated the significance of AVR3aKI-specific R3a relocalization by generating an autoactive R3a variant. We constructed R3a(D501V) (Figure 7A) by site-directed mutagenesis of the MHD motif and expressed this mutant with and without N-terminally fused YFP.
in *N. benthamiana*. In the absence of the effector, untagged R3a (D501V) is sufficient to induce HR cell death (Figure 7B). However, HR symptoms caused by R3a(D501V) alone developed a day later than HR triggered by R3a-mediated recognition of AVR3a\textsuperscript{ki} (see Supplemental Figure 8 online). Delayed HR caused by mutations in the MHD motif of resistance proteins has been observed previously (Rairdan and Moffett, 2006).

The localization of the YFP-R3a(D501V) mutant was predominantly cytoplasmic, similar to YFP-R3a when expressed in the absence of recognized effectors. This cytoplasmic localization was maintained even when YFP-R3a(D501V) was coexpressed with AVR3a\textsuperscript{ki} (Figure 7D). The observation that YFP-R3a(D501V) is largely cytoplasmic (Figure 7) suggests that HR signaling by this autoactive mutant has been uncoupled from relocalization. As with the coexpression of wild-type YFP-R3a with AVR3a\textsuperscript{ki}, YFP-R3a(D501V) does not trigger cell death symptoms (see Supplemental Figure 9 online), suggesting that downstream signaling is prevented by this fusion. Immunoblot analyses confirmed that YFP-R3a(D501V) was stable in planta, indicating that the fluorescence reports the location of intact fusion protein (Figure 7C).

### Relocalization of R3a Is Required for the Immune Response

Since relocalization of R3a in the presence of AVR3a\textsuperscript{ki} was attenuated by BFA and wortmannin treatment, we investigated the impact of these inhibitors on HR development. At 24 h after coagroinfiltration of *N. benthamiana* with *Agrobacterium* expressing R3a and AVR3a\textsuperscript{ki} (24 h prior to the onset of visible HR symptoms), we infiltrated BFA and wortmannin into the agro-infiltrated leaf area and assessed HR development. As controls (showing similar timing of HR development), we transiently coexpressed the potato resistance protein Rx1 with the corresponding coat protein (CP) of *Potato virus X* (PVX) (Bendahmane et al., 1999), and *Solanum stoloniferum* resistance gene Sto1 with the corresponding *P. infestans* avirulence effector IpiO1 (Vleeshouwers et al., 2008). In the presence of PVX-CP, Rx1 triggers an HR that is not affected by BFA and wortmannin treatment (Figure 8; see Supplemental Figure 10 online). Likewise, we did not observe suppression of the Sto1-mediated HR by BFA and wortmannin in the presence of IpiO1 (see Supplemental Figure 10 online). The infiltration of either inhibitor alone did not cause any cell death symptoms within the time frame of assessing HR development (see Supplemental Figure 10 online). In contrast with the HR mediated by Rx1 and Sto1, both inhibitors considerably attenuated R3a-triggered HR (Figure 8; see Supplemental Figure 10B online). Within the entire inhibitor-infiltrated region, the HR normally caused by R3a/AVR3a\textsuperscript{ki} did not develop.

We assessed whether BFA or wortmannin treatment would have a similar effect on the autoactive HR triggered by R3a (D501V). Inhibitors were applied from 24 h after inoculation of R3a(D501V) up to 48 h after inoculation (24 h prior to visible HR

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Figure 8. BFA and Wortmannin Significantly Attenuate HR Development Triggered by R3a in the Presence of AVR3a\textsuperscript{ki} in Contrast with R3a\textsuperscript{(D501V)}- and Rx1-Triggered HR.

(A) and (B) Transient coexpression of R3a with AVR3a\textsuperscript{ki} (left picture) and Rx1(Rx) with PVX-CP (right picture) and expression of R3a(D501V) (middle picture) by agroinfiltration in *N. benthamiana* (red circles) was followed 24 h later [48 h later for R3a(D501V)-expressing leaves] by infiltration of 20 µg/mL BFA (A) and 33 µM wortmannin (B) (areas infiltrated by inhibitors indicated as blue circles). Photos of HRs were taken 48 h [72 h for R3a(D501V)-expressing leaves] after agroinfiltration.

(C) Graph shows the percentage of inhibitor (BFA and wortmannin) infiltration sites developing a clear HR at 2 d [for R3a and Rx1] and 3 d [for R3a(D501V)] after agroinfiltration. Experiments were repeated at least three times, each with no less than four leaves from four plants. Error bars indicate ± se.
symptoms of this delayed cell death response, similar to application with wild-type R3a coexpressed with AVR3aK1). In contrast with wild-type R3a, the cell death triggered by the autoactive MHD mutant R3a(D501V) was unaffected by either BFA or wortmannin at any time point of inoculation (shown for 48 h after inoculation in Figure 8).

DISCUSSION
A vital objective of plant–pathogen interaction research is to determine how and where pathogen effectors are recognized by resistance proteins to mount a powerful immune response. General models of effector recognition by plant NB-LRR proteins are not yet well established (Caplan et al., 2008; Eltaz and Dangl, 2010). Due to the different intrinsic virulence activities of pathogen effectors that, in turn, demand various recognition and signaling capabilities from R proteins, different model systems of effector recognition processes have been recently proposed (Bernoux et al., 2011). Our molecular and microscopy analyses of the potato resistance protein R3a and the oomycete effector protein AVR3a shed light on a particular activation process of a cytoplasmic NB-LRR protein by a pathogen effector. This study shows that R3a relocalizes to compartments of the endocytic pathway only in the presence of recognized effector forms prior to HR cell death. Interestingly, R3a relocalization is accompanied by relocalization also of the recognized effector form, AVR3aK1, and is required to mount a full immune response. We provide data demonstrating that both proteins colocalize to late endosomes in host plant cells prior to HR initiation.

Relocalization of R3a to Endosomes in the Presence of Recognized Effectors Is a Prerequisite for the Immune Response

In recent years, the observation that many resistance proteins accumulate in the nucleus in the presence of their corresponding effector proteins gave rise to a model where, upon pathogen perception, nuclear accumulation of R proteins is essential for activating the signaling process that leads to transcriptional reprogramming and ultimately the HR (Wierner et al., 2007). In contrast with the nucleocytoplasmic trafficking that NB-LRR proteins like barley (Hordeum vulgare) MLA10, tobacco N, potato Rx, and Arabidopsis RPS4 and SNC1 undergo (Burch-Smith et al., 2007; Shen et al., 2007; Wirthmueller et al., 2007; Cheng et al., 2009; Slootweg et al., 2010; Tameling et al., 2010), Arabidopsis RPM1 has been shown recently to be constitutively plasma membrane localized, independent of the presence of AvrRpm1 (Gao et al., 2011), and is believed to initiate a cytosolic signaling pathway from this site. More recently, Takemoto et al. (2012) have demonstrated that a number of NB-LRR resistance proteins are constitutively associated with endomembranes, raising the likelihood that there are a number of possible subcellular locations from which the HR can be initiated. In addition, the importance of different subcellular localizations to downstream signaling events has been documented for animal NLR immune receptors, such as NOD2, which is only functional at the plasma membrane when activated (Lécine et al., 2007).

Our studies of potato R3a reveal a novel behavior: relocalization of this resistance protein from the cytoplasm to late endosomes only in the presence of recognized effector forms. The initial studies of this were based on FP–R3a fusion proteins, which are prevented from signaling the HR (Figures 1 to 3). Indeed, R3a–FP and FP–R3a(D501V) fusions were similarly prevented from eliciting HR. However, observations of the behavior of FP–AVR3a with untagged R3a, which leads to an active HR, revealed that the recognized FP–AVR3aK1, but not the unrecognized AVR3aK1, was similarly relocalized to late endosomes (Figures 4 and 5).

Studies of FP–R3a with dexamethasone-inducible AVR3aK1 (Figure 2) and of FP–AVR3aK1 with untagged R3a (Figure 4) both indicated that relocalization occurred prior to the initiation of HR, suggesting that such relocalization is an important step either in effector recognition or in activating R3a for subsequent HR signaling. Indeed, inhibitors of the endocytic cycle, wortmannin and BFA, both attenuated FP–R3a relocalization (Figure 6) and prevented R3a-mediated HR when applied 24 h prior to cell death symptoms (Figure 8), confirming the requirement for association with late endosomes for normal activation of wild-type R3a. Critically, these inhibitors had no effect on HR triggered by coexpression of Rx1 with PVX–CP or Sto1 with its cognate effector IpiO1. While we know little about the subcellular localization of Sto1, Rx1-mediated HR is dependent on nucleocytoplasmic shuttling of this resistance protein (Slootweg et al., 2010; Tameling et al., 2010). It is thus, perhaps, unsurprising that BFA and wortmannin treatments did not attenuate this HR.

The importance of the specific relocalization to late endosomes for the normal immune response of wild-type R3a was further emphasized by the behavior of the R3a(D501V) autoactive mutant form, which gave a delayed cell death response in the absence of effectors and failed to localize to late endosomes (Figure 7). This suggests that downstream signaling to trigger an HR by this form is uncoupled from effector-mediated relocalization. In keeping with this, wortmannin and BFA treatments failed to inhibit this HR (Figure 8).

Both BFA and wortmannin, which are inhibitors of different parts of the endocytic and secretory pathway (Geldner, 2004; Ebine et al., 2011; Ito et al., 2011), attenuate R3a association with late endosomes and have an effect on preexisting late endosomes with which R3a is already associated (Figure 7). Wortmannin causes deformations of late endosomes (Ebine et al., 2011) and homotypic fusions of late endosomes but also fusions between late endosomes and the trans–Golgi network to form small vacuoles (Wang et al., 2009). Treatment with BFA leads to reorganization of the ER–Golgi interaction, resulting in the formation of BFA bodies derived from modified ER cisternae (Orci et al., 1993; Uemura et al., 2004; Jaillais et al., 2008; Ebine et al., 2011). The fluorescing vesicles that we still detected after wortmannin and BFA treatments (Figure 6) may represent these late endosome fusions and BFA bodies, respectively. The assumed failure of further molecules of FP–R3a to relocalize in the presence of these inhibitors, leading to the observed cytoplasmic fluorescence, can be explained by the fact that the formation of late endosomes is inhibited by wortmannin and BFA (Geldner, 2004). Critically, the fact that we perturb normal R3a-mediated cell death with these inhibitors but not that of Rx1 or Sto1 (Figure
In this study, we show relocalization of R3a to Val gave rise to an autoactive cell death phenotype. Nevertheless, the cell death elicited by R3a(D501V) was significantly delayed compared with the effector-mediated cell death observed with wild-type R3a, a phenomenon shown also for an MHD mutant of Rx1 (Raidan and Moffett, 2006).

Recent studies of an MHD-mutated flax NB-ARC-LRR protein, M (Williams et al., 2011), showed that the M(D555V) had significantly more ATP bound than ADP, which supports the molecular switch model, in which the ADP-bound R protein is inactive and an ATP-bound R protein is active (Tameling et al., 2006; Takken et al., 2006). Williams et al. (2011) speculate that mutations within the MHD motif may prevent ATP hydrolysis that, in turn, effectively prevents the R protein entering the inactive state. Accordingly, the mutation in R3a(D501V) should cause an increase binding of ATP, either due to increased ATP binding affinity as a result of a more open NB-ARC conformation or reduced hydrolysis of ATP (Van Oijen et al., 2008), leading to autoactivity.

One interesting observation is that R3a(D501V) is cytoplasmic. Accordingly, wortmannin and BFA treatments had no significant effect on the autoactive HR. One explanation of why the autoactivity is not compromised by BFA and wortmannin could be that the HR triggered by the autoactive R3a(D501V) mutant is mediated by a signaling pathway that is independent of regulatory factors at the late endosome. An alternative explanation, which is not mutually exclusive, is that wild-type R3a is only activated after relocation to late endosomes and requires either direct or indirect interaction with AVR3a, or an AVR3a-target complex, at late endosomes to enter that active state. Following the reconformation of R3a into an active state, it is free to interact with signaling partners to trigger the HR. The site-specific activation prevents R3a from inappropriately becoming active in the absence of the effector and thus is a means to control the context of R3a activity.

**R3a and AVR3aK1 Association at Late Endosomes**

Ever since the discovery of AVR3aK1, Fluorescently tagged AVR3a in the absence of R3a is generally cytoplasmic and reveals no association with vesicles (Bos et al., 2006; Van Oijen et al., 2008; Gao et al., 2011; Williams et al., 2011). As expected, mutation of the Asp in the MHD motif of R3a to Val gave rise to an autoactive cell death phenotype. Nevertheless, the cell death elicited by R3a(D501V) was significantly delayed compared with the effector-mediated cell death observed with wild-type R3a, a phenomenon shown also for an MHD mutant of Rx1 (Raidan and Moffett, 2006).

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Moreover, coimmunoprecipitation experiments also failed to pull down an AVR3aK1-R3a complex (see Supplemental Figure 7 online). Nevertheless, Y2H is prone to false negative results, meaning that true interactions are sometimes not detected (Stellberger et al., 2010). In addition, the reconstitution of YFP during BiFC is a strong, irreversible bond. We cannot rule out a transient, direct interaction between R3a and AVR3aK1 that was undetectable in either Y2H or coimmunoprecipitation experiments but effectively maintained by the BiFC analysis. Similarly, we cannot conclude whether the interaction is mediated by an additional host protein. Indeed, HR triggered by potato R2 and *P. infestans* AVR2 was recently shown to be mediated by the host protein BSL1, indicating that the involvement of additional protein partners in the recognition between resistance proteins and oomycete avirulence proteins can occur (Saunders et al., 2012).

The host ubiquitin E3 ligase, CMPG1, is an important virulence target of AVR3a and yet is not required for R3a HR (Bos et al., 2010; Gilroy et al., 2011a), demonstrating that it is not a guardee that mediates R3a recognition of AVR3a. However, a number of host proteins, as yet unverified in planta, have been shown to interact with AVR3a using Y2H, including two components of the exocyst, Sec3 and Sec5 (Bos et al., 2010). Sec3 and Sec5 interact with each other and are involved in endocytosis and exocytosis (Sommer et al., 2005; Håla et al., 2008; Zhang et al., 2010) and thus may be associated with compartments in the endocytic pathway. Further detailed studies, beyond the scope of this work, are needed to investigate the precise nature of the interaction between R3a and AVR3a, direct or indirect, at late endosomes.

Recently, a three-dimensional structure for AVR3a was elucidated, revealing key residues involved in binding the C-terminal effector domain to phosphoinositides (PIPs) (Yaeno et al., 2011). Mutation of these residues resulted in AVR3a failing to bind to PIPs. Moreover, the mutants failed to suppress INF1-triggered cell death or stabilize CMPG1 (Yaeno et al., 2011). However, they showed that none of the mutations attenuated R3a-mediated HR, showing that PIP binding is not required for AVR3a recognition by R3a. We thus conclude that such PIP binding is also not associated with the observed relocalization to late endosomes revealed in this study.

**The Role Late Endosomes May Play in the R3a Immune Response**

A recent report (Lu et al., 2012) indicated that development of the plant-haustorium interface in a compatible *P. infestans*-host interaction is accompanied by alterations to endosomal trafficking, emphasizing the potential importance of endocytic processes in establishing susceptibility or resistance. Our investigations led us to the conclusion that late endosomes are subcellular sites where R3a triggers a defense response upon AVR3a recognition. Endosomal trafficking pathways are central regulators of plasma membrane protein homeostasis and also control multiple signaling pathways and developmental processes (Reyes et al., 2011). Apart from the literature that disagrees concerning the subcellular localization of the pentameric retromer complex (Reyes et al., 2011), indicating that proteins can be recycled from late endosomes, these compartments are nevertheless part of the
degradative sorting pathway and the question unfolds: Why would R3a relocalize to an apparent dead end of the endosomal trafficking pathway?

Importantly, endocytosis of plant plasma membrane–localized receptors has been shown to be critical for their regulation and for their signaling following perception of associated ligands (Reyes et al., 2011). This has been described for pattern recognition receptors that perceive PAMPs. Upon fig22 stimulation, the Arabidopsis flagellin LRR-receptor-like kinase receptor FLAGELLIN-SENSING2 (FLS2) is ubiquitinated, internalized, and accumulates in late endosomes, prior to its degradation (Chinchilla et al., 2006; Robatzeck et al., 2006; Salomon and Robatzek, 2006; Lu et al., 2011). Critically, endocytosis of FLS2 has been reported to be required for efficient pattern-triggered immunity (Robatzeck et al., 2006), establishing a precedent in plants for signaling from endosomes. In animals, it has been well documented that receptor internalization into late endosomes is required, in a number of cases, for signaling to occur (Andersson, 2012). This establishes late endosomes as reported sites from which signaling can be initiated to activate an immune response.

Concluding Remarks

We provide evidence for novel behavior of an NB-LRR resistance protein: its relocalization, along with the recognized effector form Avr3a, to late endosomes. We show that this event occurs prior to the HR and is a prerequisite for the development of HR cell death. Our data contribute to a more general model of R protein activation, in which HR signaling can be triggered from diverse subcellular compartments and not exclusively within the nucleus. The major challenge will be to understand the chronological order of effector recognition and R3a activation in the context of relocalization and the putative involvement of additional factors required for signaling HR and for regulation of R3a activity.

METHODS

Microbial Strains and Growth Conditions

Agrobacterium tumefaciens strain AGL1 was used in molecular cloning and transient expression experiments and was routinely cultured at 28°C in yeast extract broth medium using appropriate antibiotics (Sambrook and Russell, 2001). DNA transformations of AGL1 were conducted by electroporation (Sambrook and Russell, 2001).

Plasmid Constructs

The constructs pENTR1A-Avr3aEM, pENTR1A-Avr3aΔI, pENTR1A-Avr3aΔIV, pENTR1A-R3a, pGRAB-Avr3aEM, pGRAB-Avr3aΔI, pGRAB-Avr3aΔIV, pGRAB-R3a, pGRAB-Arv2, pCL113-Avr3aEM, pCL113-Avr3aΔI, St01-pK7WG2, and Ip01-pGR106 were described previously (Armstrong et al., 2005; Vleeshouwers et al., 2008; Bos et al., 2010; Gilroy et al., 2011b). Rx (pB1-Rx-HA) and PVX CPs (pBIN61:CP-TK) were described by Gilroy et al. (2011a).

The sequences for all the primers used in this study are shown in Supplemental Table 1 online. For conditional expression, we recombined the entry vector clones of Avr3a with pBAV105 (Vinatzer et al., 2006) to express the effector from the DEX promoter.

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Plant Material, Agroinfiltration, and Cell Death Assays

Nicotiana benthamiana was grown as described previously (Bos et al., 2010). All Agrobacterium cultures were grown overnight and, after pelleting, resuspended in sterile infiltration buffer (10 mM MES, 10 mM MgCl2, and 200 µM acetosyringone) to a final optical density at 600 nm (OD600) of 0.1 for confocal microscopy experiments (or as indicated otherwise) or 0.5 for cell death assays. For coexpression experiments, bacterial strains containing the constructs of interest, expressed from the 35S promoter, were mixed, adjusting each strain concentration to the required final OD600. After an incubation period of 2 to 4 h in darkness at room temperature, bacterial suspensions were infiltrated with a 1–mL blunt-end syringe through the abaxial leaf surface of 4- to 6-week-old plants, which were slightly wounded with a needle. On each plant, two to three leaves were used for each biological replicate; in total, at least five plants were used. FM 4-64 (3.33 µg/mL) and FM 1-43 (100 µg/mL) and Wortmannin (33 µM) (Sigma-Aldrich) were infiltrated 24 to 72 h after agroinfiltration, and bright-field/UV pictures were taken 24, 48, 72, and 96 h after inhibitor infiltration. For conditional expression of Avr3a, dexamethasone (30 µM in a solution of 0.1% Tween 20 in water) was infiltrated into leaves infected with dexamethasone-inducible vectors 4 h after initial Agrobacterium infection. Confocal and cell death imaging was done at the indicated time points after dexamethasone treatment.

Y2H Assays

Both the bait protein-encoding vector pGBK7T containing the R3a clones and the prey-encoding vector pGAD77 encoding the different Avr3a alleles were transformed into the yeast strain PJ69-4A (James et al., 1996) using the protocol described in Greff et al. (2009). Initially, yeast transformants were plated onto complete supplement medium (CSM; Formedium) lacking Leu and Trp (Leu-, Trp-) and incubated at 30°C for 3 to 4 d. Colonies were picked and cultivated overnight in 5 mL of liquid
Coimmunoprecipitation Experiments and Immunoblot Analyses

Protein extraction from yeast cells was performed as described by Kushnirov (2000). Extraction of total protein from plant samples was done by grinding leaf tissue in liquid nitrogen followed by boiling for 5 min in SDS loading buffer supplemented with 1% β-mercaptoethanol. The presence of recombinant R3a, R3a(D501V), and Avr3a fusion proteins was determined by SDS-PAGE and protein gel blotting as described previously (Boš et al., 2010). To detect fusions containing cMyc, we used α-c-Myc-horseradish peroxidase antibody (Invitrogen). For the detection of HA-containing fusions, we used α-HA from rabbit (Sigma-Aldrich). For the detection of GFP/YFP fusions, we used α-GFP from rabbit (Sigma-Aldrich, Santa Cruz Biotechnology); the latter were followed by α-mouse-horseradish peroxidase from goat (Sigma-Aldrich). Protein bands on the immunoblot were detected using ECL substrate (GE Healthcare).

Confocal Imaging

Imaging was conducted on a Leica TCS-SP2 AOBS (Leica Microsystems) using HCX APO L, ×40/0.8, and ×63×0.9 water dipping lenses. Images were at 512 × 512 resolution and taken using line-by-line sequential scanning. The optimal pinhole diameter and the same gain level for the photomultiplier tube was maintained at all times. PhotoshopCS software (Adobe Systems) was used for postacquisition image processing. The excitation wavelength for mRFP was 561 nm, and its emission was collected from 512 to 700 nm. GFP was imaged using 488 nm excitation, and its emission was collected from 500 to 530 nm. YFP was imaged using 514 nm excitation with an emission collected from 520 to 555 nm. CFP was imaged using 405 nm excitation, and its emission was collected between 455 and 490 nm. FM4-64 (Molecular Probes) was excited with the 561-nm line and emissions detected between 630 and 680 nm.

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REFERENCES


Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. FP-R3a Localizes to the Cytoplasm in the Absence of Effectors and Fails to Cause HR with AVR3aΔD or pex147-3.

Supplemental Figure 2. In the Presence of AVR3aΔD, R3a Does Not Relocalize to Chloroplasts, Nuclei, Peroxisomes, or Golgi.

Supplemental Figure 3. Cells Transiently coexpressing the YFP-R3a, CFP-PS1, and a Dexamethasone-Inducible Promoter-Driven Avr3aK1 2 h after Treatment with 30 μM Dexamethasone.

Supplemental Figure 4. YFP C-Terminally Fused to R3a and R3a(D501V) Prevents HR Development.

Supplemental Figure 5. GFP-Avr3aΔD, but Not GFP-Avr3aΔM, Relocalizes to Endosomal Compartments in the Presence of Untagged R3a.

Supplemental Figure 6. R3a and R3a Subfragments Do Not Interact with Avr3a Using Yeast Two-Hybrid Analysis.

Supplemental Figure 7. Immunoblots Showing R3a Fails to Coimmunoprecipitate with AVR3a in planta.

Supplemental Figure 8. R3a(D501V) Causes a Delayed HR Development in Comparison to Wild-Type R3a.

Supplemental Figure 9. YFP N-Terminally Fused to R3a and R3a (D501V) Prevents HR Development.

Supplemental Figure 10. HR Triggered by Sto1 and Rx1 Is Not Affected by BFA or Wortmannin Treatment.

Supplemental Table 1. Primers Used in This Study.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL databases under the following accession numbers: R3a (AY849382.1), Avr3a (PTIG_14371), Avr2 (PTIG_08943), Pex147-2 (PTIG_14368), and Pex147-3 (PTIG_14374).


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Relocalization of Late Blight Resistance Protein R3a to Endosomal Compartments Is Associated with Effector Recognition and Required for the Immune Response
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