Involvement of Adapter Protein Complex 4 in Hypersensitive Cell Death Induced by Avirulent Bacteria[1][OPEN]

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Plant immunity to avirulent bacterial pathogens is associated with subcellular membrane dynamics including fusion between the vacuolar and plasma membranes, resulting in hypersensitive cell death. Here, we report that ADAPTOR PROTEIN COMPLEX-4 (AP-4) subunits are involved in plant immunity associated with hypersensitive cell death. We isolated a mutant with a defect in resistance to an avirulent strain of *Pseudomonas syringae* pv. *tomato* (*Pto*) DC3000 *avrRpm1* from a vacuolar protein sorting mutant library of Arabidopsis (*Arabidopsis thaliana*). The mutant was identical to *gfs4-1*, which has a mutation in the gene encoding the AP-4 subunit AP4B. Thus, we focused on AP4B and another subunit, AP4E. All of the mutants (*ap4b-3*, *ap4b-4*, *ap4e-1*, and *ap4e-2*) were defective in hypersensitive cell death and resistance to *Pto* DC3000 with the type III effector AvrRpm1 or AvrRpt2, both of which are recognized on the plasma membrane, while they showed slightly enhanced susceptibility to the type-III-secretion-deficient *P. syringae* strain *hrcC*. On the other hand, both *ap4b-3* and *ap4b-4* showed no defect in resistance to *Pto* DC3000 with the type III effector AvrRps4, which is recognized in the cytosol and does not induce hypersensitive cell death. Upon infection with *Pto* DC3000 *avrRpt2*, the *ap4b-3* and *ap4b-4* leaf cells did not show fusion between vacuolar and plasma membranes, whereas the wild-type leaf cells did. These results suggest that AP-4 contributes to cell death-associated immunity, possibly via membrane fusion, after type III effector-recognition on the plasma membrane.

Pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) are two modes of plant immunity (for review, see Jones and Dangl, 2006; Tsuda and Katagiri, 2010). PTI is triggered by the recognition of microbe/pathogen-associated molecular patterns by pattern-recognition receptors. PTI acts as a basal defense response that protects plants from invasion by most potentially pathogenic microbes. ETI is induced by the specific recognition of pathogen effectors by disease resistance (R) proteins (Yang et al., 1997; Dangl and Jones, 2001). ETI is often accompanied by the hypersensitive response (HR), which is characterized by rapid and localized programmed cell death (PCD), a process referred to as hypersensitive cell death (Coll et al., 2011; Hara-Nishimura and Hatsugai, 2011).

Vacuoles play important roles in plant immunity (for review, see Iglesias and Meins, 2000; Hatsugai and Hara-Nishimura, 2010). The vacuole, which typically occupies most of the plant cell volume, contains immune-related proteins that are used against invading pathogens, as well as hydrolases and lipases that degrade cellular materials that are no longer required. Plant cells infected with avirulent bacterial pathogens discharge their vacuolar contents into the extracellular space via fusion of the vacuolar membrane to the plasma membrane, leading to the suppression of extracellular bacterial growth and hypersensitive cell death (Hatsugai et al., 2009). On the other hand, vacuolar membrane collapse is observed during infection by avirulent viruses and fungi. This process releases the vacuolar contents into the cytoplasm and leads to

[1] This work was financially supported by Grants-in-Aid for Scientific Research (grant nos. 22000014 and 15H05776 to I.H.-N. and 23570043 to N.H.) from the Japan Society for the Promotion of Science and by a National Science Foundation grant (grant no. IOS-1121425 to F.K.).

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N.H. and I.H.-N. designed the research; N.H., A.N., O.U., and K.O. performed the research; M.K. and M.N. performed ultrastructural analysis; N.H., T.S., F.K., and I.H.-N. wrote the article.

The authors responsible for the distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instruction to Authors (www.plantphysiolol.org) are Ikuko Hara-Nishimura (ihnishi@gr.bot.kyoto-u.ac.jp) and Noriyuki Hatsugai (hatsugai@umn.edu).

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www.plantphysiol.org/cgi/doi/10.1104/pp.17.01610

hypersensitive cell death as well, thereby preventing pathogen growth (Hatsugai et al., 2004, 2015; Kuroyanagi et al., 2005; Higaki et al., 2007).

Soluble vacuolar proteins, such as seed storage proteins and lytic enzymes, are synthesized on the rough endoplasmic reticulum and transported into protein storage vacuoles and lytic vacuoles, respectively, via the endomembrane system. The proteins often contain vacuolar sorting signals that can be recognized by VACUOLAR SORTING RECEPTOR 1 (VSR1) in the trans-Golgi network (TGN; Shimada et al., 2003; Fuji et al., 2007; Zouhar et al., 2010). To sort the receptors in the TGN into vacuoles, the adaptor protein (AP) complex binds to the cytosolic domains of the receptors (Robinson, 2004). We recently reported that the AP-4 complex, which is localized to the TGN subdomain, functions in VSR1-mediated vacuolar protein sorting in Arabidopsis (*Arabidopsis thaliana*; Fuji et al., 2016). AP-4 is a heterotetrameric complex consisting of two large subunits (β4 and ε), one medium subunit (μ4), and one small subunit (σ4) (Robinson, 2004).

We previously reported that a Beige and Chediak-Higashi domain protein is involved in vacuolar protein transport and is required for full-strength ETI in Arabidopsis (Teh et al., 2015). Arabidopsis *lazarus 4* (*laz4*), a suppressor mutant of *accelerated cell death11* (*acd11*), partly suppresses immunity- and PCD-related phenotypes in *acd11* (Munch et al., 2015). *LAZ4* encodes VACUOLAR PROTEIN SORTING 35B, a component of the multisubunit retromer complex required for vacuolar transport of storage proteins to protein storage vacuoles in seeds (Yamazaki et al., 2008; Munch et al., 2015). The presence of *laz4* suppresses *acd11* phenotypes, indicating that vacuolar protein transport is involved in plant immunity. However, how vacuolar protein transport contributes to plant immunity is not yet fully understood.

We previously developed a high-throughput screening system to isolate vacuolar sorting-deficient mutants in Arabidopsis, designated *green fluorescent seed* (*gfs*) mutants, using GFP as a reporter (Fuji et al., 2007). Arabidopsis seeds harboring *GFP-CT24*, encoding a signal peptide and GFP followed by the 24 C-terminal amino acids of β-conglycinin (CT24) under the control of a seed-specific promoter, accumulate this GFP fusion protein in their protein storage vacuoles (Nishizawa et al., 2003). By contrast, *gfs* mutant seeds mis-sort GFP-CT24 by secreting it from the cell, resulting in seeds exhibiting strong green fluorescence (Fuji et al., 2007). Plants likely use common systems for vacuolar protein transport in seeds and vegetative organs, because Arabidopsis mutants with a defect in vacuolar protein transport in seeds exhibit a defect in vacuolar protein transport in vegetative organs (Shimada et al., 2006; Yamazaki et al., 2008). Thus, the *gfs* mutants likely have vacuolar protein transport defects in vegetative organs.

In this study, we show that AP-4 complex is involved in plant immunity associated with fusion between the vacuolar membrane and the plasma membrane. The deficiency in AP-4 components abolished membrane fusion and hypersensitive cell death upon infection with an avirulent bacterial strain and reduced resistance to this strain. Our results provide valuable insights into the role of AP-4-dependent vacuolar protein sorting in ETI.

RESULTS

Isolation of a Vacuolar Protein Sorting Mutant That Also Has a Defect in Resistance to Avirulent Bacteria

Sixty-four M2 lines of the Arabidopsis *gfs* mutant library (Col-0 background), previously identified as mutants that mis-sort the vacuolar protein GFP-CT24 out of cells (Fuji et al., 2007), were analyzed for their susceptibility to the avirulent bacterial strain, *Pto* DC3000, expressing AvrRpm1 effector protein (*Pto* DC3000 *avrRpm1*), which is recognized by the R protein RPM1 in Col-0 (Grant et al., 1995). We identified among *gfs* mutants a mutant that exhibited chlorotic symptoms within 5 d of bacterial inoculation (*gfs4-1*; Fig. 1A).

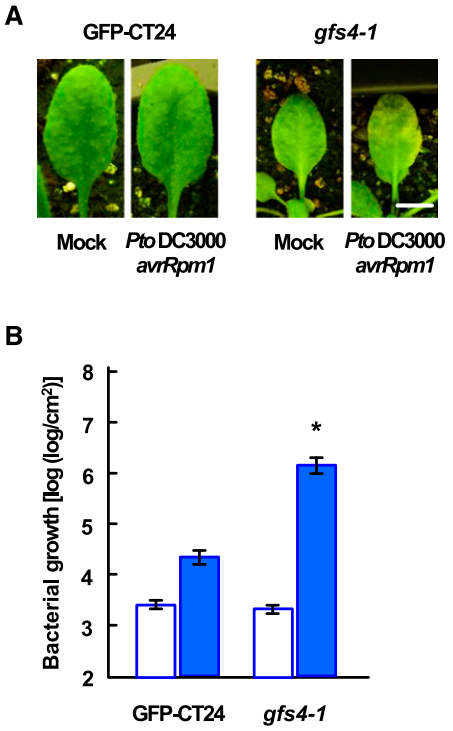


Figure 1. *gfs4-1* exhibits reduced resistance to *Pto* DC3000 *avrRpm1*. A, Leaves of GFP-CT24 and *gfs4-1* plants 5 d after mock inoculation and inoculation with *Pto* DC3000 *avrRpm1* (OD_{600} = 0.001). *gfs4-1* leaves inoculated with *Pto* DC3000 *avrRpm1* exhibit chlorotic symptoms. Bar = 0.5 cm. B, Bacterial growth immediately (white bars) and 3 d after (blue bars) inoculation with *Pto* DC3000 *avrRpm1* (OD_{600} = 0.001) in the leaves of GFP-CT24 and *gfs4-1*. Each bar represents the mean and SE of three independent experiments, each with six biological replicates. Asterisks indicate significant differences compared with GFP-CT24 plants (*$P < 0.05$, two-tailed *t* tests).

To determine whether the susceptible phenotype of the mutant is associated with increased bacterial growth in the plant, we quantified the bacterial number in leaves 3 d after inoculation. The bacterial number in mutant leaves was approximately 100-fold higher than that in GFP-CT24 control leaves (Fig. 1B). These results suggest that the vacuolar protein sorting mutant has defects in ETI induced by infection with *Pto* DC3000 *avrRpm1*.

The AP-4 Subunit AP4B Is Important for ETI Mediated by RPM1 and RPS2 but Not by RPS4

DNA sequencing analysis previously identified the vacuolar sorting mutation in gfs4-1 (Supplemental Fig. S1) to be a single nucleotide substitution resulting in a premature stop codon in the At5g11490 gene, encoding the AP-4 subunit AP4B (Fuji et al., 2016). The AP-4 complex functions in receptor-mediated vacuolar protein sorting by recognizing VSR1, a Sorting receptor used in the targeting of seed storage proteins to protein storage vacuoles in Arabidopsis (Shimada et al., 2003; Robinson, 2004; Fuji et al., 2016).

To investigate whether the defect in the *AP4B* gene is causal to the reduced ETI phenotype in *gfs4-1*, we examined whether resistance to *Pto* DC3000 *avrRpm1* was reduced in two additional mutant alleles, *ap4b-3/gfs4-3* and *ap4b-4/gfs4-4*, both of which are T-DNA insertion mutants (Supplemental Fig. S1; Fuji et al., 2016). We inoculated wild-type Col-0 and *ap4b* mutant plants with *Pto* DC3000 *avrRpm1* and counted viable bacteria immediately and at 3 d after inoculation. The number of bacteria in *ap4b-3* and *ap4b-4* plants, like that in *gfs4-1*, was appromimately100-fold higher than that in the wild-type Col-0 plants at 3 d after inoculation (Fig. 2B). The growth of the bacteria in *rpm1* plants was strongly enhanced, as expected for plants lacking the R protein (Fig. 2B). Thus, we conclude that the reduced ETI phenotype in *gfs4-1* is caused by *ap4b* mutations. Bacterial growth of virulent strain *Pto* DC3000 carrying an empty vector (*Pto* DC3000 EV) after 3 d of inoculation was slightly higher in *ap4b-3* and *ap4b-4* plants than in wild-type plants (Fig. 2A). Since the effects of *AP4B* deficiency on the bacterial growth of *Pto* DC3000 EV were much smaller than that of *Pto* DC3000 *avrRpm1*, AP4B is important for RPM1-mediated ETI.

To determine whether the requirement of AP4B for ETI is specific to *Pto* DC3000 *avrRpm1*, we examined the bacterial growth of two additional bacterial strains, *Pto* DC3000 *avrRpt2* expressing the AvrRpt2 effector protein and *Pto* DC3000 *avrRps4* expressing the AvrRps4 effector protein. *Pto* DC3000 *avrRpt2* and *Pto* DC3000 *avrRps4* are recognized by the R proteins, RPS2 and RPS4, respectively, in Col-0 plants (Bent et al., 1994; Mindrinos et al., 1994). The number of *Pto* DC3000 *avrRpt2* in *ap4b-3* and *ap4b-4* plants was approximately 100-fold higher than that in the wild-type Col-0 plants at 3 d after inoculation, but lower than the bacterial number in *rps2* control plants (Fig. 2C). By contrast, no significant difference in the growth rates of *Pto* DC3000 *avrRps4* was observed among *ap4b-3*, *ap4b-4*, and wild-type plants (Fig. 2E). The effects of *AP4B* deficiency on the bacterial growth of *Pto* DC3000 *avrRps4* were similar to those of *Pto* DC3000 EV (Fig. 2, D and E). Taken together, these results indicate that AP4B is important for RPS2-mediated ETI, as well as RPM1-mediated ETI, but not for RPS4-mediated ETI.

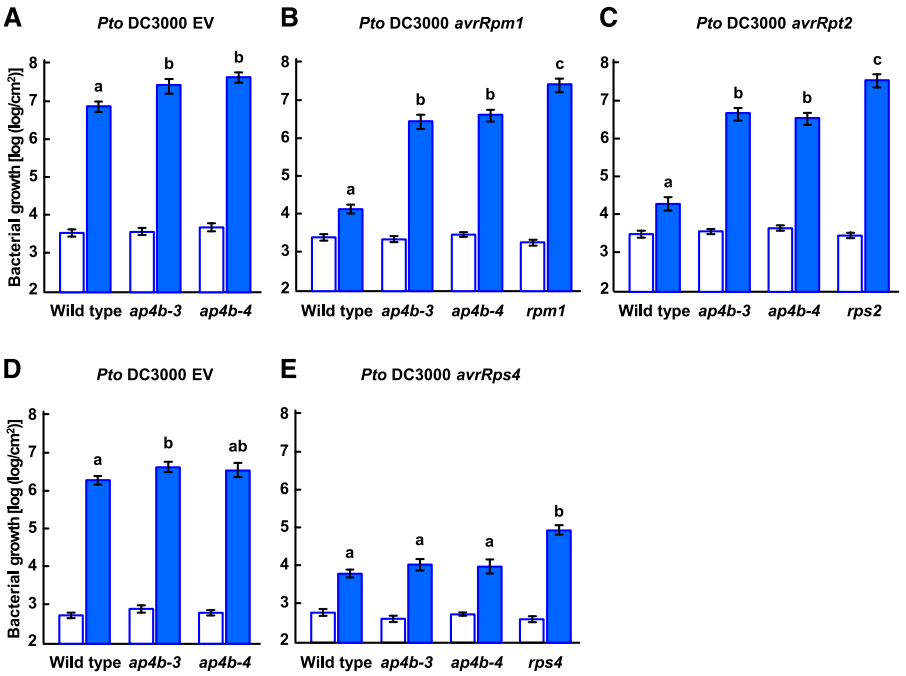


Figure 2. Deficiency of *AP4B* compromised resistance to *Pto* DC3000 *avrRpm1* and *Pto* DC3000 *avrRpt2* but not to *Pto* DC3000 *avrRps4*. A to E, Bacterial growth immediately (white bars) and 3 d after (blue bars) inoculation with *Pto* DC3000 *EV* (OD_{600} = 0.001) (A), *Pto* DC3000 *avrRpm1* (OD_{600} = 0.001) (B), *Pto* DC3000 *avrRpt2* (OD_{600} = 0.001) (C), *Pto* DC3000 *EV* (OD_{600} = 0.0001) (D), and *Pto* DC3000 *avrRps4* (OD_{600} = 0.0001) (E) in leaves of the indicated plant lines. A, B, and C were done at the same time, while D and E were done together at another time. Each bar represents the mean and SE of three independent experiments, each with six biological replicates. Different letters indicate significant differences ($P < 0.05$, two-tailed *t* tests).

AP4B Contributes to Hypersensitive Cell Death Induced by *Pto* DC3000 *avrRpm1* and *Pto* DC3000 *avrRpt2*

ETI is often associated with hypersensitive cell death (Dangl and Jones, 2001; Jones and Dangl, 2006). To determine whether *AP4B* deficiency affects hypersensitive cell death, we monitored cell death in wild-type Col-0 and *ap4b* plants using trypan blue staining at 12 h after inoculation with *Pto* DC3000 *avrRpm1*. The dead cells in wild-type plants exhibited a distinctive blue color under a light microscope (Fig. 3A, *Pto* DC3000 *avrRpm1*). The *ap4b-3* and *ap4b-4* mutant plants contained similar numbers of cells to wild-type plants in the field, but the blue color in the mutants was fainter, and the stained cells appeared to maintain their shape, in contrast to the stained cells in wild-type plants, which appeared to be shrunken (Fig. 3A, *Pto* DC3000 *avrRpm1*). These observations suggest that *AP4B* deficiency affects some aspects of the *Pto* DC3000 *avrRpm1*-induced hypersensitive cell death response. We also quantitatively monitored hypersensitive cell death based on the release of electrolytes from dead cells. Electrolyte leakage dramatically increased in wild-type plants after inoculation with *Pto* DC3000 *avrRpm1*, whereas the increase in electrolyte leakage was significantly lower in *ap4b-3* and *ap4b-4* plants than in the wild type but higher than in *rpm1* plants (Fig. 3B, *Pto* DC3000 *avrRpm1*). These results indicate that *AP4B* deficiency leads to attenuated hypersensitive cell death associated with RPM1-mediated ETI.

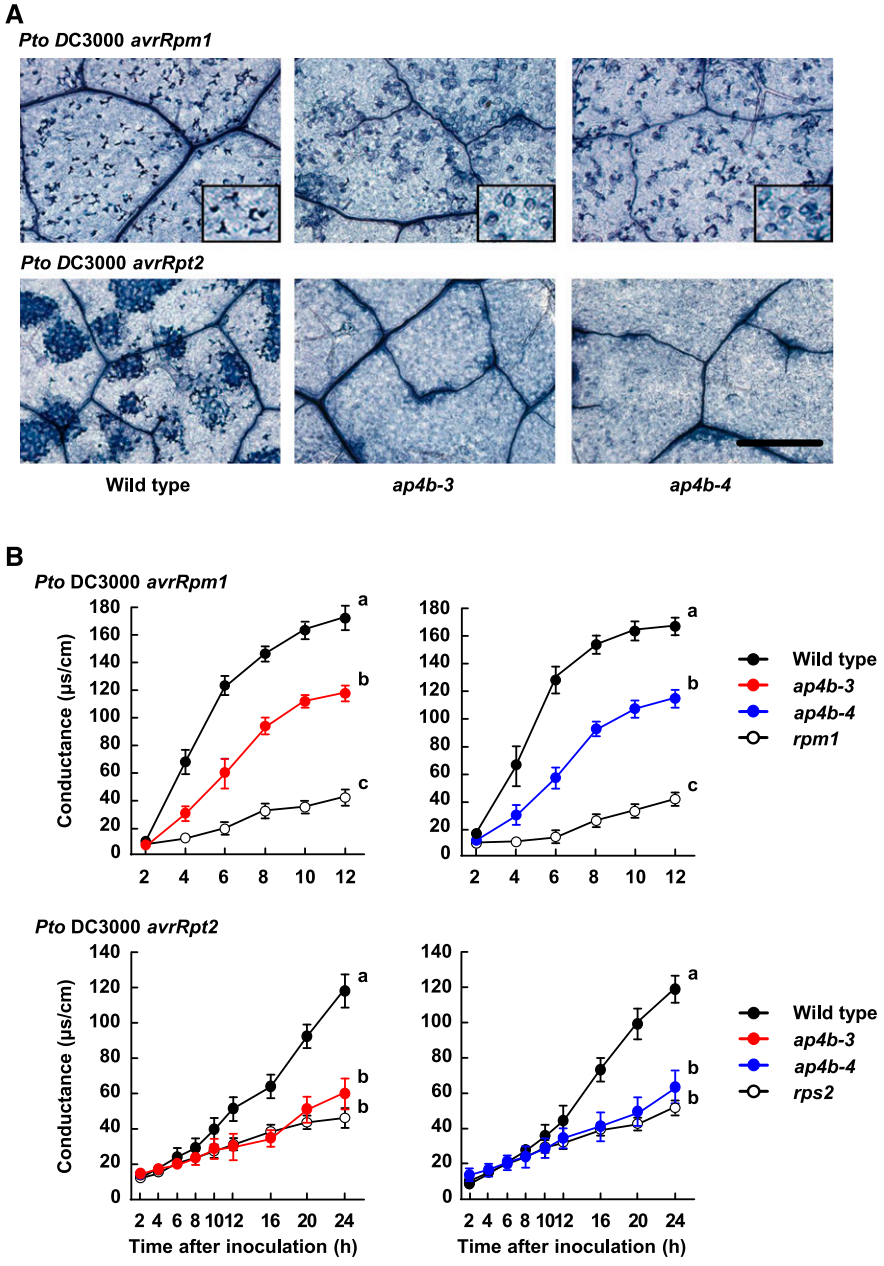


Figure 3. Hypersensitive cell death induced by *Pto* DC3000 *avrRpm1* and *Pto* DC3000 *avrRpt2* is reduced in *ap4b-3* and *ap4b-4* plants. A, Trypan blue staining of dead cells in the leaves of wild-type, *ap4b-3*, and *ap4b-4* plants at 12 h after inoculation with *Pto* DC3000 *avrRpm1* (OD_{600} = 0.001) and at 24 h after inoculation with *Pto* DC3000 *avrRpt2* (OD_{600} = 0.002). Bar = 500 μm. B, Electrolyte leakage from dying and dead cells in the leaves of wild-type, *ap4b-3*, *ap4b-4*, *rpm1*, and *rps2* plants inoculated with *Pto* DC3000 *avrRpm1* (OD_{600} = 0.1) and *Pto* DC3000 *avrRpt2* (OD_{600} = 0.1). Error bars indicate SES of three independent experiments, each with four biological replicates. Different letters indicate significant differences at 12 h (*Pto* DC3000 *avrRpm1*) and 24 h (*Pto* DC3000 *avrRpt2*) ($P < 0.05$, two-tailed *t* tests).

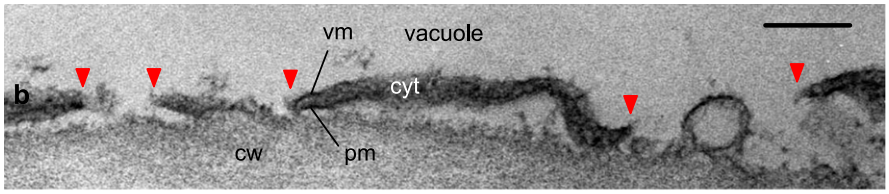
We further assessed hypersensitive cell death induced by infection with *Pto* DC3000 *avrRpt2*. Trypan blue staining revealed that *Pto* DC3000 *avrRpt2*-induced hypersensitive cell death was abolished in *ap4b-3* and *ap4b-4* mutant plants (Fig. 3A, *Pto* DC3000 *avrRpt2*). Similar results were obtained by quantitatively monitoring hypersensitive cell death based on electrolyte leakage. The rate of electrolyte leakage was much lower in *Pto* DC3000 *avrRpt2*-inoculated *ap4b-3* and *ap4b-4* plants than in wild-type plants but was the same as that in *rps2* plants (Fig. 3B, *Pto* DC3000 *avrRpt2*). These results indicate that AP4B is required for hypersensitive cell death associated with RPS2-mediated ETI.

Pto DC3000 *avrRps4*-induced hypersensitive cell death is extremely weak in wild-type Col-0 plants (Gassmann et al., 1999; Tornero et al., 2002), which made it impossible to assess the effect of *AP4B* deficiency on hypersensitive cell death during RPS4-mediated ETI.

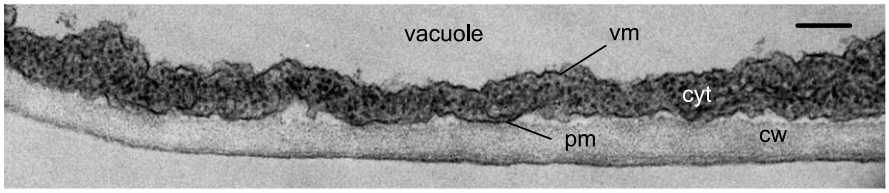
AP4B Is Required for Vacuolar Membrane Fusion to the Plasma Membrane

We previously showed that the large central vacuole fuses with the plasma membrane in response to avirulent bacterial infection, which is associated with hypersensitive cell death (Hatsugai et al., 2009). The strong inhibition of cell death in response to *Pto* DC3000 *avrRpt2* infection in the *ap4b* mutants is reminiscent of the phenotype of RNAi *ipba1* Arabidopsis lines in which the proteasome subunit gene *PBA1* is silenced. The *ipba1* lines fail to perform the membrane fusion in *Pto* DC3000 *avrRpt2*-inoculated leaves (Hatsugai et al., 2009). We investigated membrane fusion in the leaves of wild-type Col-0 and *ap4b* plants after inoculating them with *Pto* DC3000 *avrRpt2*. Vacuolar and plasma membrane fusion occurred in the leaves of wild-type Col-0 plants at 8 h after inoculation (Fig. 4A) and was detected in 81% of the examined cells (Fig. 4D), confirming a previous observation (Hatsugai et al., 2009). However, membrane fusion was not detected in the leaves of *ap4b-3* and *ap4b-4* plants (Fig. 4, B–D). These

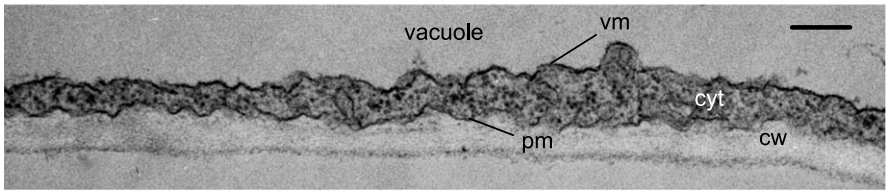
A Wild type



B *ap4b-3*



C *ap4b-4*



D

Genotype	Cells examined	Cells with membrane fusion	Frequency rate (%)
Wild type	11	9	81
ap4b-3	8	0	0
ap4b-4	8	0	0

Figure 4. Deficiency of *AP4B* suppresses fusion between the vacuolar membrane and plasma membrane in association with bacterial infection. A to C, Electron micrographs of the leaves of wild-type, *ap4b-3*, and *ap4b-4* Arabidopsis plants at 8 h after inoculation with *Pto* DC3000 *avrRpt2* (OD_{600} = 0.1). Membrane fusion between the plasma membrane and vacuolar membrane is indicated by red triangles. Bars = 200 nm. cw, cell wall; pm, plasma membrane; vm, vacuolar membrane; cyt, cytosol. D, Frequency rates of cells with fused membranes at 8 h after bacterial inoculation.

results indicate that AP4B is required for membrane fusion between the vacuolar membrane and the plasma membrane in response to *Pto* DC3000 *avrRpt2* infection.

Another AP-4 Subunit AP4E Is Also Important for ETI

AP-4 is a heterotetrameric complex: its two large subunits are AP4B and AP4E. We therefore examined whether disrupting AP4E would lead to a compromised immune response, as was observed in the *ap4b* mutant. Arabidopsis T-DNA-insertion lines *ap4e-1* and *ap4e-2*, with insertions in *AP4E*, also display abnormal vacuolar protein transport (Fuji et al., 2016). Electrolyte leakage assays following inoculation with *Pto* DC3000 *avrRpm1* and *Pto* DC3000 *avrRpt2* indicated that hypersensitive cell death was compromised in *ap4e-1* and *ap4e-2* (Fig. 5A). Consistent with this observation, the *ap4e-1* and *ap4e-2* plants exhibited substantially increased bacterial growth of *Pto* DC3000 *avrRpm1* and *Pto* DC3000 *avrRpt2* compared with wild-type plants (Fig. 5B, *Pto* DC3000 *avrRpm1* and *Pto* DC3000 *avrRpt2*). The growth rate of *Pto* DC3000 EV was slightly higher in *ap4e-1* and *ap4e-2* plants than in wild-type plants (Fig. 5B, *Pto* DC3000 EV). Since the effects of *AP4E* deficiency on the bacterial growth of *Pto* DC3000 EV were much smaller than that of *Pto* DC3000 *avrRpm1* and *Pto* DC3000 *avrRpt2*, AP4E is important for resistance to *Pto* DC3000 *avrRpm1* and *Pto* DC3000 *avrRpt2* infections. By contrast, 3 d after inoculation, the growth rates of *Pto* DC3000 *avrRps4* in *ap4e-1* and *ap4e-2* plants were not different from that in the wild type plants (Fig. 5C, *Pto* DC3000 *avrRps4*). *AP4E* deficiency on the bacterial growth of *Pto* DC3000 *avrRps4* were comparable to that

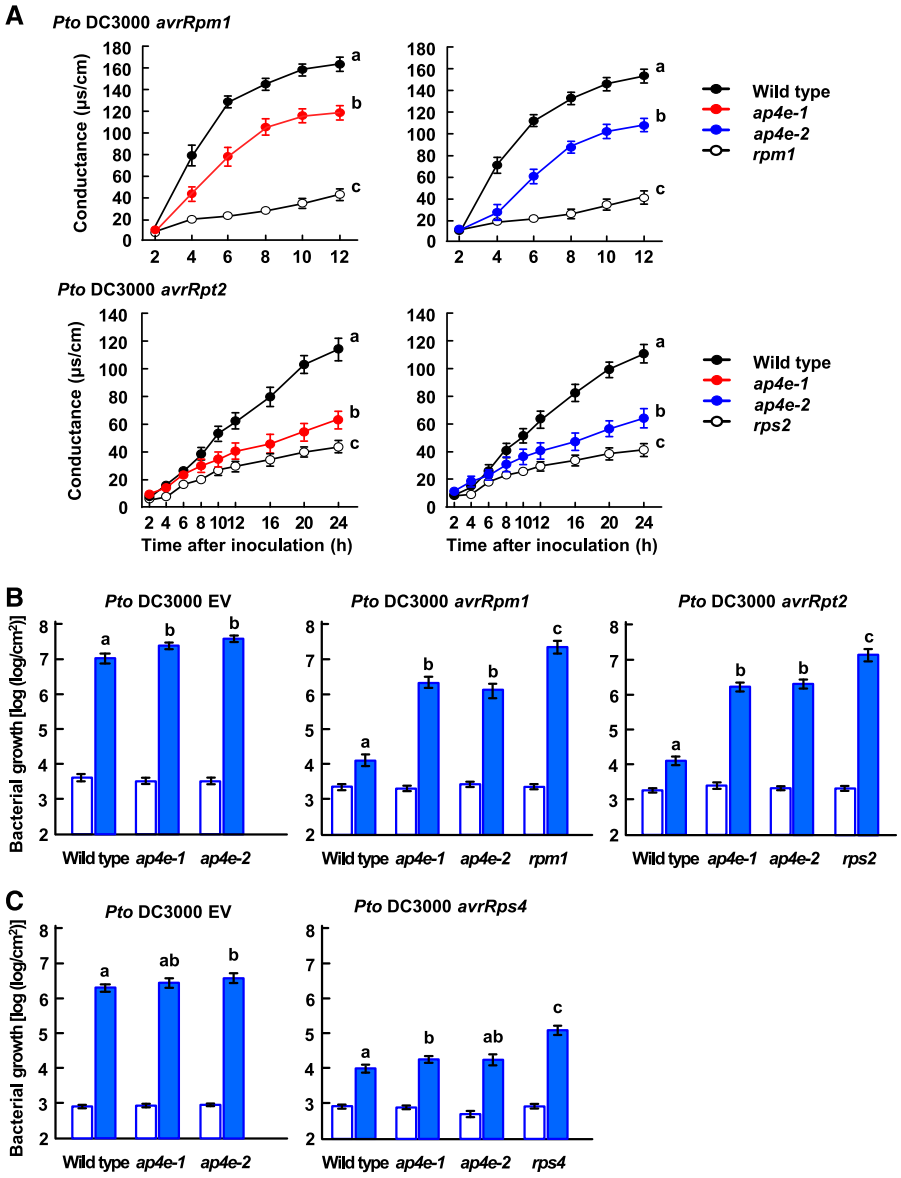


Figure 5. Both resistance and hypersensitive cell death in response to *Pto* DC3000 *avrRpm1* and *Pto* DC3000 *avrRpt2* are compromised in *ap4e-1* and *ap4e-2* plants. A, Electrolyte leakage from dying and dead cells in the leaves of wild-type, *ap4e-1*, and *ap4e-2* plants inoculated with *Pto* DC3000 *avrRpm1* (OD_{600} = 0.1) and *Pto* DC3000 *avrRpt2* (OD_{600} = 0.1). Error bars indicate SEs of three independent experiments, each with four biological replicates. Different letters indicate significant differences at 12 h (*Pto* DC3000 *avrRpm1*) and 24 h (*Pto* DC3000 *avrRpt2*) ($P < 0.05$, two-tailed *t* tests). B, Bacterial growth immediately (white bars) and 3 d after (blue bars) inoculation with *Pto* DC3000 *avrRpm1* (OD_{600} = 0.001), *Pto* DC3000 *avrRpt2* (OD_{600} = 0.001), and *Pto* DC3000 EV (OD_{600} = 0.001) in leaves of wild type, *ap4e-1*, and *ap4e-2*. C, Bacterial growth immediately (white bars) and 3 days after (blue bars) inoculation with *Pto* DC3000 EV (OD_{600} = 0.0001) and *Pto* DC3000 *avrRps4* (OD_{600} = 0.0001) in leaves of the indicated plant lines. Each bar represents the mean and SE of three independent experiments, each with six biological replicates. Different letters indicate significant differences ($P < 0.05$, two-tailed *t* tests).

of *Pto* DC3000 EV (Fig. 5C). Taken together, these results indicate that AP4E is involved in both RPM1-mediated and RPS2-mediated ETI but not involved in RPS4-mediated ETI.

AP4B and AP4E Are Weakly Involved in PTI

The enhanced bacterial growth of *Pto* DC3000 EV in the *ap4b* and *ap4e* mutants suggested that AP4B and AP4E are involved in PTI as well as ETI. To test this hypothesis, we inoculated wild-type Col-0, *ap4b*, and *ap4e* plants with *Pto* DC3000 *hrcC* and counted viable bacteria immediately and 3 d after inoculation. The *hrcC* bacterial strain cannot secrete type III effectors due to a lack of a type III secretion system component, resulting in the activation of PTI in Arabidopsis (Hauck et al., 2003). The *ap4b* and *ap4e* plants exhibited slightly but significantly higher growth of *Pto* DC3000 *hrcC* compared to wild-type plants (Fig. 6, A and B). These results indicate that AP4B and AP4E are weakly involved in PTI.

Pto DC3000 *hrcC*-activated callose deposition is a response associated with PTI. To examine the involvement of AP4B and AP4E in callose deposition, we inoculated wild-type Col-0, *ap4b*, and *ap4e* plants with *Pto* DC3000 *hrcC* and stained them for callose. *Pto* DC3000 *hrcC* inoculation induced a large number of highly localized callose deposits in the leaves of *ap4b* and *ap4e* plants as well as wild-type plants (Fig. 6, C and D). No callose deposition was observed in mock-treated plants (Fig. 6C). These results indicate that the beta and ε subunits of the AP-4 complex are not required for the callose deposition response.

DISCUSSION

AP-4 Complex Is Required for Vacuolar Membrane Fusion to the Plasma Membrane during ETI

The *ap4b* and *ap4e* mutations substantially compromised ETI mediated by RPM1 and RPS2, which is involved HR cell death. By contrast, RPS4-mediated ETI, which is independent of HR cell death, was not affected

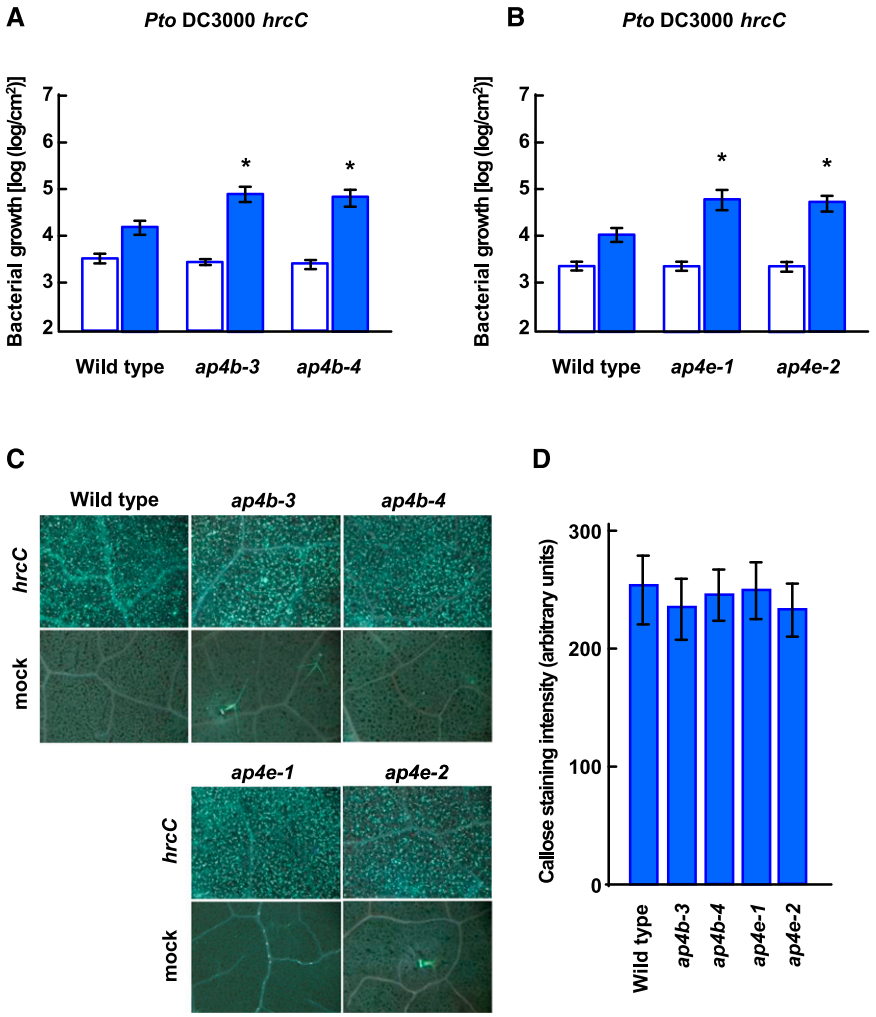


Figure 6. Deficiency of *AP4B* or *AP4E* increases bacterial growth of *Pto* DC3000 *hrcC*, whereas it does not affect callose deposition. A and B, Bacterial growth immediately (white bars) and 3 d after (blue bars) inoculation with *Pto* DC3000 *hrcC* (OD_{600} = 0.001) in leaves of the indicated plant lines. Each bar represents the mean and SE of three independent experiments, each with six biological replicates. Asterisks indicate significant differences compared with wild-type plants (*$P < 0.05$, two-tailed *t* tests). C, Callose deposition in leaves of the indicated plant lines inoculated with *Pto* DC3000 *hrcC* (OD_{600} = 0.1) (*hrcC*) and water (mock) detected by aniline blue staining at 12 h after inoculation. Bar = 500 μm. D, Quantification of callose staining intensity. Each bar represents the mean and SE from five stained regions in three different leaves.

by *ap4b* and *ap4e* mutations. These results suggest that the AP-4 complex is involved in cell death-associated immunity. The cell death-associated immune response is in some cases accompanied by vacuolar and plasma membrane fusion. These observations are consistent with the notion that AP-4 is required for membrane fusion. However, the molecular role of the AP-4 complex in membrane fusion remains unclear. The AP-4 complex may be involved in vacuolar protein sorting of immune-associated cargo proteins responsible for membrane fusion. The cargo proteins are likely vacuolar membrane proteins and soluble proteins recognized by VSR homologs, because we previously found that the AP-4 complex functions in the transport of VSR1 to the target membrane and is required for VSR1-mediated vacuolar protein transport in seed cells (Fuji et al., 2016).

Fusion of the cellular membrane with the vacuolar membrane often requires soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins (Zhang et al., 2014; Uemura and Ueda, 2014). In the animal immune response, fusion between the lysosome and plasma membrane occurs in activated cytotoxic T lymphocyte and natural killer cells to induce apoptosis/PCD (Blott and Griffiths, 2002; Luzio et al., 2007). This membrane fusion is mediated by a lysosomal SNARE protein complex containing vesicle-associated membrane protein 7 (VAMP7), a member of the R-SNARE family (Blott and Griffiths, 2002; Luzio et al., 2007; Marcet-Palacios et al., 2008; Chaineau et al., 2009). Given the evidence suggesting that SNARE proteins are targeted to the lysosome membrane through interactions with APs, including AP-4 (Martinez-Arca et al., 2003; Bennett et al., 2008; Kent et al., 2012), the AP-4 complex in plants may guide SNARE proteins to the vacuole, where they probably participate in the fusion of vacuolar and plasma membranes in response to pathogen infection. Interestingly, OsVAMP7, a rice (*Oryza sativa*) SNARE localized to the vacuolar membrane, is suggested to play a role in resistance to blast disease (Sugano et al., 2016). In addition, SNARE proteins involved in plant immune responses have also been reported, although they are mostly associated with the plasma membrane (Collins et al., 2003; Kalde et al., 2007; Uemura et al., 2012). Future identification of the cargo proteins of the AP-4 complex may provide clues about the molecular mechanism underlying membrane fusion and its regulation of HR cell death during ETI.

AP-4 Complex-Dependent Transport of Vacuolar Proteins in Plant Immunity

Plant vacuoles contain immune-related proteins as well as hydrolases and lipases (De, 2000; Carter et al., 2004). Some of these proteins are transported into vacuoles as proprotein precursors, where they are proteolytically processed to produce the respective mature/active forms (Hara-Nishimura et al., 1991; Kunze et al., 1998; Rojo et al., 2003; Shimada et al., 2003; Yamada et al., 2005). Vacuolar and plasma membrane fusion causes the discharge of accumulated mature/active vacuolar proteins into the extracellular space, leading to the suppression of extracellular bacterial growth and hypersensitive cell death (Hatsugai et al., 2009). In *ap4* mutants, vacuolar proteins may not accumulate sufficiently in the vacuole, because their proprotein precursors are not efficiently transported into vacuoles (Fuji et al., 2016). The resulting low amounts of such vacuolar proteins could result in loss of cell death and immunity to pathogen infection.

AP-4 complex-regulated vacuolar protein sorting is initiated after recognition of the VSR1 sorting receptor (Fuji et al., 2016). A homolog of VSR1 is transcriptionally induced during plant immune responses in Arabidopsis (Wang et al., 2005), although its functional relevance to plant immunity is unknown. VSR homolog(s), which could be recognized by AP-4, might be necessary to transport immune-related proteins to vacuoles, where they undergo maturation or activation.

A Possible Function of AP-4 Complex in ETI Signaling

RPM1 and RPS2 are localized to plasma membrane, while RPS4 is localized to the cytoplasm and nucleus (Axtell and Staskawicz, 2003; Mackey et al., 2003; Wirthmueller et al., 2007). This study shows that AP-4 complex influences the ETI mediated by plasma membrane-localized *R* proteins. This is consistent with our previous report that the AP-2 subunit AP2M is required for both RPM1-mediated ETI and RPS2-mediated ETI, but not for RPS4-mediated ETI (Hatsugai et al., 2016). AP2M plays a central role in clathrin-mediated endocytotic pathway from the plasma membrane to the vacuole (Di Rubbo et al., 2013; Fan et al., 2013; Kim et al., 2013; Yamaoka et al., 2013). These results suggest that the ETI-related immune-signaling components undergo endocytic internalization. Thus, AP-4 complex might be involved in vacuolar trafficking of immune-signaling components through the TGN/early endosome for degradation or turnover, resulting in efficient ETI signaling.

AP-4 Complex Is Likely a Minor Component of PTI

Fusion between the vacuolar membrane and the plasma membrane is not observed during the basal defense response (Hatsugai et al., 2009), which is consistent with the finding that PTI is generally not associated with cell death. The *ap4b* and *ap4e* mutants exhibited enhanced bacterial growth of *Pto* DC3000 *hrcC*. By contrast, callose deposition induced by this strain was intact in *ap4b* and *ap4e* mutant plants. These results indicate that although AP-4 is involved in PTI, the extent of its involvement is limited. The transport of callose to the extracellular space is mediated by the

ADP ribosylation factor guanine nucleotide exchange factor (Nomura et al., 2006; Nielsen et al., 2012). A major aspect of PTI, including callose deposition, is mediated by extracellular transport, which is not affected by deficiencies in AP-4 (Hückelhoven, 2007; Malinovsky et al., 2014; Fuji et al., 2016). These findings are consistent with the observation that *AP-4* deficient mutants did not exhibit a defect in extracellular transport (Fuji et al., 2016).

Taken together, our results provide substantive evidence to show that the AP-4 complex is required for ETI-associated HR cell death but is likely a minor component of PTI.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Col-0 was the background line for all plants used in this study. Arabidopsis *gfs* lines (Fuji et al., 2007) *ap4b-3/gfs4-3* (SAIL_796A10; Fuji et al., 2016), *ap4b-4/gfs4-4* (SAIL_781H01; Fuji et al., 2016), *ap4e-1* (SAIL_866C01; Fuji et al., 2016), *ap4e-2* (SAIL_60E03; Fuji et al., 2016), GFP-CT24 (Nishizawa et al., 2003), *rpm1-3* (Grant et al., 1995), *rps2-101C* (Mindrinos et al., 1994), and *rps4* (SALK_057697; Wirthmueller et al., 2007) were described previously. The Arabidopsis plants were grown in a controlled environment at 22°C with a 12-h photoperiod.

DNA Sequencing

DNA was isolated from F2 progeny. The nucleotide sequences of both strands were determined using an ABI Prism Big Dye Terminator Cycle Sequence Reaction Kit (Applied Biosystems) and a DNA sequencer (model 3100-Avant Genetic Analyzer; Applied Biosystems).

Bacterial Strains and Pathology Tests

Pseudomonas syringae pv. *tomato* virulent strain DC3000 carrying the empty vector pVSP61 (*Pto* DC3000 EV), congenic avirulent strains (*Pto* DC3000 expressing *avrRpm1*, *avrRpt2* or *avrRps4*), and *Pto* DC3000 *hrcC* were used in this study. Bacteria were cultured in King's B medium, washed twice in 10 mM $MgCl_2$, and resuspended at OD_{600} = 0.001 or 0.0001 for plant inoculation for the growth assays and trypan blue staining and at OD_{600} = 0.1 for the electrolyte leakage assay and electron microscopic analysis. Bacterial suspensions were inoculated into the abaxial sides of 4- to 5-week-old Arabidopsis leaves using needleless syringes. Bacterial growth was monitored as described previously (Hatsugai et al., 2009).

Trypan Blue Staining

Inoculated leaves were boiled for approximately 1 min in staining solution (1 mL lactic acid, 1 mL glycerol, 1 mL water-saturated phenol, 1 mL water, and 1 mg trypan blue) and incubated overnight at room temperature. The samples were then decolorized in 2.5 g/mL chloral hydrate for at least 1 d.

Electrolyte Leakage Assay

Electrolyte leakage from dying and dead cells was measured essentially as described (Hatsugai et al., 2009). Four disks with a 7.5-mm diameter were dissected from leaves immediately after bacterial inoculation, floated in 2 mL distilled water for 30 min, transferred to 2 mL fresh distilled water, and incubated at 22°C. Water conductance was measured with an electrical conductivity meter (B-173, Horiba).

Callose Deposition

Callose staining was performed essentially as described (Adam and Somerville, 1996). Leaves were vacuum-infiltrated with alcoholic lactophenol (one volume of phenol/glycerol/lactic acid/water [1:1:1:1] and two volumes of ethanol) and incubated at 65°C for 30 to 60 min. The leaves were transferred to fresh alcoholic lactophenol and incubated for an additional 24 h until they were completely cleared of chlorophyll. The leaf samples were rinsed in 50% ethanol, 20% ethanol, 10% ethanol, and water, stained for 30 min in 150 mM K_2HPO_4 (pH 9.5) containing 0.01% aniline blue, and observed using ultraviolet illumination at 340- to 360-nm excitation and 450- to 470-nm emission wavelengths under an Eclipse E600 microscope (Nikon). Grayscale images of callose-stained leaves were used to quantify the signal intensity using Fiji/ImageJ software ver. 1.47q (Schindelin et al., 2012).

Electron Microscopic Analysis

Arabidopsis leaves that were infected with *Pto* DC3000 *avrRpt2* were vacuum-infiltrated for 1 h in fixative, dehydrated, and embedded in Epon as described previously (Hatsugai et al., 2009). Ultra-thin sections were examined under a transmission electron microscope (model 1200EX; JEOL) at 80 kV.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative under accession numbers AP4B (At5g11490) and AP4E (At1g31730).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Arabidopsis plants used in this study.

ACKNOWLEDGMENTS

We are grateful to the ABRC at Ohio State University for providing Arabidopsis T-DNA insertion mutant seeds.

Received November 9, 2017; accepted December 8, 2017; published December 13, 2017.

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