The μ Subunit of *Arabidopsis* Adaptor Protein-2 Is Involved in Effector-Triggered Immunity Mediated by Membrane-Localized Resistance Proteins

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Endocytosis has been suggested to be important in the cellular processes of plant immune responses. However, our understanding of its role during effector-triggered immunity (ETI) is still limited. We have previously shown that plant endocytosis, especially clathrin-coated vesicle formation at the plasma membrane, is mediated by the adaptor protein-2 (AP-2) complex and that loss of the μ subunit of AP-2 (AP2M) affects plant growth and floral organ development. Here, we report that AP2M is required for full-strength ETI mediated by the disease resistance (R) genes *RPM1* and *RPS2* in *Arabidopsis*. Reduced ETI was observed in an *ap2m* mutant plant, measured by growth of *Pseudomonas syringae* pv. *tomato* DC3000 strains carrying the corresponding effector genes *avrRpm1* or *avrRpt2* and by hypersensitive cell death response and defense gene expression triggered by these strains. In contrast, *RPS4*-mediated ETI and its associated immune responses were not affected by the *ap2m* mutation. While *RPM1* and *RPS2* are localized to the plasma membrane, *RPS4* is localized to the cytoplasm and nucleus. Our results suggest that AP2M is involved in ETI mediated by plasma membrane-localized R proteins, possibly by mediating endocytosis of the immune receptor complex components from the plasma membrane.

Plant defenses that restrict pathogen growth are often mediated by resistance (R) proteins. The most prevalent class of R proteins is the nucleotide-binding leucine-rich repeat (NB-LRR) type (Dangl and Jones 2001; Tameling and Joosten 2007). Other classes are the receptor-like kinase (RLK) and receptor-like protein (RLP) types, both of which possess an extracellular LRR domain and a transmembrane domain. While members of the RLK class contain a cytoplasmic serine and threonine kinase domain, the members of the RLP class do not contain a recognizable cytoplasmic signaling domain (Dangl and Jones 2001; Tameling and Joosten 2007). These R proteins

recognize pathogen-specific signals called effectors and this recognition leads to induction of effector-triggered immunity (ETI). ETI is often accompanied by a hypersensitive response (HR), which is characterized by rapid and localized programmed cell death (hypersensitive cell death) (Hara-Nishimura and Hatsugai 2011; Jones and Dangl 2006; Tsuda and Katagiri 2010).

In *Arabidopsis*, two NB-LRR R proteins, *RPM1* and *RPS2*, interact with RIN4 at the plasma membrane (Axtell and Staskawicz 2003; Mackey et al. 2003). *RPM1* specifically detects phosphorylation of RIN4 triggered by the *Pseudomonas syringae* effector *AvrRpm1* and leads to *RPM1*-mediated ETI, whereas *RPS2* recognizes proteolytic cleavage of RIN4 by the *AvrRpt2* effector and leads to *RPS2*-mediated ETI (Axtell and Staskawicz 2003; Mackey et al. 2003). Another NB-LRR R protein, *RPS4*, confers resistance to *P. syringae* expressing *AvrRps4*. *RPS4* distributes between the cytoplasm and nucleus, and nuclear localization of *RPS4* is required for *RPS4*-mediated ETI (Wirthmueller et al. 2007).

Clathrin-mediated endocytosis (CME) is the major endocytosis pathway in animal cells. During CME, cargo proteins are captured predominantly by the adaptor protein-2 (AP-2) complex and are assembled into transport vesicles coated with clathrin. AP-2 is a heterotetrameric complex consisting two large subunits (α and β 2), a medium subunit (μ 2), and a small subunit (σ 2). For the capture of cargo proteins, μ 2 recognizes the tyrosine-based YXX Φ motif (Φ represents Leu, Ile, Phe, Met, or Val) present in the cytosolic domains of cargo proteins (Ohno et al. 1995). Multiple lines of evidence have shown that plants also use CME for uptake of various proteins from the plasma membrane, including the PIN-FORMED (PIN) auxin transporters, the water channel PLASMA MEMBRANE INTRINSIC PROTEIN2 (Dhonukshe et al. 2007), the boron transporter REQUIRES HIGH BORON1 (Takano et al. 2010), the iron transporter IRON-REGULATED TRANSPORTER1 (Barberon et al. 2011), the plant-specific endocytic SNARE VESICLE-ASSOCIATED MEMBRANE PROTEIN727 (Ebine et al. 2011), and the ligand-activated brassinosteroid receptor BRASSINOSTEROID INSENSITIVE1 (Irani et al. 2012). We and other groups have recently shown that AP-2 is involved in plant CME (Di Rubbo et al. 2013; Fan et al. 2013; Kim et al. 2013; Yamaoka et al. 2013). An insertional mutation in the *AP2M* gene encoding the *Arabidopsis* μ 2 subunit causes reduced fertility and abnormal floral organ development (Kim et al. 2013; Yamaoka et al. 2013).

AP-2 has been suggested to be involved in R protein-mediated immunity (reviewed by Geldner and Robatzek 2008; Leborgne-Castel et al. 2010; Bar and Avni 2014; Teh and Hofius 2014).

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Fig. 1. *AP2M* deficiency compromised resistance to *Pseudomonas syringae* pv. *tomato* DC3000 *avrRpm1* and DC3000 *avrRpt2* but not to DC3000 *avrRps4*. **A**, *P. syringae* pv. *tomato* DC3000 *avrRpm1*, **B**, DC3000 EV (empty vector), **D**, DC3000 *avrRpt2*, and **E**, DC3000 *avrRps4* (optical density at 600 nm = 0.0001) were inoculated into leaves of wild type, *ap2m*, *AP2M-GFP ap2m*, and the corresponding resistance gene mutants. Live bacteria were counted at 0 days postinoculation (dpi) (open bar) and 2 dpi (filled bar). Bars represent the mean and error bars the standard error from model fitting. The data were collected in three independent experiments, each of which had six or eight biological replicates. **C**, **F**, and **G**, The level of effector-triggered immunity (ETI) was estimated by subtracting $\log_{10}$ -transformed bacterial counts at 2 dpi for plants inoculated with *P. syringae* pv. *tomato* DC3000 *avrRpm1*, DC3000 *avrRpt2*, or DC3000 *avrRps4*, respectively, from the $\log_{10}$ -transformed counts for *P. syringae* pv. *tomato* DC3000 EV. The ETI level of each plant was compared with that of wild type, using a two-tailed *t* test to obtain the *P* values (Holm-Bonferroni corrected).

For example, the YXXΦ motif is present in the cytoplasmic domain of the tomato (*Lycopersicon esculentum*) RLP receptor LeEix2 (Ron and Avni 2004). Mutation of this motif in LeEix2 blocked xylanase-induced endocytosis and abolished its ability to induce HR, suggesting that the AP-2 complex might be involved in xylanase-triggered HR (Ron and Avni 2004; Sharfman et al. 2011). The YXXΦ motif is found in the cytoplasmic domain of other RLPs, such as Ve2, Cf4, and Cf9 (Jones et al. 1994; Kawchuk et al. 2001; Takken et al. 1998), but the functional relevance of the motif for their R protein-mediated immunity is still to be unraveled. Another example of the involvement of endocytosis in R protein-mediated immunity has been proposed in rice (*Oryza sativa*). During RLK Xa21-mediated race-specific resistance to the bacterial blight agent *Xanthomonas oryzae* pv. *oryzae* (Chen et al. 2010), Xa21 was internalized and probably transported via the trans-Golgi network and early endosome compartment. Additionally, cryptogin, an elicitor from the oomycete *Phytophthora cryptogea*, stimulated CME in tobacco BY-2 cells (Leborgne-Castel et al. 2008). Similarly, during infection by the hemibiotrophic fungus *Magnaporthe oryzae*, rice cells exhibited increased endocytosis (Kankanala et al. 2007). However, no direct evidence has been provided for the involvement of AP-2 in plant immune responses.

In this study, we investigated immune responses mediated by three different NB-LRR R proteins, i.e., RPM1, RPS2, and RPS4, and found that *AP2M* deficiency reduces ETI against *P. syringae* pv. *tomato* DC3000 *avrRpm1* and DC3000 *avrRpt2* but not against DC3000 *avrRps4*. Our results provide direct evidence of the role of CME in plant immunity.

RESULTS

An *ap2m* mutant showed reduced levels of ETI against *P. syringae* pv. *tomato* DC3000 *avrRpm1* and DC3000 *avrRpt2* but not to DC3000 *avrRps4*.

The Col-0 accession of *Arabidopsis* carries an *R* gene, *RPM1*, which confers resistance to *P. syringae* pv. *tomato* DC3000 *avrRpm1* (Grant et al. 1995). To investigate whether AP-2 is involved in RPM1-mediated ETI, we examined whether resistance to *P. syringae* pv. *tomato* DC3000 *avrRpm1* was impaired in an *ap2m* T-DNA insertion mutant (SAIL_165_A05) (Yamaoka et al. 2013). We inoculated wild-type and *ap2m* plants with *P. syringae* pv. *tomato* DC3000 *avrRpm1* and counted viable bacteria immediately and 2 days postinoculation (dpi). The number of bacteria in *ap2m* plants was higher than that in wild-type plants at 2 dpi but lower than the number in *rpm1* plants used as a control (Fig. 1A). Bacterial growth of the virulent strain *P. syringae* pv. *tomato* DC3000 carrying an empty vector (*P. syringae* pv. *tomato* DC3000 EV) in *ap2m* plants was only slightly increased compared with wild-type plants (Fig. 1B). The difference in $\log_{10}$ -transformed numbers of bacteria between *P. syringae* pv. *tomato* DC3000 *avrRpm1* and DC3000 EV at 2 dpi was used to estimate the impact of the *ap2m* mutation on RPM1-mediated ETI. We observed a 43% reduction of RPM1-mediated ETI in the *ap2m* plants compared with wild-type plants ($P = 8.4 \times 10^{-7}$) (Fig. 1C). An *ap2m* complementation line, which carries an *AP2M-GFP* (green fluorescent protein) transgene under the control of the endogenous *AP2M* promoter (*AP2M-GFP ap2m*) (Yamaoka et al. 2013), gave results similar to wild-type plants, confirming that the results obtained with the *ap2m* mutant were indeed the effect of the *ap2m* mutation. Thus, an *AP2M* deficiency results in compromised RPM1-mediated ETI.

To examine the effect of *AP2M* deficiency on ETI mediated by another R protein, RPS2, wild-type, *ap2m*, *AP2M-GFP ap2m*, and *rps2* plants were inoculated with *P. syringae* pv. *tomato* DC3000 *avrRpt2* (Fig. 1D). RPS2-mediated ETI was reduced

by 60% in *ap2m* plants compared with ETI in wild-type plants ($P = 1.5 \times 10^{-9}$) (Fig. 1F); this effect was complemented in *AP2M-GFP ap2m* plants. These results indicate that AP2M is involved in RPS2-mediated ETI as well as RPM1-mediated ETI.

AP2M plays a central role in CME from the plasma membrane (Di Rubbo et al. 2013) (Fan et al. 2013; Kim et al. 2013; Yamaoka et al. 2013). In contrast to plasma membrane-associated RPM1 and RPS2, RPS4 is localized to the nucleus and cytoplasm (Wirthmueller et al. 2007). It is unlikely that RPS4-mediated ETI is affected by *AP2M* deficiency. To test this hypothesis, we measured growth of *P. syringae* pv. *tomato* DC3000 *avrRps4* in wild type, *ap2m*, *AP2M-GFP ap2m*, and *rps4* plants (Fig. 1E). No significant difference in RPS4-mediated ETI was observed among wild type, *ap2m*, and *AP2M-GFP ap2m* (Fig. 1G).

Taken together, we conclude that AP2M is involved in both RPS2-mediated and RPM1-mediated ETI but not involved in RPS4-mediated ETI.

The *AP2M* deficiency reduced the amount of hypersensitive cell death during RPM1- and RPS2-mediated ETI.

ETI is often associated with hypersensitive cell death (Dangl and Jones 2001; Jones and Dangl 2006). To determine whether *AP2M* deficiency affects hypersensitive cell death, we monitored cell death in wild-type and *ap2m* plants, using trypan blue staining 12 h postinoculation with *P. syringae* pv. *tomato* DC3000 *avrRpm1*. Dead cells are seen as a distinctive blue color under a light microscope in wild-type plants (Fig. 2A). The *ap2m* mutant had clearly fewer dead cells than wild type (Fig. 2A). The number of dead cells in *AP2M-GFP ap2m* was similar to that observed in wild type, while no dead cells were observed in *rpm1 rps2*. We obtained consistent results when the cell death was quantified using an electrolyte leakage assay (Fig. 2B); the extent of cell death was high and comparable between wild type and *AP2M-GFP ap2m*, while it was clearly lower in *ap2m*, and a very low level of cell death was observed with *rpm1 rps2*.

Fig. 2. Hypersensitive cell death was reduced in the *ap2m* mutant, although reactive oxygen species production was not impaired. **A**, *Pseudomonas syringae* pv. *tomato* DC3000 *avrRpm1* and **C**, DC3000 *avrRpt2* (optical density at 600 nm [OD_{600}] = 0.001) were inoculated into leaves of wild type, *ap2m*, *AP2M-GFP ap2m*, and *rpm1 rps2*. Dead cells in the leaves at 12 h after the inoculation were visualized by trypan-blue staining. Bar = 200 μm . **B**, *P. syringae* pv. *tomato* DC3000 *avrRpm1* and **D**, DC3000 *avrRpt2* (OD_{600} = 0.1) were infiltrated into leaves of wild type, *ap2m*, *AP2M-GFP ap2m*, and *rpm1 rps2*. Electrolyte leakage from dying and dead cells in the leaves at 2 to 12 h after the inoculation was measured. Vertical bars indicate standard errors of three independent experiments, each of which had four biological replicates. **E**, *P. syringae* pv. *tomato* DC3000 *avrRpm1*, **F**, DC3000 *avrRpt2*, and **G**, DC3000 *avrRps4* (OD_{600} = 0.1) were inoculated into leaves of wild type, *ap2m*, *AP2M-GFP ap2m*, and the corresponding resistance (*R*) gene mutants. The leaves were stained with 3,3'-diaminobenzidine (DAB) 12 h (for *P. syringae* pv. *tomato* DC3000 *avrRpm1*) and 24 h (for *P. syringae* pv. *tomato* DC3000 *avrRpt2* and DC3000 *avrRps4*) after the inoculation. Bar = 200 μm . **H**, **I**, and **J**, Quantification of the DAB staining intensity. Bars represent the mean and error bars the standard deviation from five stained regions in three different leaves.

Similar results were obtained for hypersensitive cell death during RPS2-mediated ETI by both trypan blue staining (Fig. 2C) and electrolyte leakage (Fig. 2D). In both analyses, the extent of cell death was high and comparable between wild type and *AP2M-GFP ap2m*, and the level of cell death was clearly lower in *ap2m*. No or a very low level of cell death was observed with *rpm1 rps2*. Thus, hypersensitive cell death during ETI mediated by RPM1 and RPS2 is affected by the *ap2m* mutation.

The RPS4-mediated 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 *AP2M* deficiency on hypersensitive cell death during RPS4-mediated ETI.

ROS production during ETI was normal in *ap2m* mutant.

It has been proposed that ROS production is involved in hypersensitive cell death (Torres et al. 2006). To examine the involvement of *AP2M* in ROS production during RPM1-mediated immune responses, we inoculated leaves with *P. syringae* pv. *tomato* DC3000 *avrRpm1* and, then, stained them with 3,3'-diaminobenzidine (DAB), which reacts with H_2O_2 to produce brown polymerized precipitate. The brown-colored precipitate accumulated to equivalent levels in leaves of wild-type, *ap2m*, and *AP2M-GFP ap2m* plants (Fig. 2E and H). These results suggest that *AP2M* is not required for ROS production during RPM1-mediated immune responses. Similarly, deficiency of *AP2M* had no effect on ROS production in the leaves inoculated with *P. syringae* pv. *tomato* DC3000 *avrRpt2* (Fig. 2F and I) and DC3000 *avrRps4* (Fig. 2G and J).

AP2M deficiency reduced DAB gene induction levels.

Plant defense is associated with transcriptional upregulation of pathogenesis-related (*PR*) genes such as *PR1* (At2g14610) and *PR2* (At3g57260) (van Loon et al. 2006). We compared the levels of *PR1* and *PR2* mRNAs in wild-type, *ap2m*, and *AP2M-GFP ap2m* plants inoculated with *P. syringae* pv. *tomato* DC3000 *avrRpm1*. *PR1* and *PR2* expression in wild-type plants was induced 3 h after bacterial inoculation and remained elevated 12 h after the inoculation (Fig. 3A). Relative to the wild-type expression levels 12 h after the inoculation of *P. syringae* pv. *tomato* DC3000 *avrRpm1*, *PR1* and *PR2* expression levels in *ap2m* plants were statistically lower (Fig. 3A). The reduced expression of *PR1* and *PR2* in *ap2m* was rescued by expression of *AP2M-GFP*, confirming that the transcriptional defect of *PR* genes is caused by the mutation in *AP2M* (Fig. 3A). These results indicate that *AP2M* is involved in induction of *PR-1* and *PR-2* during RPM1-mediated ETI. Similar reductions in the *PR-1* and *PR-2* induced levels were observed in *ap2m* plants after *P. syringae* pv. *tomato* DC3000 *avrRpt2* inoculation (Fig. 3B). In contrast, the *ap2m* plants inoculated with DC3000 *avrRps4* showed induced levels of *PR1* and *PR2* very similar to those of wild-type and *AP2M-GFP ap2m* plants (Fig. 3C).

DISCUSSION

We have reported here that *AP2M* is partially required for both RPM1-mediated and RPS2-mediated ETI but not required for RPS4-mediated ETI. *AP2M* is the μ subunit of the evolutionarily conserved AP-2 complex, which plays a central role in CME from the plasma membrane (Di Rubbo et al. 2013; Fan et al. 2013; Kim et al. 2013; Yamaoka et al. 2013). Our results suggest that CME is involved in the signaling cascade for RPM1- and RPS2-mediated immune responses, whereas the RPS4-mediated immune response is independent of CME. Endocytosis in some cases attenuates signaling by removing activated receptors from the plasma membrane (Gagliardi et al. 2008). It is also likely that endosomes act as platforms for active signaling and that endocytosis plays a crucial role in signal propagation and amplification (Gagliardi et al. 2008). CRT1, a member of the GHLK ATPase/kinase superfamily (Dutta and Inouye 2000), physically interacted with both RPM1 and RPS2 (Kang et al. 2010). An *Arabidopsis* mutant lacking CRT1 displayed compromised resistance to *P. syringae* pv. *tomato* DC3000 *avrRpm1* and DC3000 *avrRpt2* (Kang et al. 2010). CRT1 localized to endosomes. This implies that the interactions of CRT1 with RPM1 and RPS2 occur in endosomes (Kang et al. 2010), whereas it has been suggested that

Fig. 3. Induced mRNA levels of two pathogenesis-related (*PR*) genes in *ap2m* leaves were lower than those in wild-type leaves. **A**, *Pseudomonas syringae* pv. *tomato* DC3000 *avrRpm1*, **B**, DC3000 *avrRpt2*, and **C**, DC3000 *avrRps4* (optical density at 600 nm = 0.1) were inoculated into leaves of wild type, *ap2m*, and *AP2M-GFP ap2m* and tissue was harvested immediately (0 h) and 3, 6, and 12 h after the inoculation. Each bar represents the log₂ of the mean expression value and the error bars represent the standard error from fitting a model to the data collected in three independent biological replicates. The log₂-transformed expression values were normalized using the control gene *ACTIN2* (At2g18780). Asterisks indicate significant differences compared with wild-type plants (* indicates $P < 0.05$, ** indicate $P < 0.01$, two-tailed *t* tests adjusted with the Holm-Bonferroni correction).

RPM1 functions on the plasma membrane (Gao et al. 2011). RPM1 and RPS2 have been shown to form complexes on the plasma membrane with plasma membrane-associated proteins (Qi and Katagiri 2009). These reports suggest that RPM1, RPS2, or other receptor complex components can relocate from the plasma membrane to endosomes and mediate immune signal transduction in endosomes. In support of this hypothesis, the potato NB-LRR immune receptor R3a localizes to endosomes for recognition of the *Phytophthora infestans* effector AVR3a(KI) (Engelhardt et al. 2012). Endosomal localization of these immune receptors might be mediated by the AP-2 complex. Future identification of the cargo proteins of AP-2 will likely unravel the role of AP-2-dependent endocytosis in the plant immune response. However, it should be noted that the effect of the *ap2m* mutation on RPM1- and RPS2-mediated ETI and its associated responses was partial. While endosomal localization of one or more immune signaling components may be important to express full-strength RPM1- and RPS2-mediated ETI, there appears to also be one or more other mechanisms that mediate signaling downstream of the plasma membrane.

AP2M deficiency compromised ETI against *P. syringae* pv. *tomato* DC3000 *avrRpm1* and DC3000 *avrRpt2*, which was associated with both weakened hypersensitive cell death and *PR* gene induction. In contrast, ROS production was intact in the *ap2m* mutant during ETI. There are at least three hypotheses to explain these observations. One possible explanation is that the effector recognition functions are normal in the *ap2m* mutants but the downstream pathway of ROS production is impaired. Recent studies suggest that endocytosis plays a role in immune responses downstream of ROS production. Cryptogein stimulated CME in a ROS-dependent manner in tobacco BY-2 cells. This event was abolished in a cell line with impaired ROS production (Leborgne-Castel et al. 2008). Another study showed that inhibition of ROS produced in response to flg22 prevented FLS2 endocytosis (Serrano et al. 2007). These observations may suggest that AP2M functions in the pathway between ROS production and CME. A second possible explanation is that the signaling pathway downstream of effector recognition is divided into two separate branches, one affected by the *ap2m* mutation and the other leading to ROS production. Consistent with this notion, the ROS production was strongly reduced but the HR was only moderately affected in an *Arabidopsis rhbohD* mutant in response to *P. syringae* pv. *tomato* DC3000 *avrRpm1* (Torres et al. 2002). Similarly, *Nbrboh*-silenced *Nicotiana benthamiana* plants exhibited reduced elicitor-mediated ROS production but did not show an obvious change in HR or *PR* gene expression compared with nonsilenced plants (Zhang et al. 2009). In *Arabidopsis*, a defect in proteasome function did not affect ROS production but abolished HR associated with ETI (Hatsugai et al. 2009). Thus, ROS production may be mediated by a different signaling pathway than cell death and other responses during ETI. A third possible explanation is based on the observation that the effect of the *ap2m* mutation is quantitative. Both RPM1-mediated and RPS2-mediated ETI were only partially abolished in the *ap2m* mutant, unlike in the corresponding *R* gene mutants. A weak signal for ETI must still remain even in the *ap2m* mutant. This weak signal might be sufficient to fully induce ROS production as the signal-dose response for ROS production may have a low saturation point. Other ETI-associated responses were, however, affected; they may have higher saturate points in the signal-dose response.

Taken together, our results demonstrate that AP2M is important but not absolutely required for plant immune responses. They also suggest that AP2M is required for full resistance mediated by membrane-localized *R* proteins.

MATERIALS AND METHODS

Plant materials and growth conditions.

Arabidopsis thaliana accession Col-0 was the background of all the plants used in this study. *Arabidopsis ap2m* (SAIL_165_A02) (Yamaoka et al. 2013), *rpm1-3* (Grant et al. 1995), *rps2 101C* (Mindrinos et al. 1994), *rpm1-3/rps2 101C* (Mackey et al. 2003), *rps4* (SALK_057697) (Wirthmueller et al. 2007), and *AP2M-GFP ap2m* (Yamaoka et al. 2013) were previously described. *Arabidopsis* plants were grown in a controlled environment at 22°C with a 12-h photoperiod and 75% relative humidity.

Bacterial strains and preparation of inocula.

P. syringae pv. *tomato* DC3000 was grown overnight in King's B medium supplemented with 50 µg of rifampicin per milliliter. *P. syringae* pv. *tomato* DC3000 strains carrying the empty vector (pVSP61), *avrRpm1*, *avrRpt2*, or *avrRps4* were grown overnight in King's B medium supplemented with 50 µg of rifampicin and 30 µg of kanamycin per milliliter. The bacteria were harvested by centrifugation, were washed, and were diluted to the desired density with water.

Bacterial growth assay.

Bacterial growth assays were performed as described previously (Tsuda et al. 2008). In brief, bacterial suspensions of the indicated density were infiltrated into leaves of 4- to 5-week-old plants using a needleless syringe. CFU per square centimeter of leaf surface area were counted from leaf tissue collected 2 days after infiltration. A mixed-effect model was fit to log₁₀-transformed bacterial count data, using the lme function from the nlme package in the R environment. The model $S_{grf} = GT_{gt} + R_r/F_f + \varepsilon_{grf}$, where $S = \log_{10}$ -transformed bacterial number, $GT = \text{plant_genotype:treatment interaction}$ (different treatments represent different bacterial strains), and random factors, $R = \text{biological replicate}$ (from independent experiments); $F = \text{planting flat nested within each replicate}$, and $\varepsilon = \text{residual}$. The mean estimates of the genotype:treatment interactions were used as the modeled log₁₀-transformed bacterial counts. Bacterial growth was calculated as the difference in modeled log₁₀-transformed bacterial counts between each mutant genotype and wild-type genotype. Growth values of effector and empty vector strains were compared using two-tailed *t* tests. For the *t* tests, the standard errors appropriate for each comparison of differences were calculated using the variance and covariance values obtained from the model fitting. *P* values obtained from the *t* tests were adjusted using the Holm-Bonferroni multiple testing correction.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis.

Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's protocol. qRT-PCR was performed using a Superscript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen) and a Roche LightCycler480 machine. Primers for qRT-PCR are listed in Supplementary Table S1. The relative log₂ expression values were obtained by subtracting the cycle threshold (*C_t*) values for each gene from the corresponding *C_t* values of the *Actin2* gene. A mixed-effect model was fit separately to the relative log₂ expression data for each gene, using the lme function from the nlme package in the R environment. The model $\Delta C_{t_{gti}} = GTI_{gti} + R_r/T/I_i + \varepsilon_{gti}$, where $GTI = \text{genotype:treatment:time interaction}$, and random factors, $R = \text{biological replicate}$ (from independent experiments), $T = \text{treatment nested within replicate}$, $I = \text{time nested within treatment}$, and $\varepsilon = \text{residual}$. The mean estimates of the genotype:treatment:time interactions were used as the modeled log₂ expression values. Modeled log₂ expression values for the mutant genotypes were

compared with those of the wild-type genotype, using two-tailed *t* tests. For these *t* tests, the relevant standard error for each comparison was calculated using the variance and covariance values obtained from the model fitting. For each gene independently, *P* values obtained from the *t* tests were adjusted for multiple hypothesis testing using the Holm-Bonferroni correction.

Trypan blue staining.

Inoculated leaves were boiled for approximately 1 min in the staining solution (1 ml of lactic acid, 1 ml of glycerol, 1 ml of water-saturated phenol, 1 ml of water, and 1 mg of trypan blue) and were incubated overnight at room temperature. Then, samples were decolorized in 2.5 g of chloral hydrate per milliliter for at least 1 day.

Electrolyte leakage assay.

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

Reactive oxygen species (ROS) detection.

ROS was visualized in leaf tissues by staining with DAB (Sigma-Aldrich), as described previously (Thordal-Christensen et al. 1997). Leaves were vacuum-infiltrated with DAB solution. The leaf samples were placed in a plastic box with high humidity overnight at room temperature. Leaves were fixed (60% [vol/vol] ethanol, 20% [vol/vol] lactic acid, and 20% [vol/vol] glycerol), were decolorized in 2.5 g chloral hydrate per milliliter, and were visualized with a light microscope. The grayscale images of the DAB-stained leaves were used to quantify the signal intensity using Fiji/ImageJ software ver. 1.47q (Schindelin et al. 2012).

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