ASPARTATE OXIDASE Plays an Important Role in Arabidopsis Stomatal Immunity[1][W][OA]

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Perception of pathogen-associated molecular patterns (PAMPs), such as bacterial flagellin (or the peptide flg22), by surface-localized receptors activates defense responses and subsequent immunity. In a previous forward-genetic screen aimed at the identification of Arabidopsis (*Arabidopsis thaliana*) *flagellin-insensitive* (*fin*) mutants, we isolated *fin4*, which is severely affected in flg22-triggered reactive oxygen species (ROS) bursts. Here, we report that *FIN4* encodes the chloroplastic enzyme ASPARTATE OXIDASE (AO), which catalyzes the first irreversible step in the de novo biosynthesis of NAD. Genetic studies on the role of NAD have been hindered so far by the lethality of null mutants in NAD biosynthetic enzymes. Using newly identified knockdown *fin* alleles, we found that AO is required for the ROS burst mediated by the NADPH oxidase RBOHD triggered by the perception of several unrelated PAMPs. AO is also required for RBOHD-dependent stomatal closure. However, full AO activity is not required for flg22-induced responses that are RBOHD independent. Interestingly, although the *fin4* mutation dramatically affects RBOHD function, it does not affect functions carried out by other members of the RBOH family, such as RBOHC and RBOHF. Finally, we determined that AO is required for stomatal immunity against the bacterium *Pseudomonas syringae*. Altogether, our work reveals a novel specific requirement for AO activity in PAMP-triggered RBOHD-dependent ROS burst and stomatal immunity. In addition, the availability of viable mutants for the chloroplastic enzyme AO will enable future detailed studies on the role of NAD metabolism in different cellular processes, including immunity, in Arabidopsis.

The first active layer of the plant immune system is based on the recognition of conserved elicitors, called pathogen-associated molecular patterns (PAMPs), by plasma membrane pattern recognition receptors (PRRs). PAMP-triggered immunity (PTI) is sufficient to fend off most microbes. Successful pathogens must evade or suppress PTI to cause disease, and among other strategies they do so by secreting effector proteins into plant cells. Some of these effectors can be recognized directly or indirectly by intracellular immune receptors, called R proteins, leading to effector-triggered immunity. These events illustrate the dynamic arms race between plants and their pathogens.

In the plant model Arabidopsis (*Arabidopsis thaliana*), the leucine-rich repeat (LRR) receptor kinases (RKs) FLAGELLIN SENSING2 (FLS2) and EF-TU RECEPTOR (EFR) are well-characterized PRRs that bind the bacterial PAMPs flagellin (or the derived peptide flg22) and EF-Tu (or the derived peptides elf18/26), respectively (Gómez-Gómez and Boller, 2000; Zipfel et al., 2006). In addition, the LysM domain-containing RK CHITIN ELICITOR RECEPTOR KINASE1 (CERK1) is required for the response to the fungal PAMP chitin and to the bacterial PAMP peptidoglycan, most likely as part of a multiprotein recognition complex with LysM domain-containing receptor-like proteins (Miya et al., 2007; Wan et al., 2008; Gimenez-Ibanez et al., 2009a, 2009b; Willmann et al., 2011). One of the earliest events triggered by the binding of flg22 or elf18/elf26 to FLS2 or EFR is the heteromerization with the regulatory LRR receptor-like kinase SOMATIC EMBRYOGENESIS RECEPTOR KINASE3/BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE1 (SERK3/BAK1; hereafter referred to as BAK1), leading to phosphorylation of both proteins and initiation of downstream signaling (Chinchilla et al., 2007; Heese et al., 2007; Schulze et al., 2010; Roux et al., 2011). Additionally, FLS2, EFR, CERK1, and BAK1 interact with BOTRYTIS-INDUCED KINASE1 (BIK1) a cytoplasmic kinase that positively regulates several PAMP-induced immune responses (Lu et al., 2010; Zhang et al., 2010). PAMP-induced responses include activation of the mitogen-activated protein kinase (MAPK) pathways MEKK-MKK4/5-MPK3/6 and MEKK1-MKK1/2-MPK4/11 as well as an independent pathway involving the calcium-dependent protein kinases CDPK4/5/6/11 (Tena et al., 2011; Bethke et al., 2012; Zhang et al., 2012). These signaling cascades lead to the transcriptional activation of defense genes via the WRKY transcription factors WRKY22/29 and WRKY25/33 (Nicaise et al., 2009). Other responses associated with

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PTI include ion fluxes, a burst of reactive oxygen species (ROS), stomatal closure, callose deposition at the cell wall, and seedling growth inhibition (Boller and Felix, 2009).

In plants, the NADPH oxidase respiratory burst oxidase homologs (RBOHs) are the most studied ROS-producing enzymes. In Arabidopsis, the *RBOH* genes belong to a family of 10 members, although experimental characterization has been carried out only for four of them (Marino et al., 2012), namely *RBOHB*, *RBOHC*, *RBOHD*, and *RBOHF*. This limited knowledge has nevertheless illustrated that RBOH-generated ROS play an essential role for several cellular functions, including seed germination (RBOHB; Müller et al., 2009), root hair development (RBOHC/RHD2; Foreman et al., 2003), and biotic and abiotic stress responses (RBOHD/F; for review, see Marino et al., 2012). Importantly, RBOHD is the main enzyme responsible for the rapid production of apoplastic ROS upon PAMP perception (Nühse et al., 2007; Zhang et al., 2007). Recently, apoplastic peroxidases, such as PRX33 and PRX34, have also been shown to contribute to flg22- and elf26-triggered ROS bursts (Daudi et al., 2012; O'Brien et al., 2012). Given the diverse roles of ROS produced in different environmental conditions, tight regulation and specificity of ROS production is expected, although the mechanisms underlying such regulation are still unknown.

A cellular response induced by the perception of PAMPs, such as flg22 or LPS, is the closure of stomata within a few hours, which restricts pathogen entry into plant tissues (Melotto et al., 2008). In nature, plants rely on PAMP-induced stomatal closure to prevent invasion by bacterial pathogens. Therefore, in order to invade the apoplast and cause disease, a successful pathogen has to evolve virulence factors to impede stomatal closure and/or trigger stomatal reopening. A well-studied suppressor of stomatal closure is the bacterial phytotoxin CORONATINE (COR), produced by several pathovars of *Pseudomonas syringae* (Bender et al., 1999). Supporting the importance of stomatal closure for plant immunity, a COR-deficient (COR^-) derivative of the Arabidopsis pathogen *P. syringae* pv *tomato* (*Pto*) DC3000 is unable to colonize the apoplast efficiently or cause disease when surface inoculated (Melotto et al., 2006). The fact that Arabidopsis *fls2* mutant plants are more susceptible to a COR^- *Pto* DC3000 mutant further illustrates the importance of FLS2 function for antibacterial stomatal immunity (Nekrasov et al., 2009; Zeng and He, 2010). Mechanistically, flg22- or LPS-induced stomatal closure depends on RBOHD, nitric oxide, the OST1 kinase, inhibition of inward potassium channels via the G-protein α-subunit GPA1, as well as the stress hormones abscisic acid (ABA) and salicylic acid (SA; Melotto et al., 2006; Mersmann et al., 2010; Zeng and He, 2010; Zhang et al., 2010). PAMP-induced stomatal closure is negatively regulated by RIN4-mediated regulation of the proton ATPases AHA1/2 (Liu et al., 2009). Additionally, recent reports have shown that stomatal immunity is subject to complex and multilayered regulation involving the ATP-binding cassette transporter family protein AtGCN20/AtABCF3/SCORD5 (Zeng et al., 2011) and the L-type lectin receptor-like kinases LecRK-VI.2 and LecRK-V.5 that play contrasting roles in PAMP-induced stomatal closure (Desclos-Theveniau et al., 2012; Singh et al., 2012).

Our knowledge of the molecular components involved in PTI signaling and of the subsequent immunity to pathogens is still limited. In recent years, several forward genetic screens have been carried out in Arabidopsis to identify additional components of PTI. The independent *elf18-insensitive* (*elfin*) and *priority in sweet life* (*psl*) mutant screens based on the seedling growth inhibition assay identified numerous *efr* alleles and revealed a specific requirement for endoplasmic reticulum quality control and *N*-glycosylation for EFR biogenesis (Li et al., 2009; Lu et al., 2009; Nekrasov et al., 2009; Saijo et al., 2009). The *elfin* genetic screen further identified the novel *bak1* allele, *bak1-5*, which revealed that the functions of BAK1 in PTI, brassinosteroid-mediated growth, and cell death control could be mechanistically uncoupled (Schwessinger et al., 2011). In addition to the *elfin* screen, we have also performed a genetic screen aimed at the identification of mutants impaired in the ROS burst triggered by flg22 (*flagellin-insensitive* [*fin*] mutants). *fin1* and *fin2* correspond to the *fls2* and *bak1* alleles, respectively (Boutrot et al., 2010). The detailed analysis of the *fin3* mutant, which carries an 11-amino acid deletion in the C terminus of EIN2 that is a key regulator of ethylene signaling, coupled with the characterization of other ethylene signaling mutants, revealed an unexpected direct role for this hormone in the transcriptional regulation of *FLS2* (Boutrot et al., 2010).

Here, we report that *FIN4* encodes the chloroplastic enzyme ASPARTATE OXIDASE (AO), which catalyzes the first irreversible step in the de novo biosynthesis of nicotinamide adenine dinucleotide (NAD). *fin4* corresponds to a viable knockdown mutant that is impaired in the RBOHD-dependent PAMP-induced ROS burst and stomatal closure. In contrast, RBOHD-independent PAMP-induced responses are not affected in *fin4* mutants. Interestingly, *fin4* plants were not affected in the functions of RBOHC and RBOHF in root hair elongation and ABA-induced stomatal closure. Consistent with the loss of PAMP-induced stomatal closure in the mutant, *fin4* was affected in stomatal immunity to *P. syringae*. Our results demonstrate an unambiguous role for NAD metabolism in plant innate immunity and an unexpected specificity for the requirement of optimal NAD levels between distinct RBOH isoforms in Arabidopsis.

RESULTS

fin4 Carries an Insertion in the *AO* Gene

In a previous forward-genetic screen aimed at the identification of Arabidopsis *fin* mutants using a

collection of transfer-DNA (T-DNA) lines (Boutrot et al., 2010), we isolated *fin4*, which is severely affected in flg22-triggered ROS bursts, producing only about 20% to 40% of the total ROS produced during 40 min after treatment with 100 nM flg22 when compared with wild-type ecotype Columbia (Col-0; Fig. 1, A, D, and E). The overall temporal pattern of the ROS burst, however, was not affected in the *fin4-1* mutant (Fig. 1A). To identify the causative mutation in *fin4* (hereafter referred to as *fin4-1*), we used an adapter ligation-mediated PCR method (O'Malley et al., 2007). As a result, we identified a T-DNA insertion in the promoter (845 nucleotides upstream of the ATG) of the *AO* gene (At5g14760; Fig. 1B) that encodes the first enzyme of the de novo NAD biosynthesis pathway in the chloroplast (Fig. 2A; Katoh et al., 2006).

To confirm that the *fin* phenotype is indeed caused by the mutation in *AO*, we obtained additional Arabidopsis lines carrying T-DNA insertions in the *AO* locus. One of them (SALK_013920, referred to as *fin4-2*; Fig. 1B) is embryo lethal in homozygosis (Katoh et al., 2006). Nevertheless, using another line

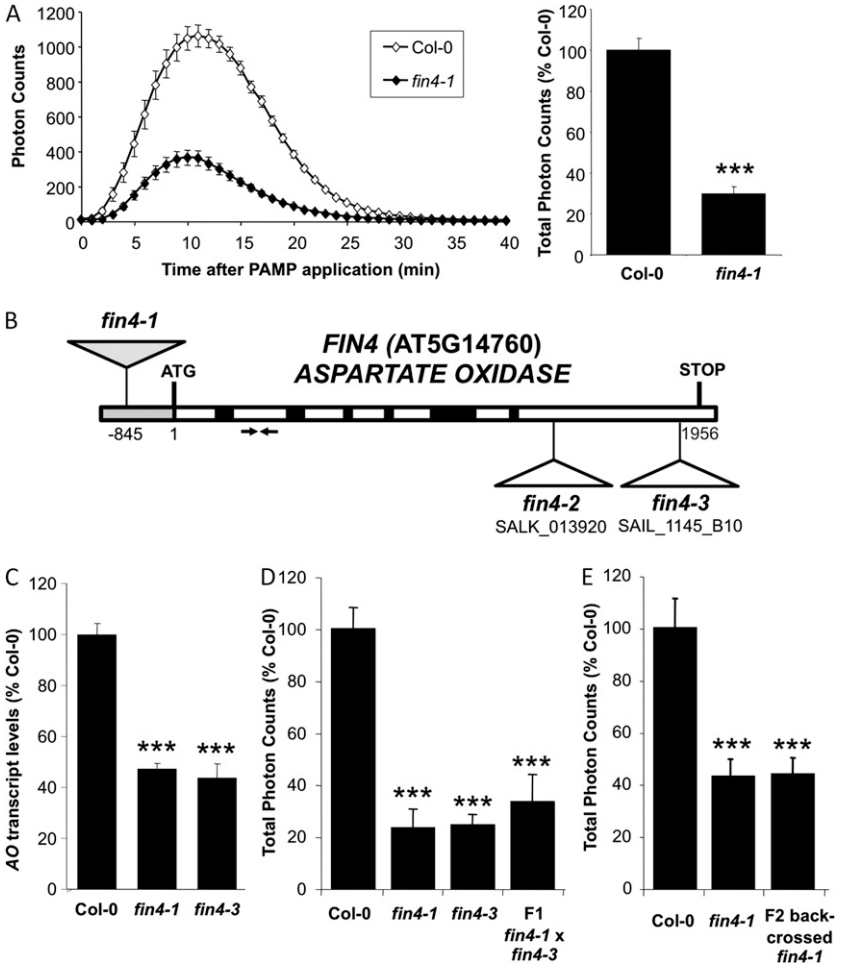

Figure 1. *fin4-1* carries a mutation in the *AO* gene. A, Oxidative burst triggered by 100 nM flg22 in wild-type Col-0 and *fin4-1* mutant plants measured in a luminol-based assay as relative light units. Values are averages ± SE ($n = 24$). Asterisks indicate significant differences compared with wild-type values at $P < 0.001$. B, Schematic representation of the *AO* gene (*At5G14760*) with positions of the *fin4-1*, *fin4-2* (SALK_013920), and *fin4-3* (SAIL_1145_B10) mutations. The gray box represents the promoter region, while the white and black boxes represent exons and introns, respectively. Numbers indicate nucleotide positions relative to the first nucleotide of the coding sequence. Arrows indicate primers used for qRT-PCR experiments. C, qRT-PCR analysis of *AO* expression in wild-type Col-0, *fin4-1*, and *fin4-3*. Values are averages ± SE ($n = 3$). Asterisks indicate significant differences compared with wild-type values at $P < 0.001$. D, Allelism test of the *fin4-1* and *fin4-3* mutants. Values correspond to the total amount of photons emitted in a luminol-based assay as relative light units during a 40-min period after treatment. Values are averages ± SE ($n = 24$). Asterisks indicate significant differences compared with wild-type values at $P < 0.001$. E, Oxidative bust triggered by 100 nM flg22 in wild-type Col-0, *fin4-1* mutant plants, and homozygous *fin4-1* mutant plants backcrossed to wild-type Col-0 (F2). Values correspond to the total amount of photons emitted in a luminol-based assay as relative light units during a 40-min period after treatment. Values are averages ± SE ($n = 12$). Asterisks indicate significant differences compared with wild-type values at $P < 0.001$. All experiments were performed at least twice with similar results.

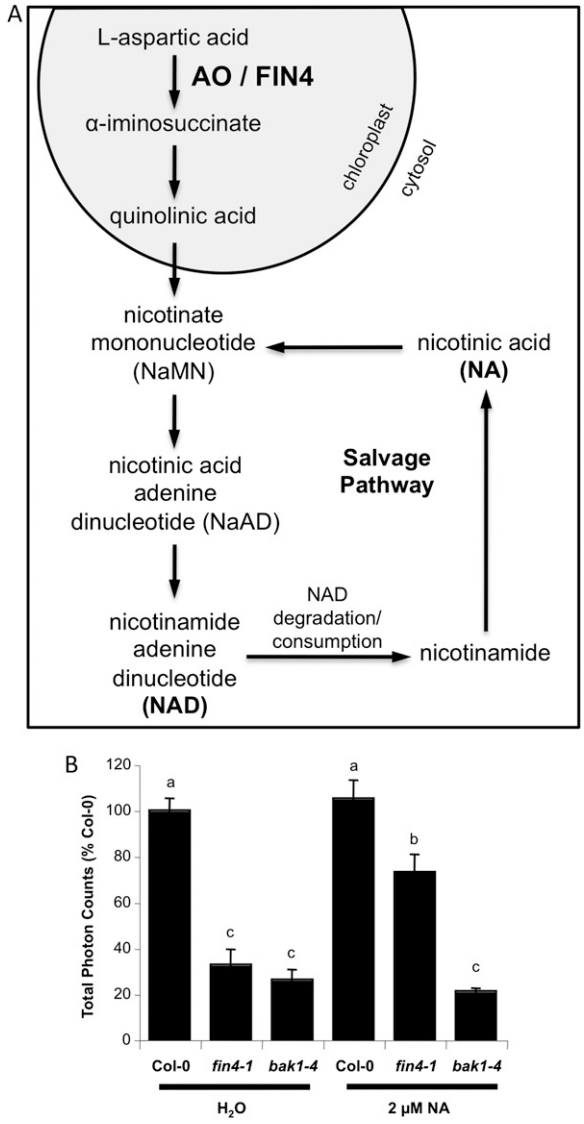

Figure 2. Exogenous NA complements the reduced flg22-triggered ROS burst in *fin4-1*. A, Schematic representation of the de novo NAD biosynthesis pathway. B, Oxidative burst triggered by 100 nM flg22 in wild-type Col-0, *fin4-1*, and *bak1-4* mutant plants with exogenous application of 2 μM NA or water (mock control) 24 h before elicitation. Values correspond to the total amount of photons emitted in a luminol-based assay as relative light units during a 40-min period after treatment. Values are averages ± SE (n = 24). Letters indicate significant differences at $P < 0.05$. All experiments were performed three times with similar results.

(SAIL_1145_B10, referred to as *fin4-3*; Fig. 1B), we were able to identify viable homozygous plants carrying a T-DNA insertion toward the 3′ end of the *AO* gene (Fig. 1B; Supplemental Fig. S1). Quantitative real-time (qRT)-PCR experiments showed that both *fin4-1* and *fin4-3* mutant plants show an approximately 45% reduction of *AO* transcript levels when compared with the wild type (Fig. 1C). Consistently, homozygous *fin4-3* plants showed a clear reduction in flg22-triggered ROS burst similar to that observed in *fin4-1* (Fig. 1D; illustrated here as the total amount of photons produced during 40 min after treatment), indicating that mutations in the *AO* locus cause the *fin* phenotype. Additionally, an allelism test between *fin4-1* and *fin4-3* performed in the F1 confirmed that the *fin* phenotype was linked to mutations at the *AO* locus (Fig. 1D). To further confirm that the mutation in the *AO* gene was the actual cause of the ROS-burst phenotype of the originally isolated mutant and to completely reject the possibility that the *fin* phenotype is caused by second-site mutations, we performed a backcross with Col-0 wild-type plants and identified homozygous *fin4-1* mutants in the F2, which showed a clear reduction in flg22-triggered ROS bursts (Fig. 1E). These results confirmed that the reduced flg22-induced ROS burst observed in *fin4-1* and *fin4-3* was linked to mutations in the *AO* gene.

Exogenous Nicotinic Acid Partially Complements the flg22-Triggered ROS Burst in *fin4-1*

NAD is an essential cofactor in energy metabolism and electron transfer. In plants, de novo biosynthesis of NAD starts with the oxidation of L-Asp to α-iminosuccinate, catalyzed by AO (Katoh et al., 2006). Subsequent reactions are catalyzed by quinolinate synthase and quinolate phosphoribosyltransferase (QPT) and generate nicotinate mononucleotide, which undergoes additional enzymatic steps to generate NAD (Fig. 2A; Katoh et al., 2006). Therefore, a likely hypothesis was that the impaired flg22-induced ROS burst is due to reduced NADH levels in *fin4-1*. We tested if treatment of the *fin4-1* mutant with nicotinic acid (NA), which can be converted into a substrate of the NAD biosynthesis pathway downstream of the step catalyzed by AO (Zheng et al., 2004, 2005; Wang and Pichersky, 2007), could complement the *fin4* phenotype. While exogenous pretreatment with 2 μM NA for 24 h had no effect on the ROS burst triggered by 100 nM flg22 in the Col-0 wild type or *bak1-4*, it significantly increased the flg22-triggered ROS burst in *fin4-1* when compared with the water control treatment (Fig. 2B). These results suggest that the reduced flg22-induced ROS burst of the *fin4-1* mutant is caused by deficient AO activity and subsequent NAD availability.

AO Is Required for the PAMP-Induced RBOHD-Dependent ROS Burst and Stomatal Closure

Next, we tested if AO activity was generally required for the PAMP-induced ROS burst in Arabidopsis. In addition to the perception of bacterial flagellin (or its surrogate peptide flg22) by FLS2, Arabidopsis also perceives other microbial PAMPs. The bacterial elongation factor Tu (or its surrogate peptide elf18) is perceived by the LRR-RK EFR (Zipfel et al., 2006), while the perception of fungal chitin depends on

the LysM-RK CERK1 (Miya et al., 2007; Wan et al., 2008). As in the case of flg22, the perception of elf18 or chitin triggers a rapid ROS burst in an RBOHD-dependent manner (Nühse et al., 2007; Zhang et al., 2007; Ranf et al., 2011; Segonzac et al., 2011). Treatment of *fin4-1* and *fin4-3* mutant plants with 100 nM elf18 or 1 mg mL^{-1} chitin led to the production of a ROS burst that was significantly reduced when compared with wild-type plants (Fig. 3). The reduction observed in both *fin4* mutant alleles after elf18 or chitin treatment was similar to that observed after flg22 treatment (Figs. 1, A, D, and E, and 3). These results demonstrate that AO is required for the ROS burst triggered by unrelated PAMPs perceived by different surface-localized PRRs.

Given the function of AO in the RBOHD-mediated ROS burst, we tested if AO is required for additional RBOHD-dependent responses triggered by PAMPs. The production of ROS by RBOHD is required for PAMP-induced stomatal closure (Mersmann et al., 2010; Fig. 4). Treatment of wild-type adult leaves with 5 μM flg22 or elf18 for 1 h induced a significant reduction in the stomatal aperture (Fig. 4). In contrast, *fin4-1* and *fin4-3* mutant leaves were unable to close stomata when treated with flg22 or elf18, similar to *rbohD* mutant leaves (Fig. 4; Supplemental Fig. S2A), demonstrating the biological relevance of the previously observed reduction in ROS bursts.

AO Is Not Required for RBOHD-Independent flg22 Responses

PAMP-induced responses divide into two distinct branches downstream of receptor activation and the early Ca^{2+} burst: one branch leads to the RBOHD-dependent ROS burst, while the other leads to the activation of MAPK and CDPK cascades and transcriptional changes (Galletti et al., 2008, 2011; Lu et al., 2009; Ranf et al., 2011; Segonzac et al., 2011). Notably, the ROS burst triggered by RBOHD is not required for MAPK activation (Ranf et al., 2011; Segonzac et al., 2011). Given the role of NAD in cellular signaling (Berger et al., 2004; Koch-Nolte et al., 2009, 2011), we sought to determine if AO is acting upstream or independently of RBOHD and, therefore, if it is required for RBOHD-independent PTI signaling events.

Treatment of Arabidopsis wild-type 2-week-old seedlings with 1 μM flg22 results in the activation of MAPKs within 5 min (Fig. 5A). A similar activation was observed in *fin4-1*, *fin4-3*, and *rbohD* seedlings (Fig. 5A). Within minutes, flg22 treatment of Arabidopsis seedlings induced massive transcriptional changes (Zipfel et al., 2004). Accordingly, a drastic increase in transcript levels for the marker genes *WRKY22*, *SIRK/FRK1*, and *NHL10* can be observed within 60 min of treatment with 100 nM flg22 in wild-type seedlings (Fig. 5B). Treatment of *fin4-1* or *rbohD* seedlings led to a similar induction of these flg22-induced transcripts (Fig. 5B). Together, these results revealed that AO is not required for flg22-induced MAPK activation and induction of the selected marker genes.

A late response associated with the activation of PTI by flg22 (and elf18) is the inhibition of seedling growth (Gómez-Gómez et al., 1999; Zipfel et al., 2006). The growth inhibition triggered by a range of flg22 concentrations (20–100 nM) was comparable in wild-type, *fin4-1*, and *rbohD* seedlings (Fig. 5C). Altogether, these results indicate that AO is not required for RBOHD-independent early and late responses to flg22.

AO Is Not Required for RBOHF or RBOHC Function

Different members of the RBOH family are specialized in cell growth, plant development, or responses to abiotic stresses and biotic interactions (for review, see Marino et al., 2012). RBOH-dependent ROS production is essential for multiple cellular functions, and we wanted to test if AO deficiency also affects the functions of RBOHs other than RBOHD.

The hormone ABA is produced during osmotic stress and induces ROS bursts and stomatal closure in an RBOHD/RBOHF-dependent manner (Kwak et al., 2003; Bright et al., 2006), although it has been recently shown that RBOHF plays a predominant role in ABA-induced stomatal closure (Zhang and Mou, 2009). To test if *fin4* affects ABA-induced stomatal closure, we compared the stomatal aperture of wild-type, *fin4-1*, *rbohD*, and *rbohD rbohF* leaves after treatment with 5 μM ABA for 1 h. Interestingly, all genotypes responded similarly to ABA, with the exception of *rbohD rbohF* (Fig. 6).

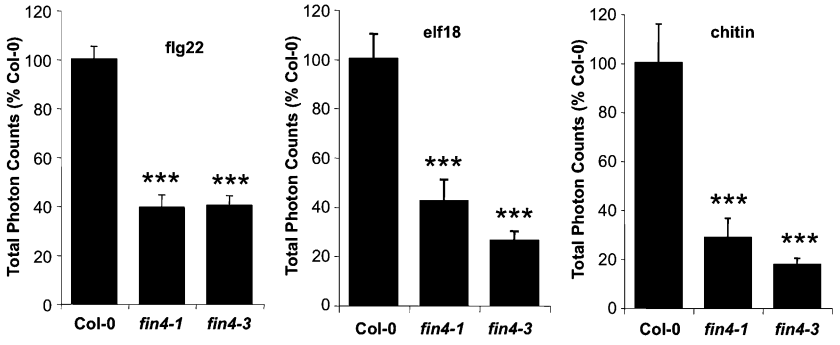

Figure 3. AO is required for elf18 and chitin-induced ROS. Oxidative burst was triggered by 100 nM flg22, 100 nM elf18, or 1 mg mL^{-1} chitin in wild-type Col-0, *fin4-1*, and *fin4-3* mutant plants. Values correspond to the total amount of photons emitted in a luminol-based assay as relative light units during a 40-min period after treatment. Values are averages ± SE ($n = 24$). Asterisks indicate significant differences compared with wild-type values at $P < 0.001$. All experiments were performed three times with similar results.

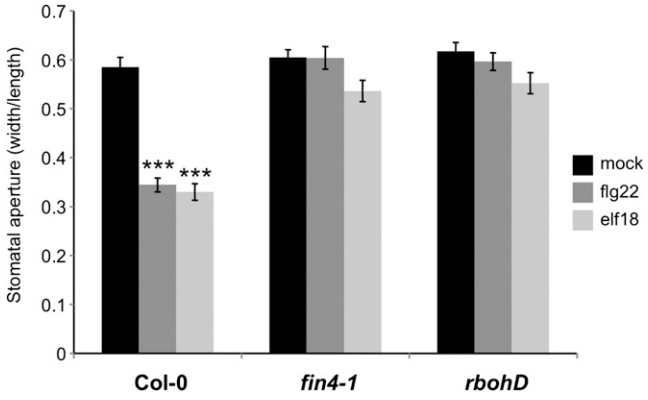

Figure 4. AO is required for PAMP-induced stomatal closure. Stomatal aperture is shown for wild-type Col-0, *fin4-1*, and *rbohD* mutant adult leaves, measured 1 h after mock, 5 μM flg22, or 5 μM elf18 treatment. Values are averages ± SE (n = 26). Asterisks indicate significant differences compared with mock-treated samples at $P < 0.001$. All experiments were performed four times with similar results.

ROS have also been shown to regulate cell growth and the elongation of root hairs in an RBOHC/RHD2-dependent manner (Foreman et al., 2003). Accordingly, *rbohC* mutant plants display impaired root hair elongation (Foreman et al., 2003). When we compared root hair elongation in wild-type, *fin4-1*, and *rbohD* seedlings, we observed that root hairs developed normally in the *fin4-1* and *rbohD* mutants (Fig. 7).

Together, these results confirmed that ABA-induced stomatal closure and root hair elongation are RBOHD independent and revealed that reduced AO levels do not affect RBOHF-mediated ABA-induced stomatal closure and RBOHC-mediated root hair elongation.

AO Is Required for Stomatal Immunity

Given that AO is required for flg22-induced stomatal closure (Fig. 4) and that flg22 perception by FLS2 plays an essential role in the stomatal response triggered by *Pto* DC3000 in Arabidopsis (Zeng and He, 2010), we sought to determine if the *fin4* mutant is impaired in stomatal immunity to *Pto* DC3000. COR is a bacterial toxin that contributes to stomatal reopening and invasion of the apoplast by *Pto* DC3000 (Melotto et al., 2006). Thus, *COR*$^-$ *Pto* DC3000 mutants are less virulent on wild-type plants when surface inoculated (Melotto et al., 2006) but have a partially restored virulence on mutant plants affected in flagellin perception or in PAMP-induced stomatal closure (Melotto et al., 2006; Liu et al., 2009; Nekrasov et al., 2009; Zeng and He, 2010; Zeng et al., 2011; Desclos-Theveniau et al., 2012; Singh et al., 2012). Interestingly, *fin4-1* and *fin4-3* mutant plants were significantly impaired in stomatal closure and more susceptible than wild-type plants after surface inoculation with *Pto* DC3000 *COR*$^-$ (Fig. 8; Supplemental Fig. S2). Surprisingly, despite being impaired in flg22-induced stomatal closure, *rbohD* mutant plants were not more susceptible to *Pto*

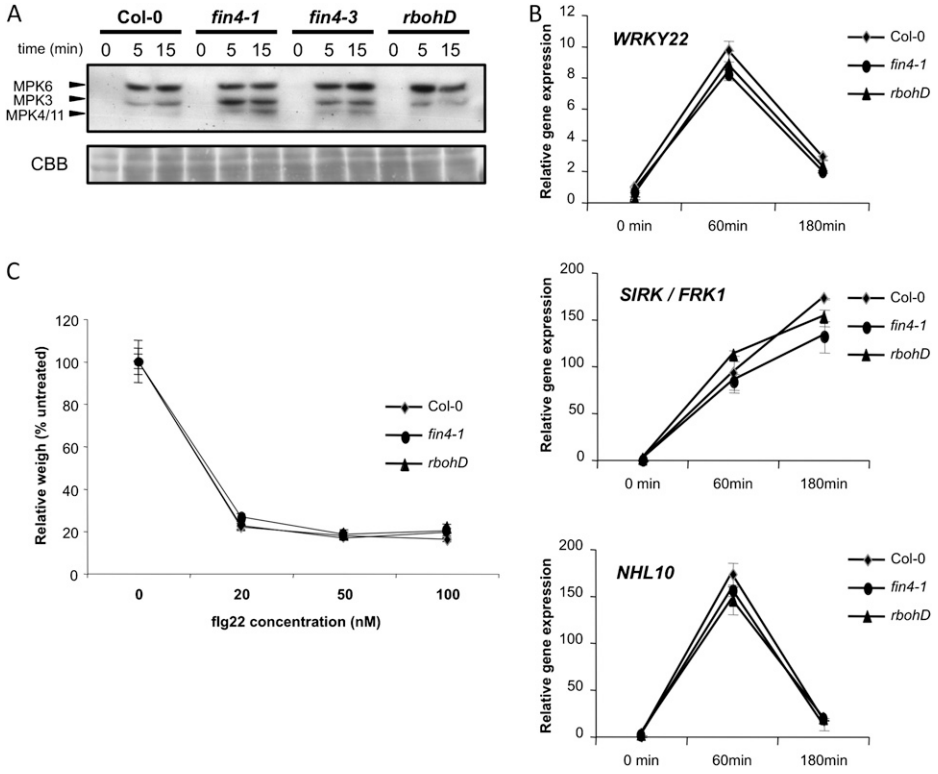

Figure 5. AO is not required for RBOHD-independent flg22 responses. A, MAPK activation assay in wild-type Col-0, *fin4-1*, *fin4-3*, and *rbohD* seedlings after treatment with 1 μM flg22. CBB, Coomassie Brilliant Blue. B, qRT-PCR analysis of PAMP-induced transcripts in wild-type Col-0, *fin4-1*, and *rbohD* seedlings after treatment with 100 nM flg22. Gene expression values are relative to the *U-box* housekeeping gene (At5g15400) and are normalized to untreated Col-0 plants. Values are averages ± SE (n = 3). C, Growth inhibition of wild-type Col-0, *fin4-1*, *fin4-3*, and *rbohD* seedlings treated with different concentrations of flg22 for 8 d. Values are averages ± SE (n = 12). All experiments were performed three times with similar results.

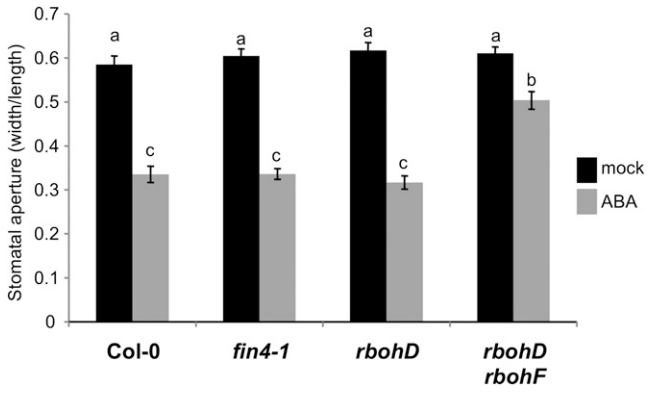

Figure 6. AO is not required for RBOHF function in ABA-induced stomatal closure. Stomatal aperture is shown for wild-type Col-0, *fin4-1*, *rbohD*, and *rbohD rbohF* leaves measured 1 h after mock or 5 μM ABA treatment. Values are averages ± SE (n = 26). Different letters indicate significantly different values at $P < 0.05$. All experiments were performed four times with similar results.

DC3000 *COR*$^-$ than wild-type plants (Fig. 8B). Hence, AO is required for stomatal immunity to *Pto* DC3000.

DISCUSSION

Our genetic screen for *fin* mutants has proven successful for the identification of novel components required for PTI. We have previously documented a direct role for ethylene signaling in the regulation of *FLS2* transcription (Boutrot et al., 2010). In this study, we revealed a requirement for the chloroplastic AO enzyme, which is involved in NAD metabolism, in PAMP-triggered ROS bursts, and in stomatal immunity.

NAD is an essential cofactor in energy metabolism and electron transfer. Additionally, NAD and its metabolites also function in cell signaling pathways, contributing to ion fluxes and protein modifications, such as ADP ribosylation and NAD-dependent protein deacetylation (Berger et al., 2004; Koch-Nolte et al., 2009, 2011). Additionally, several reports suggest that NAD may be involved in plant defense responses (Dutilleul et al., 2003, 2005; Zhang and Mou, 2009; Djebbar et al., 2012; Pétriacq et al., 2012). However, despite the relevance of NAD in plant signaling, genetic studies on the role of NAD have been hindered so far by the lethality of knockout mutants in the chloroplastic enzymes AO, quinolinate synthase, and QPT, involved in the first steps of de novo NAD biosynthesis (Katoh et al., 2006). Therefore, studies on the role of NAD in plant biological processes have so far relied on exogenous application of NAD or NADP (Zhang and Mou, 2009) or inducible overproduction of NAD in planta based on the overexpression of a QPT homolog, *nadC*, from *Escherichia coli* (Pétriacq et al., 2012).

In this work, we report that *FIN4* encodes AO, and we found that two alleles, *fin4-1* and *fin4-3*, correspond to knockdown mutants (Fig. 1, A and B). Unlike the previously described knockout *AO* allele (referred to as *fin4-2* in this work; Katoh et al., 2006), *fin4-1* and *fin4-3* are viable and fertile plants and displayed no obvious developmental phenotypes other than a small reduction in size when compared with wild-type plants (Supplemental Fig. S1). Therefore, the analysis of these novel alleles provides a unique opportunity to study the importance of de novo NAD biosynthesis for several cellular processes, including innate immunity. First, we found that AO is required for RBOHD-dependent ROS burst triggered by the perception of several unrelated PAMPs that are perceived by distinct PRRs in Arabidopsis (Figs. 1, A, D, and E, and 3). The reduced PAMP-triggered ROS burst could be attributed to deficient NAD levels, as a flg22-induced ROS burst could be partially complemented in *fin4-1* by exogenous application of NA (Fig. 2), which can be converted into a substrate of the NAD biosynthesis pathway downstream of the step catalyzed by the AO (Zheng et al., 2004, 2005; Wang and Pichersky, 2007).

In addition to the PAMP-induced ROS burst, *fin4-1* was also completely impaired in PAMP-triggered stomatal closure (Fig. 4), a response that also depends on RBOHD (Mersmann et al., 2010; Fig. 4). Notably, other flg22-induced responses that do not depend on RBOHD, such as MAPK activation, accumulation of marker gene transcripts, or seedling growth inhibition, were not affected in *fin4-1* seedlings (Fig. 3). This is

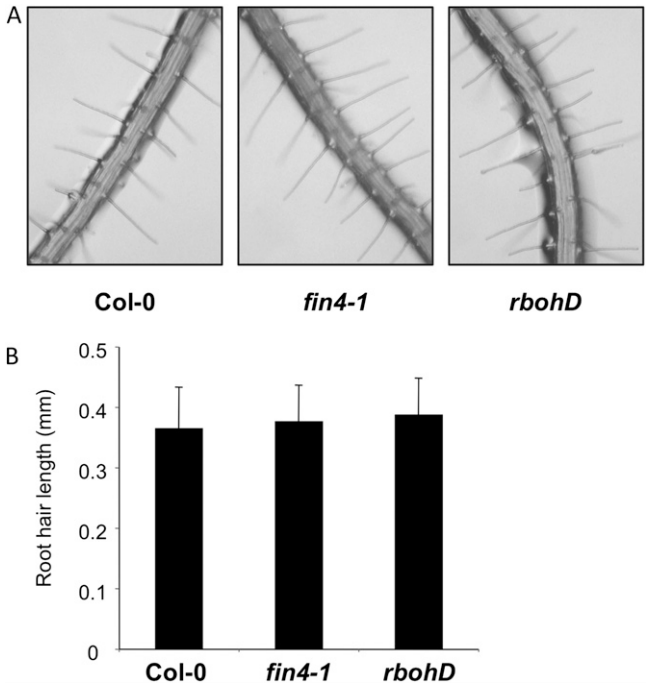

Figure 7. AO is not required for RBOHC function in root hair elongation. A, Photographs of root hairs in wild-type Col-0, *fin4-1*, and *rbohD* seedlings. B, Root hair length measurements. Values are averages ± SE (n = 26). All experiments were performed three times with similar results.

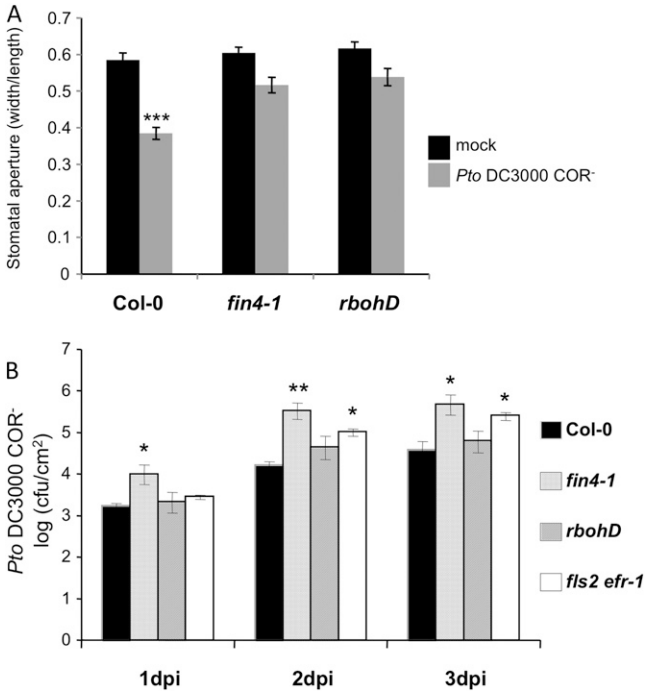

Figure 8. AO is required for stomatal immunity. A, Stomatal aperture of wild-type Col-0, *fin4-1*, and *rbohD* leaves, measured 1 h after mock treatment or inoculation with *Pto* DC3000 *COR*$^-$ (OD_{600} = 0.1). Values are averages ± SE (n = 26). Asterisks indicate significant differences compared with mock-treated wild-type values at $P < 0.001$ (***). B, Growth of surface-inoculated *Pto* DC3000 *COR*$^-$ (OD_{600} = 0.1) in wild-type Col-0, *fin4-1*, *rbohD*, and *fls2 efr-1* mutant plants 1, 2, or 3 d post inoculation (dpi). Values are averages ± SE (n = 4). Asterisks indicate significant differences compared with wild-type values at $P < 0.05$ (*) or $P < 0.01$ (**). cfu, Colony-forming units. All experiments were performed three times with similar results.

consistent with previous reports that PTI signaling undergoes an early branching downstream of PRR activation and that RBOHD is not required for MAPK activation and the expression of early PAMP-induced genes (Galletti et al., 2008; Lu et al., 2009; Ranf et al., 2011; Segonzac et al., 2011). Importantly, these results also illustrate that *fin4-1* is not generally affected in PAMP responses.

RBOHs are plant NADPH oxidases that utilize NADPH as a cytosolic electron donor to generate superoxide from oxygen in the apoplast. As we could only observe an impairment of RBOHD-dependent PAMP-induced responses in *fin4-1*, we wondered if the NAD deficiency associated with reduced AO levels was not generally affecting RBOH functions in general. To our surprise, we found that *fin4-1* was not affected in root hair elongation, which is RBOHC/RHD2 dependent (Foreman et al., 2003), or in ABA-induced stomatal closure, which is mostly RBOHF dependent (Kwak et al., 2003; Bright et al., 2006; Zhang and Mou, 2009; Figs. 6 and 7). Therefore, it may be possible that RBOHD-dependent ROS bursts and stomatal closure are more sensitive to a scarcity of redox resources in comparison with other cellular functions mediated by other members of the RBOH family.

The major evidence for an important role of AO in plant immunity comes from the observation that *fin4-1* mutant plants are more susceptible to spray inoculation with *Pto* DC3000 *COR*$^-$ (Fig. 8). Notably, a recent study using transgenic overexpression of the bacterial QPT homolog *nadC* from *E. coli* in Arabidopsis has shown that inducible NAD overproduction leads to increased SA levels and resistance to *Pto* DC3000 expressing the effector AvrRpm1 (Pétriacq et al., 2012). Surprisingly, the enhanced SA levels associated with the inducible overaccumulation of NAD in the *nadC* plants did not increase the basal resistance to wild-type *Pto* DC3000 (Pétriacq et al., 2012). Conversely, we tested if a deficient NAD synthesis pathway in the *fin4-1* mutant would correlate with an increased susceptibility to bacteria. However, we did not detect an increased susceptibility of *fin4-1* plants upon infection with bacterial strains other than *Pto* DC3000 *COR*$^-$, such as wild-type *Pto* DC3000 or *Pto* DC3000 *(avrRpm1)* (Supplemental Fig. S3). These results suggest that the inability of the *fin4-1* mutant to trigger PAMP-induced stomatal closure is the major factor causing the increased susceptibility toward *Pto* DC3000 *COR*$^-$.

Interestingly, the *rbohD* mutant, which is also deficient in PAMP-triggered stomatal closure (Mersmann et al., 2010; Figs. 4 and 8A), did not display increased susceptibility to *Pto* DC3000 *COR*$^-$ in our surface inoculation assays (Fig. 8). This result is in agreement with several previous reports that failed to demonstrate a clear role for RBOHD-generated ROS in resistance to *P. syringae* (Torres et al., 2002; Zhang et al., 2007; Mersmann et al., 2010; Segonzac et al., 2011; Chaouch et al., 2012). This may be explained by the recent observation that *rbohD* mutant plants accumulate higher levels of antimicrobial compounds (such as camalexin and scopoletin) than wild-type plants upon inoculation with *Pto* DC3000 (Chaouch et al., 2012), which may compensate for the initial increased bacterial entry through open stomata in this mutant. In contrast, in the same study, *rbohF* mutant plants were shown to be more susceptible to *Pto* DC3000 (Chaouch et al., 2012).

fin4-1 leaves are completely blocked in their ability to close their stomata in response to flg22 or elf18 (Fig. 4), despite being only partially affected in their PAMP-induced ROS production (Figs. 1 and 3). This suggests that a threshold of ROS levels is required to trigger stomatal closure and/or that AO plays additional roles in stomatal closure independently of RBOHD-generated ROS. NAD is a substrate for mono- and poly-ADP-ribosylation catalyzed by poly-ADP-ribose polymerases (Vanderauwera et al., 2007), and poly-ADP-ribosylation has been previously linked to PAMP-induced responses (Briggs and Bent, 2011). However, inhibition of poly-ADP-ribosylation did not affect the flg22-induced ROS burst (Adams-Phillips et al., 2010), suggesting that the defects of *fin4-1* leaves in PAMP-induced ROS

bursts and stomatal closure are not linked to defects in poly-ADP-ribosylation.

Interestingly, both the ATP-binding cassette transporter SCORD5/AtGNC20/AtABCF3 and the L-type lectin receptor kinase LecRK-VI.2 are required for PAMP-induced stomatal closure and for stomatal immunity to *Pto* DC3000 *COR*$^-$, while they are dispensable to ABA-induced stomatal closure (Zeng et al., 2011; Singh et al., 2012). In that sense, their mutants share the same phenotypes as *fin4-1*. However, unlike *fin4-1*, *scord5* and *lecrk-vi.2* mutants are not affected in PAMP-induced ROS burst (Zeng et al., 2011; Singh et al., 2012). These recent results, including ours, further illustrate the complexity underlying stomatal immunity and point to the AO activity as a novel additional layer of the specific regulation of PAMP-induced stomatal immunity.

Intriguingly, our demonstration that NAD metabolism significantly contributes to immunity echoes recent reports illustrating the negative regulation of immunity by Nudix hydrolases that use NAD derivatives as substrates. Notably, AtNUDT7 is induced by pathogens and the null AtNUDT7 mutant is more resistant to *Pto* DC3000 and to the oomycete *Hyaloperonospora arabidopsidis*, suggesting that it is a negative regulator of immunity (Bartsch et al., 2006; Ge et al., 2007). Similarly, the effector Avr3b from the oomycete pathogen *Phytophthora sojae* carries functional Nudix hydrolase motifs that are required for the suppression of PTI by this pathogen (Dong et al., 2011). A similar strategy to target NAD metabolism and subsequent NAD-derived metabolites may be used by other pathogens, as, for example, the genome of the pathogenic bacterium *Ralstonia solanacearum* encodes several type III-secreted effectors that carry a Nudix motif (Tamura et al., 2005).

The availability of the viable mutants *fin4-1* and *fin4-3* in the chloroplastic enzyme AO should allow future detailed studies on the role of NAD metabolism in different cellular processes, including immunity, in Arabidopsis.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) Col-0 was used as the wild-type control. Arabidopsis plants used in this study were grown as one plant per pot at 20°C to 21°C with a 10-h photoperiod in environmentally controlled chambers or on plates containing Murashige and Skoog (MS) medium (including vitamins; Duchefa) and 1% sucrose supplemented with 0.8% agar the first 5 d at 22°C and with a 16-h photoperiod. The original *fin4* mutant line (*fin4-1*) belongs to the first set of 10,000 T-DNA SALK lines (Alonso et al., 2003) screened by Boutrot et al. (2010). The *fin4-3* T-DNA insertion line (SAIL_1145_B10) was obtained from the Nottingham Arabidopsis Stock Centre. Other mutant lines used in this study are *rbohD* (Torres et al., 2002), *rbohD rbohF* (Torres et al., 2002), and *fls2c efr-1* (Nekrasov et al., 2009).

Identification of the *fin4-1* Mutant

To sequence the T-DNA junctions in *fin4-1*, we followed the original protocol for the adapter ligation-mediated PCR method (O'Malley et al., 2007). The sequencing of the obtained fragments (of 715 and 250 bp from the left and right borders, respectively) identified a T-DNA insertion in At5g14760 starting 843 bp upstream of the ATG. The following specific PCR primers were used to confirm the T-DNA positions: FIN4LP (5′-GAAGCCACCACTTACAAATCAA-3′) and FIN4RP (5′-GAGCTCTCTGATCTAAGCACGA-3′).

Chemicals

The flg22 and elf18 peptides were purchased from Peptron, and chitin oligosaccharide was purchased from Yaizu Suisankagaku Industry. NA and ABA were purchased from Sigma-Aldrich.

Measurement of ROS Generation

Oxidative burst measurement was performed as described previously (Gimenez-Ibanez et al., 2009a). ROS were elicited with flg22 (100 nM), elf18 (100 nM), or chitin (1 mg mL^{-1}). Twenty-four leaf discs from 12 5-week-old plants were used for each condition. Luminescence was measured over 40 min using the High-Resolution Photon Counting System (HRPCS218; Photek) coupled to an aspherical wide lens (Sigma). For NA supplementation, leaf discs were incubated in presence of NA (2 μM) throughout the experiment.

Stomatal Aperture Measurements

Leaf discs from 4- to 5-week-old plants were exposed to white light for 2 h while submerged in a solution containing 50 mM KCl, 10 μM $CaCl_2$, 0.01% Tween 20, and 10 mM MES-KOH, pH 6.15, to induce stomatal aperture. Subsequently, 5 μM ABA, 5 μM flg22, 5 μM elf18, *Pto* DC3000 *COR*$^-$ (optical density at 600 nm [OD_{600}] = 0.1), or mock solution was added to the buffer, and the samples were incubated under the same conditions for 1 h. Abaxial leaf surfaces were observed with a microscope (Leica DMR), and stomatal aperture was measured using ImageJ software.

MAPK Activation

MAPK assays were performed on six 2-week-old seedlings grown in liquid medium. Seedlings were then elicited with 1 μM flg22 for 5 or 15 min and frozen in liquid nitrogen. MAPK activation was monitored by western blot with antibodies that recognize the dual phosphorylation of the activation loop of MAPK (pTEpY). Phospho-p44/42 MAPK (Erk1/2; Thr-202/Tyr-204) rabbit monoclonal antibodies from Cell Signaling were used according to the manufacturer's protocol. Blots were stained with Coomassie Brilliant Blue to verify equal loading.

RNA Isolation and qRT-PCR

Gene expression assays were performed on four 2-week-old seedlings grown in liquid medium. Seedlings were then elicited with 100 nM flg22 for 60 or 180 min and frozen in liquid nitrogen. Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA samples were treated with Turbo DNA-free DNase (Ambion) and quantified with a Nanodrop spectrophotometer (Thermo Scientific). Complementary DNA synthesis and qRT-PCR were realized as described by Boutrot et al. (2010). Primers used for quantitative PCR are as follows: *U-box* (At5g15400) forward, 5′-TGCGCTGCCAGATAATACACTATT-3′, and reverse, 5′-TGCTGCCCAACATCAGGTT-3′ (Nemhauser et al., 2004); *AO* (At5g14760) forward, 5′-TGCAAAGCAAGGAACAGTCG-3′, and reverse, 5′-CCAAAGGGCATAACACAGCA-3′; *SIRK/FRK1* (At2g19190) forward, 5′-ATCTTCGCTTGGAGCTTCTC-3′, and reverse, 5′-TGCAGCGCAAGGACTAGAG-3′ (He et al., 2006); *WRKY22* (At4g01250) forward, 5′-GATCATCTAGCGGTGGGAGA-3′, and reverse, 5′-TATTCCTCCGGTGGTAGTGG-3′; and *NHL10* (At2g35980) forward, 5′-TTCCTGTCCGTAACCCAAAC-3′, and reverse, 5′-CCCTCGTAGTAGGCATGAGC-3′ (Boudsocq et al., 2010).

Seedling Growth Inhibition Assay

Seedling growth inhibition was assessed as described previously (Nekrasov et al., 2009). In brief, 5-d-old Arabidopsis seedlings were grown in liquid MS medium containing 1% sucrose supplemented with flg22. Seedlings were weighted 8 d after treatment.

Root Hair Length Measurements

Root hair length measurements were performed as described by Foreman et al. (2003). Arabidopsis seedlings were grown on MS medium solidified with 0.5% (w/v) Phytagel at 24°C under continuous illumination. The plates were inclined at an angle of 80° to allow the roots to grow along the surface of the medium. Five-day-old seedlings were observed and photographed with a dissecting binocular microscope, and measurements were done using ImageJ software.

Bacterial Infection Assays

The *Pseudomonas syringae* pv *tomato* (*Pto*) DC3000 (Cuppels, 1986) and *Pto* DC3000 *COR*$^-$ (Brooks et al., 2004) strains were grown in overnight culture in Luria-Bertani medium supplemented with appropriate antibiotics. Cells were harvested by centrifugation, and pellets were resuspended in 10 mM $MgCl_2$ to an OD_{600} of 0.1. Immediately prior to spraying, Silwet L-77 was added to bacteria to 0.02% (v/v). Bacteria were sprayed onto leaf surfaces, and plants were maintained uncovered. For syringe inoculation of *Pto* DC3000 expressing the heterologous effector AvrRpm1 (Bisgrove et al., 1994), bacteria were similarly grown and harvested. Cell pellets were resuspended in 10 mM $MgCl_2$ to an OD_{600} of 0.0002 and infiltrated using a needleless syringe into three leaves each of four plants per genotype. Samples were taken 1, 2, or 3 d postinoculation using a cork borer (0.38 cm^2) to cut leaf discs from three leaves per plant and four plants per genotype. Leaf discs were ground in 10 mM $MgCl_2$, diluted, and plated on Luria-Bertani agar with appropriate selection. Plates were incubated at 28°C and colonies were counted 2 d later.

Statistical Analysis

Statistical significances based on *t* test and one-way ANOVA were determined with Prism 5.01 software (GraphPad Software).

Supplemental Data

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

Supplemental Figure S1. *fin4-1* and *fin4-3* homozygous mutants are viable plants.

Supplemental Figure S2. The independent allele *fin4-3* is also impaired in stomatal immunity.

Supplemental Figure S3. *fin4-1* plants are not more susceptible to *Pto* DC3000 or *Pto* DC3000 expressing AvrRpm1.

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