Ethylene Signaling Regulates Accumulation of the FLS2 Receptor and Is Required for the Oxidative Burst Contributing to Plant Immunity[1][W]

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Reactive oxygen species (ROS) are potent signal molecules rapidly generated in response to stress. Detection of pathogen-associated molecular patterns induces a transient apoplastic ROS through the function of the NADPH respiratory burst oxidase homologs D (RbohD). However, little is known about the regulation of pathogen-associated molecular pattern-elicited ROS or its role in plant immunity. We investigated ROS production triggered by bacterial flagellin (flg22) in Arabidopsis (*Arabidopsis thaliana*). The oxidative burst was diminished in ethylene-insensitive mutants. Flagellin Sensitive2 (FLS2) accumulation was reduced in *etr1* and *ein2*, indicating a requirement of ethylene signaling for FLS2 expression. Multiplication of virulent bacteria was enhanced in Arabidopsis lines displaying altered ROS production at early but not late stages of infection, suggesting an impairment of preinvasive immunity. Stomatal closure, a mechanism used to reduce bacterial entry into plant tissues, was abolished in *etr1*, *ein2*, and *rbohD* mutants. These results point to the importance of flg22-triggered ROS at an early stage of the plant immune response.

A rapid and transient increase in reactive oxygen species (ROS), termed an "oxidative burst," is often associated with responses to abiotic and biotic stresses and could trigger changes in stomatal aperture or programmed cell death in defense against pathogens (Kwak et al., 2003; Torres and Dangl, 2005). ROS production can occur extracellularly through activities of plasma membrane-resident NADPH oxidases (Kangasjärvi et al., 2005; Torres and Dangl, 2005). In plants, Rboh proteins, which are homologs of mammalian NADPH oxidase 2, were shown to be the predominant mediators of apoplastic ROS production (Torres et al., 1998; Galletti et al., 2008). Respiratory burst oxidase homologs D and F (RbohD and RbohF) were identified by mutation to be the responsible oxidases in Arabidopsis (*Arabidopsis thaliana*) defense responses (Torres et al., 2002). While most ROS generated in response to avirulent *Pseudomonas syringae* bacteria and *Hyaloperonospora* oomycete pathogens depend on RbohD function, the induced cell death response by these pathogens appears to be mostly regulated by RbohF. Cell death provoked upon infection with the necrotizing fungus *Alternaria*, however, is under the control of RbohD (Pogány et al., 2009). The contribution of NADPH oxidases to plant immunity was also described in barley (*Hordeum vulgare*) and tobacco (*Nicotiana benthamiana*), where resistance to powdery mildew fungi and the oomycete *Phytophthora infestans*, respectively, was dependent on Rboh functions (Yoshioka et al., 2003; Trujillo et al., 2006).

An early layer of active plant defense is mediated by pattern recognition receptors, which sense microbes according to conserved constituents, so-called pathogen-associated molecular patterns (PAMPs). These initiate a plethora of defense responses referred to as PAMP-triggered immunity (Boller and Felix, 2009). The Arabidopsis receptor kinase Flagellin Sensitive2 (FLS2) recognizes and physically interacts with flg22, the elicitor-active epitope of bacterial flagellin (Felix et al., 1999; Gomez-Gomez and Boller, 2000; Chinchilla et al., 2006). FLS2 is plasma membrane localized and expressed throughout the plant (Robatzek et al., 2006). FLS2 requires the receptor kinase BRI1-Associated Kinase1 (BAK1), which forms a heteromeric complex upon flg22 binding (Chinchilla et al., 2007). Subsequently, a rapid and transient flg22-stimulated oxidative burst occurs that is dependent on RbohD (Zhang et al., 2007). In addition, flg22 triggers early responses, such as ethylene biosynthesis, activation of mitogen-activated protein (MAP) kinase cascades, and changes in gene expression (Felix et al., 1999; Asai et al., 2002; Zipfel et al., 2004). Late flg22 responses include the accumulation of salicylic acid (SA), callose deposition, and an arrest of seedling growth (Gomez-Gomez et al., 1999; Mischina and Zeier, 2007). This collectively contributes to plant immunity (Zipfel et al., 2004; Melotto et al., 2006).

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Little is known about the regulatory components of FLS2-activated early flg22 responses and their relevance in plant resistance to pathogens. Here, we investigated flg22-triggered ROS production in Arabidopsis seedlings and have identified ethylene signaling as a critical component of the oxidative burst in response to flg22, partly through promoting the accumulation of FLS2. We further provide evidence that the flg22-triggered oxidative burst is required for resistance to bacterial infection at the point of pathogen entry through stomata.

RESULTS

ROS Production in Response to Flg22 Is Diminished in Ethylene-Insensitive Mutants

Although flg22-induced resistance to bacterial infection was reported to be independent of single hormones known to play key roles in plant defense (Zipfel et al., 2004), recent studies support a role for each of the SA, ethylene, and jasmonic acid pathways in PAMP-triggered immunity (Tsuda et al., 2009; Wang et al., 2009). However, PAMP-elicited bacterial resistance corresponds to a late flg22 response. To investigate the involvement of different hormone, stress, and kinase signaling pathways in early PAMP-triggered responses, we monitored the flg22-induced oxidative burst in intact seedlings of a collection of known mutants (Supplemental Fig. S1). Nearly all mutants tested were able to mount a wild-type-like oxidative burst. A slight increase in ROS production could be detected in mutants impaired in SA signaling, while some mutants involved in abscisic acid responses, and *rcd1*, a regulator of ROS-responsive cell death and stress-induced ethylene biosynthesis (Overmyer et al., 2000), were slightly reduced. A significant reduction in the flg22-elicited oxidative burst was detected in *dnd1* mutants (Supplemental Fig. S1), which are lowered in cell death (Clough et al., 2000). Most strikingly, ethylene-insensitive mutants displayed a severe decrease in flg22-triggered ROS levels (Fig. 1A; Supplemental Fig. S2). A strong dominant allele of the ethylene receptor mutant, *etr1-1*, was almost unresponsive, whereas a partially ethylene-insensitive allele, *etr1-3*, displayed a partially compromised flg22-elicited oxidative burst. Also, flg22-elicited ROS production was nearly abolished in the ethylene-insensitive mutant *ein2-1*.

Ethylene is a known component of plant immunity and accumulates upon flg22 treatment (Gomez-Gomez et al., 1999). Its recognition is mediated by a family of membrane receptors including ETR1, which acts in concert with the Raf-like kinase CTR1 to negatively regulate ethylene responses (Wang et al., 2002). Upon ethylene perception, the downstream component EIN2 activates the transcription factor EIN3, which drives transcriptional changes in ethylene-responsive genes. Mutants in *CTR1* causing constitutive ethylene responses (Kieber et al., 1993), or *ETO1*, leading to overproduction of ethylene (Wang et al., 2004), were dispensable for flg22-triggered ROS (Supplemental Fig. S1). These findings suggest that an active ethylene signaling pathway is required for the flg22-induced ROS production. By contrast, *ein3-1* mutants produced nearly wild-type-like ROS levels upon flg22 elicitation (Supplemental Fig. S1). This might be explained by the less pronounced ethylene insensitivity in *ein3* loss-of-function alleles due to functional redundancy within the *EIN3*-like gene family (Chao et al., 1997; Bleecker and Kende, 2000).

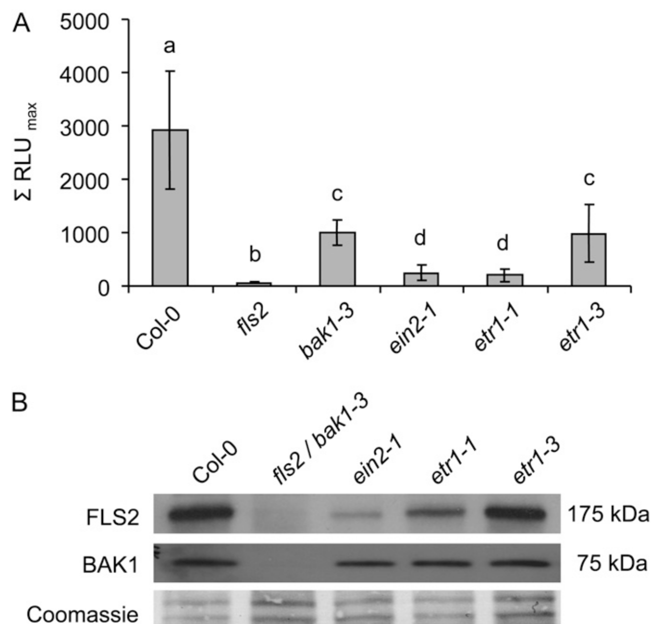

Figure 1. Flg22-stimulated oxidative burst and FLS2 abundance in ethylene-insensitive mutants. A, Flg22-triggered ROS production was monitored in liquid-grown intact seedlings of the indicated genotypes over time. Depicted are average values ($n = 18$); error bars represent ±SD. Similar results were obtained in multiple independent experiments. Letters indicate significant differences at $P < 0.05$. B, Immunoblot analysis of the indicated genotypes using specific anti-FLS2 and anti-BAK1 antibodies. Coomassie Brilliant Blue staining is shown for equal loading. Several independent experiments revealed similar results.

Ethylene Signaling Contributes to FLS2 Expression

Reduced ROS generation in response to flg22 might be a result of lowered FLS2 or BAK1 abundance. Indeed, we detected reduced FLS2 steady-state levels in the strong *ein2-1* and *etr1-1* mutants (Fig. 1B) that could explain the compromised flg22-induced oxidative burst in these lines. However, the partial ethylene-insensitive allele, *etr1-3*, which exhibited a reduced flg22-induced oxidative burst, accumulated FLS2 to levels observed in the wild type. This suggests that additional regulatory mechanisms underlie flg22-triggered ROS production. No differences in BAK1 abundance were observed in all three mutant alleles when compared with Columbia (Col-0) wild-type seedlings (Fig. 1B). Differences in FLS2 steady-state

levels between mutants correlated with differences in *FLS2* transcript accumulation (Fig. 2). Similarly, induced *FLS2* transcript accumulation upon flg22 treatment was lower in the ethylene-insensitive mutants when compared with the Col-0 wild type. This was confirmed by public database analysis (Supplemental Table S1). The *BAK1* steady-state transcript levels remained unaltered in *etr1-1*, *etr1-3*, and *ein2-1* seedlings. Since flg22-induced ROS is dependent on the NADPH oxidase RbohD (Zhang et al., 2007), we also analyzed *RbohD* transcript levels in the ethylene-insensitive mutants, which were unaltered (Fig. 2).

Our data revealed that ETR1 and EIN2 could regulate the expression of FLS2 at the level of transcription or translation. A possible transcriptional regulation of *FLS2* expression is supported by database searches, which revealed that *FLS2* transcript levels increase upon ethylene/1-aminocyclopropane-1-carboxylic acid treatment and decrease in the presence of aminoethoxyvinylglycine, an inhibitor of ethylene biosynthesis (Supplemental Table S2). Several ethylene response elements, which are cis-regulatory elements responsible for ethylene-induced transcriptional changes (Supplemental Table S3; Kosugi and Ohashi, 2000; Hao et al., 2003), can be found within the *FLS2* promoter region, which was previously shown to confer mutant complementation (Zipfel et al., 2004). Thus, the expression of *FLS2* appears to be under the control of basal ethylene signaling, which influences FLS2 steady-state levels.

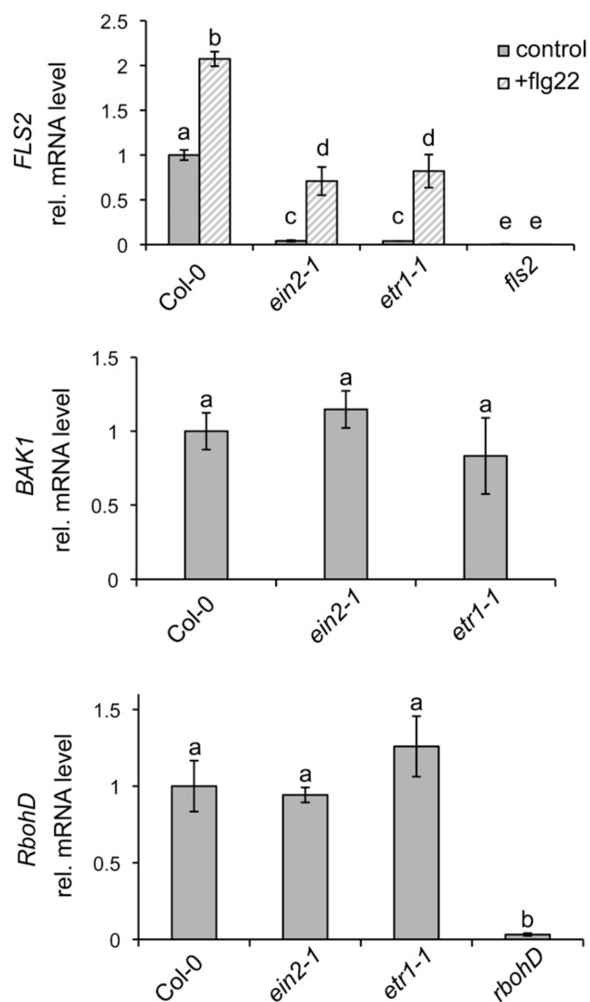

Figure 2. *FLS2* expression levels in ethylene-insensitive mutants. Quantitative real-time PCR monitoring of *FLS2*, *BAK1*, and *RbohD* transcript levels in the indicated genotypes is shown. *Tubulin* was used as a control. Depicted are average values of two independent experiments ($n = 6$); error bars represent ±SD. Letters indicate significant differences at $P < 0.05$.

Differential Behavior of Flg22 Responses in Ethylene-Insensitive Mutants

Flg22-triggered callose deposition, a late PAMP response, was shown to depend on *EIN2* (Clay et al., 2009). By contrast, when tested for flg22-elicited seedling growth arrest, sensitivity to flg22 in the ethylene-insensitive mutants occurred as in the wild type (Fig. 3A). Also, flg22 activation of signaling MAP kinases, representing another early FLS2-mediated response and involved in ethylene biosynthesis (Liu and Zhang, 2004; Boller and Felix, 2009), remained unaltered in *etr1-1*, *etr1-3*, and *ein2-1* seedlings (Fig. 3B). The impaired oxidative burst, but wild-type-like activation of MAP kinases and seedling growth arrest in flg22-stimulated ethylene-insensitive mutants, suggest different genetic requirements for individual flg22 responses.

Ethylene is an important regulator of plant development and growth (Ecker, 1995), and ethylene signaling mutants exhibit enhanced steady-state levels of ethylene compared with wild-type plants (Kende, 1993; Supplemental Fig. S3). Therefore, we monitored flg22-triggered ROS production in fully expanded leaves of adult plants and found that the oxidative burst was impaired in the ethylene-insensitive mutants (Supplemental Fig. S4). Remarkably, when we used excised leaf material as described by Felix et al. (1999), the strong ethylene-insensitive *etr1-1* and *ein2-1* mutants produced almost wild-type-like levels of flg22-triggered ROS (Fig. 4A). Wounding alone did not induce ROS production in this assay. To further exclude conditional developmental differences, we examined leaf discs from seedlings. All tested mutants displayed wild-type-like ROS levels upon flg22 treatment (Supplemental Fig. S5), and FLS2 protein was present at similar levels in wounded *etr1-1*, *ein2-1*, and Col-0 wild type (Fig. 4B). Notably, *FLS2* expression is enhanced upon wounding (Supplemental Table S2). This result suggests that wounding reverts the compromised flg22-induced oxidative burst in the ethylene-insensitive mutants and points to a stimulus-dependent regulation of flg22-triggered defense responses.

Ethylene Signaling Contributes to Plant Immunity

Ethylene has diverse functions in plant-microbe interactions (Van Loon et al., 2006). It is important

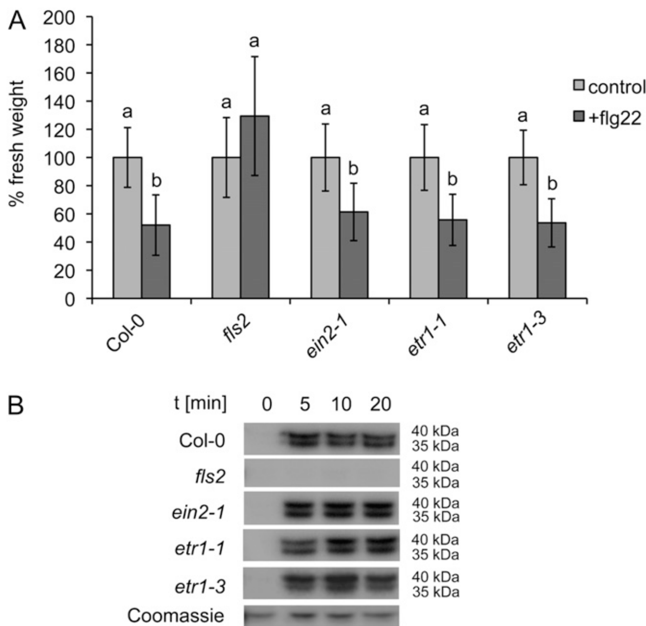

Figure 3. Flg22-stimulated early and late responses in ethylene-insensitive mutants. A, Seedling growth of the indicated genotypes was measured in the absence or presence of 100 nM flg22. Error bars represent ±SD. Letters indicate significant differences at $P < 0.05$. Similar results were obtained in three independent experiments. B, Flg22-induced MPK6 and MPK3 activation was determined by in-gel MAP kinase assays at the indicated time points and seedling genotypes. Coomassie Brilliant Blue staining is shown for equal loading.

for defense against necrotrophic fungi (Chagué et al., 2006), but its contribution to bacterial resistance remains unclear. Unaltered, reduced, or enhanced bacterial numbers have been reported depending on infection conditions (Bent et al., 1992; Pieterse et al., 1998). Our data demonstrated that among the flg22 responses tested, the ethylene-insensitive mutants were most impaired in ROS production. Due to the rapid and transient nature of the oxidative burst, we reasoned that differences in pathogen proliferation might be most apparent at early infection time points after exposure to a weak bacterial pathogen. FLS2-mediated immunity is suppressed by the effectors AvrPto and AvrPtoB (Göhre et al., 2008; Shan et al., 2008); therefore, we used the invasive but disarmed *Pseudomonas syringae* pv *tomato* DC3000 (PtoDC3000) ΔAvrPto/ΔAvrPtoB strain (Rosebrock et al., 2007). To avoid interference with wounding, we surface inoculated the set of ethylene-insensitive mutants and found that *ein2-1* and *etr1-1* allowed elevated bacterial multiplication compared with wild-type plants at 1 d post infection (Fig. 5). This is in agreement with the recently reported increased susceptibility of *ein2-1* mutants to PtoDC3000 infection (Clay et al., 2009). Bacterial growth rates at later time points were similar or even lower than wild-type levels in ethylene-insensitive mutants (Fig. 5). Moreover, the *etr1-3* mutant allele, which confers partial insensitivity to ethylene and still allowed a partial flg22-induced oxidative burst, facilitated bacterial multiplication similar to wild-type plants early during infection. This suggests that ethylene contributes to preinvasive immunity, possibly through regulation of PAMP-triggered ROS production, but has a different role after disease establishment. At 3 d post infection, *etr1-3* mutants displayed enhanced bacterial resistance compared with Col-0, unraveling differential effects of altered ethylene signaling in plant immunity.

Ethylene-Insensitive Mutants Are Impaired in PAMP-Triggered Stomatal Closure

Plants respond to bacterial invasion with closure of stomata in order to prevent pathogens from entering into leaf tissues. It has been shown that stomatal closure is induced upon perception of PAMPs and that successful pathogens need to overcome this level of preinvasive immunity (Melotto et al., 2006). Plant immunity in the ethylene-insensitive mutants was compromised at early but not late stages of bacterial infection; therefore, we analyzed stomatal behavior in the ethylene-insensitive mutants. As a measure of stomatal closure, we monitored the aperture of the inner sites of guard cells in *etr1-1* and *ein2-1* plant leaves in the absence or presence of flg22 (Lemichez et al., 2001; Desikan et al., 2006; Supplemental Fig. S6A). Elicited leaves of Col-0 exhibited reduced stomatal aperture, which was not observed in *fls2* mutants (Fig. 6). The ethylene-insensitive mutants *etr1-1* and *ein2-1* were not able to reduce stomatal aperture in response to flg22, and the partial ethylene-insensitive allele *etr1-3* displayed weak flg22-induced stomatal closure (Fig. 6). A defect in stomatal closure upon bacterial detection is likely the cause of enhanced susceptibility early

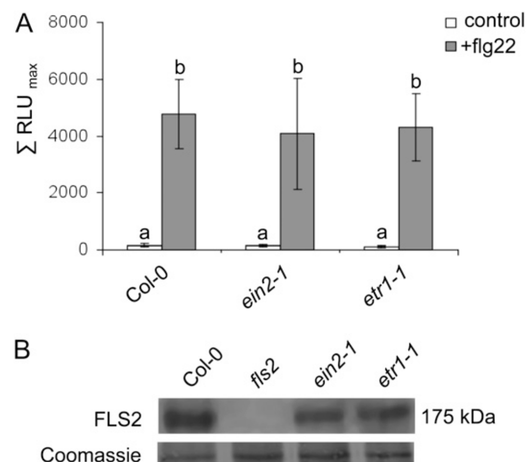

Figure 4. Flg22-induced oxidative burst in leaf discs. A, Mature leaves of the indicated genotypes were excised into discs of approximately equal sizes and monitored for flg22-mediated ROS production. Depicted are average values ($n = 8$); error bars represent ±SD. RLU, Relative light unit. Letters indicate significant differences at $P < 0.05$. B, FLS2 immunoblot analysis. Coomassie Brilliant Blue staining is shown for equal loading.

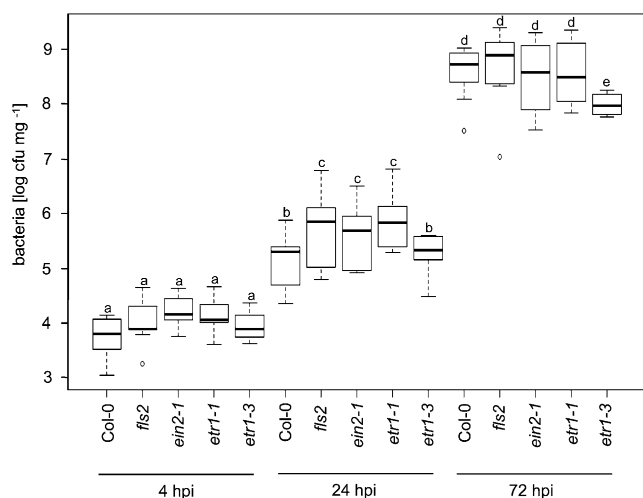

Figure 5. Bacterial growth in ethylene-insensitive mutants. Plants of the indicated genotypes were surface inoculated with PtoDC3000 ΔAvrPto/ΔAvrPtoB, and bacterial multiplication was monitored at 4, 24, and 72 h post infection (hpi). Shown are average values of three independent experiments (n = 16); error bars represent ±SD. Letters indicate significant differences at $P < 0.05$.

in the infection process in ethylene-insensitive mutants. Like flg22 treatment, incubation with PtoDC3000 ΔAvrPto/ΔAvrPtoB bacteria reduced stomatal aperture in wild-type plants but not in *etr1-1* and *ein2-1* mutants and only weakly in *etr1-3* mutants. By contrast, stomatal closure occurred in PtoDC3000 ΔAvrPto/ΔAvrPtoB-infected *fls2* mutants, likely due to the presence of other PAMPs than bacterial flagellin. Stomatal closure plays an important role in the FLS2-mediated immunity against PtoDC3000 infection (Zeng and He, 2010). FLS2 expression in the epidermis and in guard cells was described previously (Robatzek et al., 2006; Supplemental Fig. S6B). Database analysis in addition revealed that *BAK1*, *RbohD*, and *ETR1* are expressed in guard cells, and *BAK1*, *ETR1*, and *EIN2* are expressed in the epidermis, consistent with a role in PAMP-triggered stomatal closure (Supplemental Table S1).

rbohD Mutants Are Compromised in Flg22-Triggered Stomatal Closure and Immunity

Since *etr1-1* and *ein2-1* were strongly diminished in flg22-induced ROS accumulation but not MAP kinase activation, we reasoned that flg22-induced stomatal closure requires an oxidative burst. Although both RbohD and RbohF were reported to regulate plant defense responses (Torres et al., 2002), RbohD alone was sufficient for the PAMP-triggered oxidative burst (Zhang et al., 2007). Our results confirmed that *rbohD*, but not *rbohF* mutants, are impaired in flg22-elicited ROS (Supplemental Fig. S7A). Moreover, Arabidopsis seedlings overexpressing *RbohD* accumulated higher ROS levels (Supplemental Fig. S7A). Other flg22 responses, such as seedling growth arrest and MAP kinase activation, were not affected by interference with *RbohD* function, suggesting that these responses occur independent of the flg22-triggered oxidative burst (Supplemental Fig. S6, B and C). But unlike *etr1-1* and *ein2-1* mutants, FLS2 steady-state levels remained unaltered in *rbohD* and 35S::*RbohD* lines (Supplemental Fig. S8A), indicating that RbohD is an essential and rate-limiting component of the flg22-induced ROS production. Stomatal aperture was not affected by flg22 treatment in *rbohD* mutants (Fig. 6). Also, no stomatal closure was detected in *rbohD* mutants when incubated with PtoDC3000 ΔAvrPto/ΔAvrPtoB bacteria. By contrast, *rbohF* mutants and 35S::*RbohD* lines showed reduced stomatal aperture upon flg22 stimulation and bacterial infection. This demonstrates that stomatal closure provoked by flg22 and PtoDC3000 ΔAvrPto/ΔAvrPtoB infection specifically depends on the *RbohD*-mediated oxidative burst.

The strong *etr1-1* and *ein2-1* and the *rbohD* mutants were all impaired in flg22-triggered ROS production and stomatal closure. However, in contrast to the

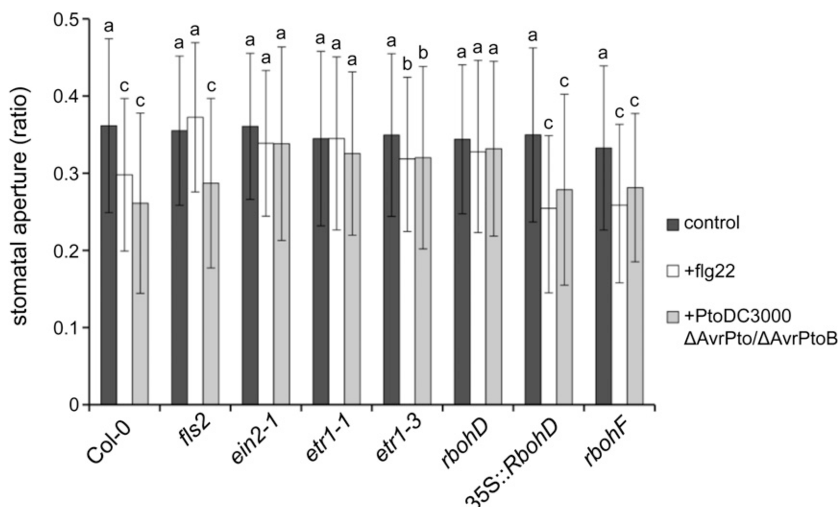

Figure 6. Flg22-triggered stomatal closure. Leaves of the indicated seedlings were untreated, stimulated with flg22, or infected with PtoDC3000 ΔAvrPto/ΔAvrPtoB, and stomatal aperture was measured microscopically as width to length ratio. Depicted are average values; error bars represent ±SD (n = 140 stomata of three independent experiments). Letters indicate significant differences at $P < 0.1$ (b) or $P < 0.05$ (c).

ethylene-insensitive lines, *rbohD* mutants were unaffected in FLS2 accumulation. To test whether the *rbohD* mutants were also compromised in immunity, we similarly investigated bacterial growth at early time points by spray infection of disarmed PtoDC3000 ΔAvrPto/ΔAvrPtoB. We detected significantly increased bacterial proliferation in the *rbohD* loss-of-function mutant (Fig. 7). Enhanced susceptibility was not observed at later time points. The precise role of PAMP-induced ROS in plant immunity has remained unclear, although it is a closely associated reaction of PAMP perception. Besides being antimicrobial, ROS might serve as signaling molecules promoting rapid plant defense responses in a cell autonomous or non-autonomous manner (D'Autréaux and Toledano, 2007; Miller et al., 2009). Our results provide evidence that PAMP-triggered ROS production through RbohD is important for plant defense at early stages of bacterial infection.

DISCUSSION

Among the mutant collection surveyed in this study, mutants impaired in ethylene sensing were identified as strongly diminished in the flg22-triggered oxidative burst. This is in accordance with findings that ethylene-insensitive mutants did not accumulate callose in response to flg22, whereas other hormone signaling mutants reacted like the wild type (Clay et al., 2009; Millet et al., 2010). Altogether, ethylene sensing is required for flg22-induced ROS production and callose deposition but not for flg22-triggered MAP kinase activation, seedling growth arrest, and induced resistance (Figs. 1 and 3; Zipfel et al., 2004; Adams-Phillips et al., 2008; Clay et al., 2009). It is evident from these findings that there are different genetic requirements for individual PAMP responses. However, different response assays likely differ in sensitivity, and the possibility that genetic redundancy obscures the importance of other than ethylene signaling pathways cannot be excluded. Indeed, the *dde2/ein2/pad4/sid2* quadruple mutant defective in SA, jasmonic acid, and ethylene signaling displayed reduced responsiveness in flg22-induced resistance and seedling growth arrest (Tsuda et al., 2009). Noteworthy, *etr1-1* represents a dominant ethylene-insensitive mutant allele, while *ein2-1* and *ein3-1* are loss-of-function alleles conferring complete and reduced ethylene insensitivity, respectively (Kieber et al., 1993; Chao et al., 1997; McCourt, 1999).

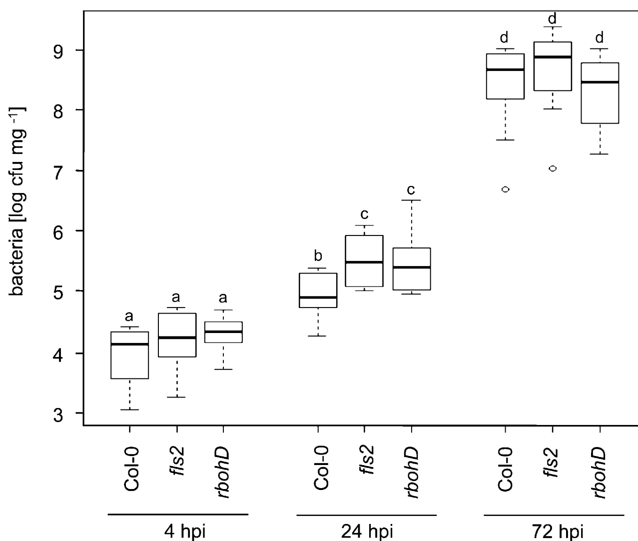

Figure 7. Bacterial growth in plants with altered ROS accumulation. Plants of the indicated genotypes were surface infected with PtoDC3000 ΔAvrPto/ΔAvrPtoB, and bacterial multiplication was determined at 4, 24, and 72 h post infection (hpi). Shown are average values of three independent experiments ($n = 16$); error bars represent ±SD. Letters indicate significant differences at $P < 0.05$.

FLS2, but not BAK1, steady-state levels were significantly lower in plants carrying strong *etr1-1* and *ein2-1* alleles. Moreover, *etr1-1* and *ein2-1* mutants accumulated reduced amounts of *FLS2* transcripts. Ethylene signaling, therefore, is necessary for basal expression of FLS2. Recently, *ein3-4*, a semidominant ethylene-insensitive mutant allele, was shown to accumulate less callose upon flg22 treatment and concomitantly allow enhanced bacterial multiplication compared with wild-type plants (Chen et al., 2009). This is in accordance with data obtained in our study and provides evidence that FLS2 abundance may be downstream of EIN3. However, plants carrying the weak *etr1-3* allele, which were reduced in flg22-induced ROS production, accumulated wild-type-like FLS2 levels. This suggests that ethylene signaling is not only required for proper FLS2 expression but also for the oxidative burst response.

Remarkably, wounding appears to counteract defects in *etr1-1* and *ein2-1* mutants, because both wild-type-like FLS2 levels and the flg22-triggered oxidative burst were recovered in ethylene-insensitive mutants when leaf discs instead of whole seedlings were used. EIN3-like transcription factors are transcriptionally altered in response to wounding and involved in the regulation of wound-induced genes in rice (*Oryza sativa*; Hiraga et al., 2009). In addition, wound signaling is mediated by MAP kinase activation via MPK6 and involves ethylene (Schweighofer et al., 2007). Ethylene signaling is required for flg22-triggered dissociation of MPK6 and the ethylene response factor ERF104 (Nühse et al., 2000; Bethke et al., 2009). Genetic interference with *ERF104* function altered the growth arrest in seedlings treated with flg22. It is likely, therefore, that wounding primes ethylene-insensitive mutants competent to flg22-induced ROS production by at least partly utilizing the same signaling components.

In this study, we observed that ethylene signaling through *ETR1* and *EIN2* contributes to flg22-dependent stomatal closure. The *etr1-1* and *ein2-1* mutants were also impaired in stomatal closure when incubated with PtoDC3000 ΔAvrPto/ΔAvrPtoB bacteria and accordingly facilitated enhanced bacterial growth com-

pared with the wild type at early stages of infection. Increased susceptibility of ethylene-insensitive mutants was described before for nonhost pathogens and when bacteria were surface inoculated (Knoester et al., 1998; Pieterse et al., 1998; Clay et al., 2009). Previously reported unaltered immunity of ethylene-insensitive mutants in flg22-induced resistance might be due to hand inoculation (Zipfel et al., 2004). Bacterial growth rates were comparable between mutants and the wild type later during infection, indicating a different requirement of ethylene signaling at this stage. Notably, the bacterial strain PtoDC3000 ΔAvrPto/ΔAvrPtoB is disarmed in virulence but still able to produce coronatine and therefore is able to reopen stomata and colonize leaf tissues (Melotto et al., 2006; Zhang et al., 2008).

We revealed that *RbohD* contributes to restricting bacterial numbers at an early stage of infection. PAMP perception is particularly relevant at the level of preinvasive immunity. It can induce stomatal closure and thereby hinder the entry of pathogens into plant tissues (Melotto et al., 2006; Zeng and He, 2010). We identified *RbohD* as specifically required for flg22- and bacteria-induced stomatal closure. This defect in PAMP-triggered stomatal closure, which would largely compromise preinvasive immunity, is likely to be the cause of elevated bacterial numbers at early but not late stages of infection. An interaction between ROS and stomatal aperture in response to flg22 is consistent with a reduced oxidative burst in mutants devoid of *OST1*, another mediator of PAMP-induced stomatal closure and indicated to phosphorylate RbohF, which might also be postulated for RbohD (Melotto et al., 2006; Nühse et al., 2007; Sirichandra et al., 2009).

Regulation of stomatal closure in plant immunity also involves nitric oxide and GPA1, the G-subunit of heterotrimeric G protein (Melotto et al., 2006; Zhang et al., 2008; Zeng and He, 2010). GPA1-dependent ROS production through RbohD/F is necessary for stomatal closure triggered by treatment with exogenous calmodulin (Li et al., 2009). Thus, ROS production is an intrinsic component regulating stomatal aperture in various stress responses. It is notable that the functions of Coronatine Insensitive1, previously identified as a crucial component of PAMP-triggered stomatal closure (Melotto et al., 2006), or Abscisic acid Insensitive1 (ABI1) and ABI2, key regulators of stomatal closure upon abiotic stress (Assmann et al., 2001), appear to be dispensable for flg22-induced oxidative burst.

The ETR1 receptor was previously proposed as a point of convergence for ethylene and ROS signaling in stomatal function (Desikan et al., 2005, 2006), and a role for EIN2 in ROS signaling was demonstrated in ozone-dependent accumulation of ROS (Overmyer et al., 2000). Altogether, our data revealed an interaction between flg22-induced ROS production and signaling through ethylene, a hormone that itself accumulates in response to flg22. We propose that ethylene signaling contributes to basal expression of FLS2 and provide evidence for RbohD-dependent PAMP-triggered stomatal closure, which is required for proper plant immunity at early stages of infection.

MATERIALS AND METHODS

Plants and Growth Conditions

Unless stated otherwise, Arabidopsis (*Arabidopsis thaliana*) genotypes (Col-0, *fls2*, *bak1-3*, *etr1-1*, *etr1-3*, *ein2-1*, *rbohD*, and mutant plants listed in Supplemental Fig. S1) were grown on soil or Jiffy pellets (Jiffy Products International). For bacterial growth assays, plants were grown for 2 weeks in a controlled-environment chamber under short-day conditions (65% humidity and 23°C/22°C day/night temperatures); for ROS assays using leaf discs, plants were grown for a further 2 weeks under greenhouse conditions (21°C/19°C day/night temperatures).

Oxidative Burst Measurements

Oxidative burst was measured in intact seedlings (Khairullin and Akhmetova, 2001), and flg22 treatment was performed as outlined below (Supplemental Fig. S9). Arabidopsis seedlings were grown under sterile conditions on 96-well microtiter plates (467 μmol of light, 60% humidity, and 21°C/19°C day/night temperatures), each in 100 μL of Murashige and Skoog medium supplied with Nitch vitamins for 14 d under short-day conditions. In order to reduce variation between individual samples, growth medium was exchanged with water containing 10 nM flg22, incubated for 1 h, replaced with water, and further incubated for 1 h. ROS production was triggered with 100 nM flg22 applied together with 20 μM luminol and 1 μg per 100 μL of horseradish peroxidase. Luminescence was measured by a Centro LB 960 microplate luminometer (Berthold Technologies). Each plate was measured over a period of 40 min in 13 cycles. ROS detection using leaf discs from soil-grown adult and seedling plants was performed as described by Gomez-Gomez et al. (1999). All ROS measurements were repeated at least three times with similar results. The measurement values flanking the maximal level of ROS production, representing a time interval of 3.5 min, were determined, and their sum was calculated and referred to as Σ RLU_{max} (where RLU = relative light units). Statistical analysis was done using Student's *t* test.

Seedling Growth Arrest

Flg22-triggered seedling growth arrest was essentially done as described by Gomez-Gomez et al. (1999). The mean of seedling growth without flg22 treatment was set at 100%, and percentage growth in the presence of 100 nM flg22 was calculated. Statistical analysis was done using Student's *t* test.

Protein Detection and MAP Kinase Assays

Protein extraction and immunoblot analyses were essentially done as described previously (Häweker et al., 2010). In-gel MAP kinase assays were carried out as reported by Chinchilla et al. (2007). Briefly, 17-d-old in vitro-grown seedlings were treated with 100 nM flg22, and samples were harvested at intervals. Total protein was extracted and subjected to SDS-polyacrylamide gels supplied with 0.25 mg mL^{-1} myelin basic protein as kinase substrate. MAP kinase activation was determined by phosphoimaging (Typhoon 8600 PhosphorImager and Image Eraser; Molecular Dynamics).

Quantitative Real-Time PCR Analysis

Arabidopsis genotypes were grown in vitro as described for ROS measurements of seedlings. RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) followed by TURBO DNase (Applied Biosystems) treatments to remove genomic DNA. Subsequent cDNA synthesis was done using the SuperScript II RNase H^- Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was performed in the presence of qPCR SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich). The following primer pairs were used for PCR amplification: *FLS2* (5′-ACTCTCCTCCAGGGGCTAAGGAT-3′ and 5′-AGCTAACAGCTCTCCAGGGATGG-3′), *BAK1* (5′-ACCGCCTCCTATCTC-TCCTACACC-3′ and 5′-CTGGGTCCTCTTCAGCTGGTACA-3′), *RbohD* (5′-GCCGAGCCGTATCTCCATTC-3′ and 5′-TCCAATGCCGAGACCTACGA-3′),

and *Tubulin4* (5′-AGGGAAAGGAAGAGAGGAAG-3′ and 5′-GCTGGCTA-ATCCTACCCTTTGG-3′). PCR amplification was monitored in triplicate using the Chromo4 detection system (Bio-Rad). Amplification of *Tubulin4* (At1g04820) served as an internal control for normalization, and gene transcript levels were calculated with reference to expression in the Col-0 wild type and averaged to 1. Statistical analysis was done by Student's *t* test using R software (http://www.R-project.org).

Bacterial Infections

Bacterial infection assays were modified from Zipfel et al. (2004). Briefly, *Pseudomonas syringae* pv *tomato* DC3000 ΔAvrPto/ΔAvrPtoB (Rosebrock et al., 2007) was spray inoculated onto leaf surfaces of 2-week-old seedlings at 10^8 colony-forming units mL^{-1}. All aerial parts were harvested 4, 24, and 72 h after pathogen treatment and surface sterilized. Two seedlings from each of 16 samples were pooled, and bacteria were extracted by grinding in 10 mM $MgCl_2$. Samples were diluted and plated on medium containing appropriate antibiotics. For each Arabidopsis genotype, eight samples, each comprising two seedlings, were analyzed. The results of three independent experiments were combined, and statistical analysis (ANOVA and subsequent posthoc test by Tukey's honestly significant difference) was done using R software (http://www.R-project.org).

Stomatal Aperture

Two-week-old seedlings were grown in vitro under standard conditions and then transferred to 100 mE m^{-2} s^{-1} light for at least 3 h in order to ensure that most stomata were open. The seedlings were vacuum infiltrated 10 min in a water solution without or with 3 μM flg22 or 10^8 colony-forming units mL^{-1} PtoDC3000 ΔAvrPto/ΔAvrPtoB (Rosebrock et al., 2007) and incubated for 2 h. The lower epidermis of six leaves of independent seedlings was mounted on glass slides and imaged using a Zeiss Axiophot microscope. Stomatal aperture of at least 140 stomata was determined as the ratio between width and length (Lemichez et al., 2001; Morillon and Chrispeels, 2001) using ImageJ software. Statistical analysis (Student's *t* test) was done using R software.

Additional information about materials and methods used are provided in Supplemental Materials and Methods S1.

Supplemental Data

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

Supplemental Figure S1. Overview of mutants with known roles in plant defense and stress signaling tested for flg22-induced ROS production.

Supplemental Figure S2. Oxidative burst in ethylene-insensitive mutants.

Supplemental Figure S3. Steady-state accumulation of ethylene.

Supplemental Figure S4. Oxidative burst in ethylene signaling mutants at adult stage.

Supplemental Figure S5. Flg22-induced ROS in wounded seedling material.

Supplemental Figure S6. Imaging of stomata.

Supplemental Figure S7. Flg22-stimulated responses in lines with altered ROS production.

Supplemental Figure S8. FLS2 accumulation in lines with altered ROS production.

Supplemental Figure S9. Flg22-stimulated oxidative burst.

Supplemental Table S1. Transcript abundance of genes involved in plant defense and stress signaling in different mutant backgrounds.

Supplemental Table S2. Transcript abundance of genes involved in plant defense and stress signaling upon various treatments.

Supplemental Table S3. Potential transcription factor binding sites within 1,000 bp of FLS2 promoter sequence.

Supplemental Materials and Methods S1. Oxidative burst measurements in intact adult plants; ethylene measurements; microscopic analysis; reverse transcription-PCR analysis.

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