Cell Wall Damage-Induced Lignin Biosynthesis Is Regulated by a Reactive Oxygen Species- and Jasmonic Acid-Dependent Process in Arabidopsis

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Cell Wall Damage-Induced Lignin Biosynthesis Is Regulated by a Reactive Oxygen Species- and Jasmonic Acid-Dependent Process in Arabidopsis[1][C][W][OA]

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The plant cell wall is a dynamic and complex structure whose functional integrity is constantly being monitored and maintained during development and interactions with the environment. In response to cell wall damage (CWD), putatively compensatory responses, such as lignin production, are initiated. In this context, lignin deposition could reinforce the cell wall to maintain functional integrity. Lignin is important for the plant's response to environmental stress, for reinforcement during secondary cell wall formation, and for long-distance water transport. Here, we identify two stages and several components of a genetic network that regulate CWD-induced lignin production in Arabidopsis (*Arabidopsis thaliana*). During the early stage, calcium and diphenyleneiodonium-sensitive reactive oxygen species (ROS) production are required to induce a secondary ROS burst and jasmonic acid (JA) accumulation. During the second stage, ROS derived from the NADPH oxidase RESPIRATORY BURST OXIDASE HOMOLOG D and JA-isoleucine generated by JASMONIC ACID RESISTANT1, form a negative feedback loop that can repress each other's production. This feedback loop in turn seems to influence lignin accumulation. Our results characterize a genetic network enabling plants to regulate lignin biosynthesis in response to CWD through dynamic interactions between JA and ROS.

The plant cell wall is an essential component of different biological processes, including cell morphogenesis and plant-pathogen interactions (Hématy et al., 2009; Szymanski and Cosgrove, 2009). During these processes, the cell wall has to maintain its integrity while also having to meet different functional requirements. In yeast (*Saccharomyces cerevisiae*), a cell wall integrity maintenance mechanism has evolved that monitors the functional integrity of the wall and initiates changes in wall composition and/or structure and cellular metabolism if necessary (Levin, 2005). In yeast, plasma membrane proteins, such as MID1 (a component of a stretch-activated Ca^{2+}-permeable channel) and the plasma membrane protein WSC1 have been implicated in the process (Levin, 2005). They can sense membrane distortion or cell wall damage (CWD) and mediate responses via the small G-protein RHO1, a mitogen-activated protein (MAP) kinase cascade, and transcription factors, such as SWI4 and RLM1 (Levin, 2005). The available evidence suggests that a similar cell wall monitoring mechanism may operate in plants (Seifert and Blaukopf, 2010). Accordingly, the Arabidopsis (*Arabidopsis thaliana*) proteins MID1 COMPLEMENTING-ACTIVITY1 (MCA1) and MCA2 partially complement the lethal phenotype of the *mid1* yeast strain (Nakagawa et al., 2007; Yamanaka et al., 2010). Expression of MCA1 and 2 in yeast enhances Ca^{2+} influx into the cytoplasm upon hypoosmotic shock, and roots of the double mutant *mca1 mca2* exhibit reduced Ca^{2+} uptake (Yamanaka et al., 2010). Both proteins share certain structural features. In the N-terminal region, they have motifs similar to regulatory regions in rice (*Oryza sativa*) protein kinases, an EF-hand-like and coiled-coil motif (Yamanaka et al., 2010). The C-terminal region contains two to four putative transmembrane domains and a Cys-rich domain of unknown function (PLAC8 motif; Galaviz-Hernandez et al., 2003).

Cellulose microfibrils represent the main load-bearing components of the plant cell wall, enabling it to resist the turgor pressure of the cell. Well-established methods to

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cause specific CWD in plants are inhibition of cellulose biosynthesis during primary cell wall formation by the herbicide isoxaben or through mutations reducing the activity of the cellulose synthase complex both during primary and secondary cell wall formation (Scheible et al., 2001; Hématy et al., 2007; Hernández-Blanco et al., 2007). Reduction in cellulose biosynthesis induces production of the phytohormones jasmonic acid (JA), salicylic acid, and ethylene, enhances pathogen resistance, and leads to changes in cell wall composition/structure, as well as causing ectopic lignin production (Ellis et al., 2002; Caño-Delgado et al., 2003; Manfield et al., 2004; Hernández-Blanco et al., 2007; Hamann et al., 2009). Similar responses have been observed in plants exposed to other forms of biotic and abiotic stress (Fujita et al., 2006).

CWD-induced lignin deposition in Arabidopsis is reduced in a loss-of-function mutant for the NADPH oxidase *RESPIRATORY BURST OXIDASE HOMOLOG D* (*RBOHD*) and enhanced in *JASMONIC ACID RESISTANT1* (*JAR1*) mutant seedlings (Hamann et al., 2009). These observations implicate reactive oxygen species (ROS) and JA-based signaling processes in the regulation of CWD-induced lignin deposition. Ten *RBOH* genes have been identified in Arabidopsis (Torres et al., 2005). The interaction between RBOHD and F seems to fine-tune the spatial control of ROS production during pathogen infection (Torres et al., 2006). RBOHD-derived ROS have been shown to act both as a local and systemic signal during the response to pathogen infection (Grant et al., 2000b; Torres et al., 2006). The enzymatic activity of RBOHD is synergistically regulated through phosphorylation and Ca^{2+} binding (Torres et al., 2005; Ogasawara et al., 2008; Miller et al., 2009). OXIDATIVE SIGNAL-INDUCIBLE1 (OXI1), a Ser/Thr kinase transiently activated by wounding and ROS, is necessary for translation of ROS signals to downstream targets such as MAP KINASE3 and 6 (Rentel et al., 2004). OXI1 has also been implicated in the response to the oomycete *Hyaloperonospora arabidopsidis* and *Pseudomonas syringae* pv *tomato* DC3000 (Rentel et al., 2004; Petersen et al., 2009).

Jasmonate has been shown to be an important regulator of plant responses to wounding and environmental stress (Fonseca et al., 2009a). The biosynthetic processes giving rise to JA-Ile, the biologically active form of JA, are well established (Fonseca et al., 2009b). *ALLENE OXIDE SYNTHASE* (*AOS*) encodes a cytochrome P450 that catalyzes the formation of an unstable allene oxide, which gives rise to 12-oxophytodienoic acid, a precursor of JA (Hofmann and Pollmann, 2008; Gfeller et al., 2010). JAR1 is required for conjugation of JA to Ile, leading to active JA-Ile (Suza and Staswick, 2008). JA-Ile is perceived by the SCF^{COI1} E3 ubiquitin complex (Chini et al., 2007; Thines et al., 2007; Katsir et al., 2008). Within this complex, CORONATINE INSENSITIVE1 (COI1) binds JA-Ile and controls the interaction between the complex and the JASMONATE ZIM-motif proteins (Feng et al., 2003; Yan et al., 2009). These proteins control the activity of transcription factors like MYC2 that regulate JA-dependent defense and wound responses (Boter et al., 2004; Lorenzo et al., 2004; Chini et al., 2007). However, understanding of the processes inducing and modulating JA biosynthesis is limited.

The receptor-like kinase *THESEUS1* (*THE1*) was originally isolated as a suppressor of *procuste*, a mutation that causes a reduction in cellulose biosynthesis during primary cell wall formation (Hématy et al., 2007). The kinase has been implicated in the response to CWD because mutations in *THE1* reduce CWD-induced lignin production, whereas THE1 overexpression enhances it.

During lignin biosynthesis *p*-coumaryl, coniferyl, and sinapyl alcohols are produced, which are randomly cross-linked in the cell wall to form a lignin polymer (Vanholme et al., 2010). The biosynthetic routes giving rise to the different monolignols have been characterized, and a large number of transcription factors have been identified that regulate the process (Zhong and Ye, 2007). However, the mechanisms regulating the activity of the transcription factors are poorly understood. Cross-linking of the monolignols in the wall is brought about by laccases and/or peroxidases (Cai et al., 2006; Demont-Caulet et al., 2010). While laccases use oxygen to oxidize their metal enabling the catalytic phenol oxidation, peroxidases use hydrogen peroxide. The hydrogen peroxide for this process may derive from NADPH oxidases, but this remains to be confirmed. Lignin induction has been correlated with cold, drought, or light stresses as well as mechanical injuries in a large number of different plant species, such as poplar (*Populus* spp.), rice, pine (*Pinus*), Arabidopsis, and soybean (*Glycine max*; Moura et al., 2010). In parallel, it has been shown that expression of lignin biosynthetic genes can be correlated with lignin production in response to infection by pathogens; for example, in Arabidopsis challenged with *Xanthomonas campestris* and in *Linum usitatissimum* cell cultures inoculated with *Botrytis cinerea* (Lauvergeat et al., 2001; Hano et al., 2006). While lignin biosynthesis itself is well characterized, the mechanism regulating ectopic lignin deposition in response to environmental stimuli is not well understood.

Previous work suggested that ROS- and JA-mediated signaling may regulate ectopic lignin production induced by CWD (Hamann et al., 2009). To determine how the different signaling cascades interact to regulate the response to CWD, we initiated a systematic analysis using mutants involved in JA production (*aos* and *jar1*) or perception (*coi1*), ROS biosynthesis (*rbohD*, *rbohF*, and *rbohDF*) or signaling (*oxi1*), or implicated in CWD perception (*mca1* and *the1*) (Nakagawa et al., 2007). We examined how the mutants affect isoxaben-induced lignin deposition, as well as JA and ROS production, and finally extended these results by pharmacological inhibition of JA-, ROS-, and Ca^{2+}-based signaling processes, allowing a careful temporal analysis of CWD-induced ROS and JA signaling as well as lignin production.

Our results suggest that the response to CWD can be divided into an early and a late stage. Ca^{2+} and diphenyleneiodonium (DPI)-sensitive generation of ROS are required during the early stage to initiate a subsequent RBOHD-dependent ROS burst and JA production. Notably, a negative feedback loop between JA and ROS regulates lignin production in the elongation zone of the primary root of Arabidopsis seedlings during the late stage of CWD, revealing an unexpectedly complex biphasic regulation of the response to plant CWD.

RESULTS

ROS and JA Signaling Have Opposite Effects on CWD-Induced Lignin Deposition

It has been shown previously that isoxaben-induced CWD causes lignin deposition in the primary root elongation zone of Arabidopsis seedlings (Hamann et al., 2009). To determine how the different signals discussed above affect the response to CWD, seedlings mutant for *JAR1*, *AOS*, *COI1*, *THE1*, *OXI1*, *RBOHD*, *RBOHF*, or *MCA1* were treated with isoxaben and assessed for lignin deposition in the primary root after 12 h. To detect lignin deposition, phloroglucinol was used. Phloroglucinol is a histochemical staining method that specifically detects 4-*O*-linked hydroxy-cinnamyl aldehydes, which form part of the lignin polymer (Pomar et al., 2002). None of the mock-treated seedlings showed any lignin deposition in the root tip (Fig. 1; data not shown). Mutations reducing JA (*aos*), JA-Ile production (*jar1*), or perception (*coi1*) caused enhanced lignin deposition, whereas mutants impaired in ROS production (*rbohD*, *rbohF*, and *rbohDF*) or ROS signal translation (*oxi1*) either reduced (*rbohD*, *rbohF*, and *oxi1*) or prevented (*rbohDF*) lignin deposition (Fig. 1). *mca1* roots also exhibited reductions in lignin deposition (Fig. 1), which were similar to those observed in *the1* seedlings (Nakagawa et al., 2007). To illustrate the variability observed, Supplemental Figure S1 shows five root tips of isoxaben-treated seedlings for Columbia-0 (Col-0) and five of the genotypes examined.

To summarize, lignin deposition is enhanced in mutants impaired in JA biosynthesis or signaling, whereas it is decreased (or absent) in mutants impaired in CWD perception and ROS signaling/perception. These observations suggest that JA functions as a repressor of CWD-induced lignin production, while ROS is required for lignin biosynthesis. It also implicates the putative stretch-activated membrane channel MCA1 in the CWD response.

ROS Negatively Regulates CWD-Induced JA Production

The effects on lignin production observed in the JA biosynthesis and signaling mutants together with previously published observations (Ellis et al., 2002; Hamann et al., 2009) raised the question of how quickly CWD induces JA production. Time-course experiments revealed that JA production was already increased after 4 to 5 h in isoxaben-treated seedlings, whereas no increase was detectable in mock-treated seedlings (Fig. 2A). To determine if mutations modifying CWD-induced lignin deposition also affect JA production, the JA concentration was measured after 7 h of isoxaben treatment in these mutants. This time point was selected since lignin deposition is already detectable at this time and JA concentration is strongly increased in isoxaben-treated Col-0 seedlings (Fig. 2A; Hamann et al., 2009). JA was undetectable in any of the mock-treated wild-type and mutant seedlings or in the isoxaben-treated *aos* seedlings (Supplemental Fig. S2A). JA concentration was similar to Col-0 in isoxaben-treated *oxi1* and *mca1* but enhanced to varying degrees in *rbohD*, *rbohF*, *rbohDF*, *jar1*, and *the1* seedlings (Fig. 2B; Supplemental Fig. S2A). These observations suggest that RBOHD(F)-derived ROS and THE1 function as repressors of JA production in response to CWD.

JA is not detectable in *aos* and increased in *jar1* seedlings, and both mutants show enhanced lignin deposition in response to CWD. This suggests that AOS is required to generate CWD-induced JA, and, if JAR1 is not functional, JA accumulates in *jar1* seedlings. A similar observation has been made previously in *jar1* leaves (Suza and Staswick 2008). It also suggests that JA-Ile is the active compound for inhibition of CWD-induced lignin deposition. The reason being that it cannot be generated in *aos* (because the precursor JA is missing) or *jar1* seedlings (because the conjugation of JA to Ile is not possible), and both mutants exhibit enhanced CWD-induced lignin deposition.

CWD-Induced ROS Production Requires RBOHD and THE1 But Is Inhibited by OXI1

The phenotypic effects of the *rboh* and *oxi1* mutants on lignin deposition and JA production implicated

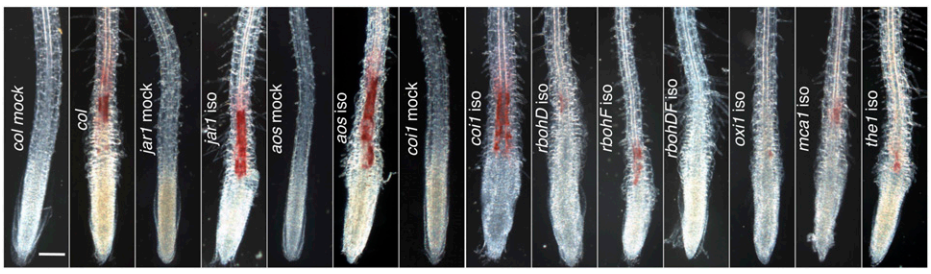

Figure 1. Lignin deposition in Col-0 and mutant Arabidopsis seedlings. Dark-field images of primary root tips stained with phloroglucinol for lignin deposition after 12 h. Genotypes and treatments of seedlings are shown in the pictures. Bar = 200 μm.

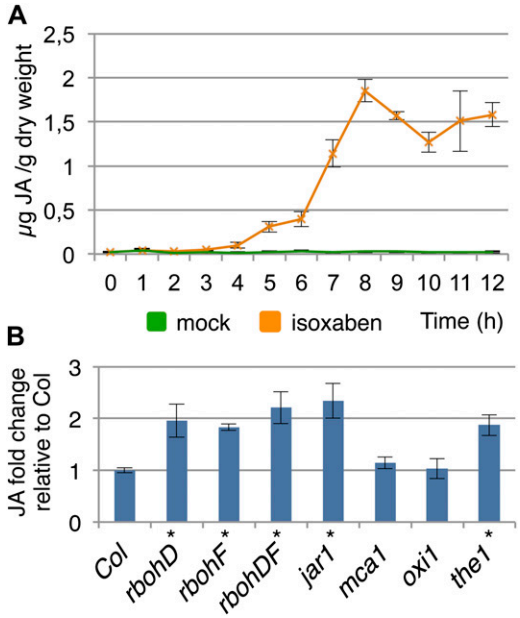

Figure 2. JA production in mock- or isoxaben-treated Col-0 and mutant seedlings. A, JA concentration in mock-treated (green) or isoxaben-treated (orange) Col-0 seedlings. B, JA concentration in different mutant seedlings (blue) after 7 h of isoxaben treatment normalized to isoxaben-treated Col-0 seedlings (*Col*). Significance: $*P < 0.05$. The others are not significantly different from Col-0 isoxaben-treated seedlings.

ROS-based signaling processes in the response to CWD. To determine if CWD induces ROS production, a luminol-based method detecting H_2O_2 was used for in vivo quantification (Keppler et al., 1989). ROS production was detected in roots of Col-0 seedlings after 3 to 4 h of isoxaben treatment (Figure 3A; Supplemental Fig. S4). To determine if any of the genes implicated in the CWD response affect ROS production, *rbohD*, *rbohF*, *rbohDF*, *oxi1*, *the1*, *mca1*, *aos*, and *jar1* seedlings were mock or isoxaben treated, and ROS generation was quantified. No ROS production was detected in mock-treated seedlings (Fig. 3B). No significant differences in comparison to isoxaben-treated Col-0 were observed in *rbohF*, *aos*, *jar1*, or *mca1* seedlings (Fig. 3B). However, *rbohD*, rboh*DF*, and *the1* seedlings exhibited significantly reduced ROS production, whereas it was enhanced in *oxi1* (Figure 3B; Supplemental Fig. S4). These experiments show that isoxaben treatment induces ROS production and that RBOHD and THE1 are required for ROS production, whereas OXI1 seems to limit it.

Manipulation of Ca^{2+}, ROS, and JA Signaling Inhibits CWD-Induced Lignin Deposition

Data presented above suggest that ROS- and JA-mediated processes, together with genes implicated in CWD and mechanoperception, influence CWD-induced lignin production. To clarify the role of the different signaling cascades in response to CWD, pharmacological cotreatments combining isoxaben with different chemicals were performed. *mca1* and *rbohD* seedling root tips exhibited reduced lignin deposition in response to CWD. Overexpression of MCA1 enhances hypoosmotic shock-induced Ca^{2+} influx into the cytoplasm, and Ca^{2+} has been shown to regulate RBOHD activity in Arabidopsis (Nakagawa et al., 2007; Ogasawara et al., 2008). DPI acts as an inhibitor of flavin-containing ROS-generating enzymes like NADPH oxidases (Hancock and Jones, 1987). Accordingly, the effects of calcium antagonists (EGTA and $LaCl_3$) and DPI on lignin deposition were examined. In parallel, seedlings were also treated with methyl-jasmonate (MeJA), which forms a precursor for JA-Ile. Notably, DPI, EGTA, MeJA, and $LaCl_3$ inhibited lignin deposition in a concentration-dependent manner (Fig. 4; data not shown). The results show that it is possible to reproduce the phenotypic effects of the mutations in RBOHD, RBOHF, THE1, and MCA1 by pharmacological intervention. These observations confirm that (intact) Ca^{2+} signaling and DPI-sensitive ROS production are essential for CWD-induced lignin deposition. In addition, the inhibition of lignin deposition by MeJA is in accordance with the opposite effect observed in the *aos*, *jar1*, and *coi1* seedlings (compare Fig. 1 with Fig. 4). Importantly, none of the substances employed induced detectable cell death after 12 h at the concentrations used (Supplemental Fig. S3). These observations provide additional support for the concept that JA- and ROS-based processes jointly regulate lignin deposition in a Ca^{2+}-dependent manner.

MeJA, Ca^{2+} Signaling Inhibitors, and DPI Inhibit CWD-Induced ROS Production

To resolve the question of JA- and Ca^{2+}-based signaling processes affecting ROS production, seedlings were treated with MeJA, EGTA, and $LaCl_3$ alone or combined with isoxaben, and ROS production was measured. Unexpectedly, 1 mM MeJA alone induced ROS production (light-blue trace, Supplemental Fig. S4A). However, the temporal pattern of the response was different from isoxaben-induced ROS and may have resulted from nonspecific stress (Supplemental Fig. S5A). We therefore decided to use only 0.1 mM MeJA in this experiment since it did not induce ectopic ROS production (Supplemental Fig. S5A). Cotreatments of isoxaben with EGTA, $LaCl_3$, and MeJA prevented ROS production at the lowest concentrations employed (Fig. 5; Supplemental Fig. S4B). To confirm independently that ROS detected after 3 to 4 h is generated by a DPI-sensitive enzyme (possibly an NADPH oxidase), seedlings were cotreated with isoxaben and DPI. DPI inhibited ROS production in a concentration-dependent manner (Supplemental Fig. S5B). These experiments demonstrate that Ca^{2+}-dependent processes (signaling or components of the cell wall) are involved in ROS induction by CWD and that both MeJA and DPI can inhibit ROS generation.

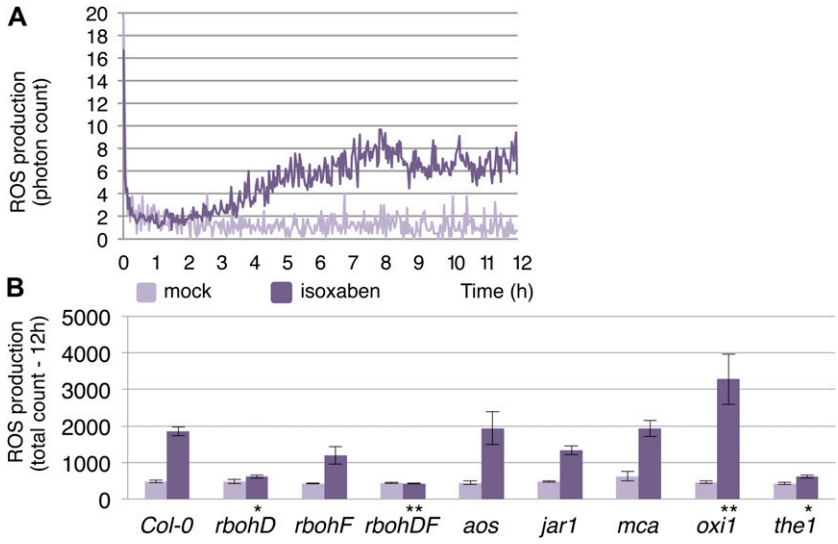

Figure 3. ROS generation in mock- or isoxaben-treated Col-0 and mutant seedlings. A, ROS production in Col-0 seedlings either mock treated (light gray) or isoxaben treated (dark gray). B, ROS production in wild-type and mutant seedlings either isoxaben-treated (dark gray) or mock treated (light gray). Significance: $*P < 0.05$; $**P < 0.001$. The others are not significantly different from Col-0 isoxaben. [See online article for color version of this figure.]

CWD-Induced JA Production Is Inhibited by Ca^{2+} Signaling Inhibitors and DPI

We proceeded to determine how the different chemicals alone or in combination with isoxaben affect JA production. Treatment with 10 mM EGTA, 10 mM $LaCl_3$, or 10 μM DPI individually either did not cause (DPI) or caused only minor JA accumulation (EGTA and $LaCl_3$; Supplemental Fig. S2B). However, EGTA, $LaCl_3$, and DPI all inhibited isoxaben-induced JA production in a concentration-dependent manner (Fig. 6; Supplemental Fig. S2B). These results show that Ca^{2+}-based and DPI-sensitive processes are required for isoxaben-induced JA production. ROS production seems to be more sensitive to EGTA and $LaCl_3$ treatment than JA biosynthesis because complete inhibition of ROS production was observed at concentrations of 1 mM $LaCl_3$ and 2.5 mM EGTA, at which isoxaben still induced JA production (compare Supplemental Figs. S2 and S5). These observations suggest that different Ca^{2+}-dependent processes may regulate CWD-induced JA and ROS production.

Delayed Addition of DPI Mimics the Effects of *rbohdf* on JA Production

The DPI effect on JA biosynthesis was unexpected since increased JA production was detected in *rbohD* and *rbohF* mutant seedlings after 7 h of isoxaben treatment (Fig. 2B), suggesting that RBOHD(F)-derived ROS inhibit JA production. Therefore, the possibility that the CWD response might consist of a two-stage process was considered. ROS could have opposite functions in the different phases and being produced by different DPI-sensitive ROS-generating enzymes. Accordingly, time-course experiments were performed in which DPI addition was delayed relative to isoxaben addition, and JA concentrations were measured. The measurements showed that delaying DPI addition by 3 to 4 h removed the inhibitory effect previously observed (Fig. 7A; Supplemental Fig. S2C). The enhanced JA levels recorded after the delayed pharmacological interventions suggest that the DPI treatment mimics loss of the RBOHD and F activities (compare Figs. 7A and 2B). Since Ca^{2+} antagonists inhibited CWD-induced JA accumulation, similar to DPI, the same experimental approach was used to assess the impact of delayed addition of a calcium antagonist ($LaCl_3$). Interestingly, a somewhat similar effect was observed. JA was detectable at later time points but did not recover to the same extent as that observed following delayed DPI addition (Fig. 7A; Supplemental Fig. S2C). The same experimental design was used to assess the effects of calcium antagonists on isoxaben-induced ROS production. In contrast with the results for JA production, delaying addition of $LaCl_3$ and EGTA still prevented ROS production (Supplemental Fig. S6).

Additionally, the effect on lignin deposition by delayed addition of DPI, $LaCl_3$, and EGTA was determined. All three substances apparently inhibited lignin

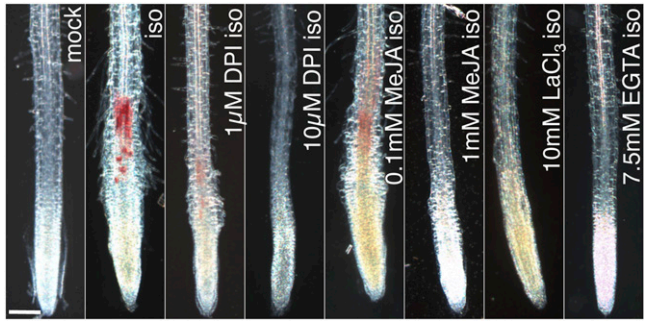

Figure 4. Effects of Ca^{2+} inhibitors, MeJA and DPI, on CWD-induced lignin production in Col-0 seedlings. Dark-field images of phloroglucinol stained primary root tips from Col-0 seedlings treated for 12 h as described on the pictures. Bar = 200 μm.

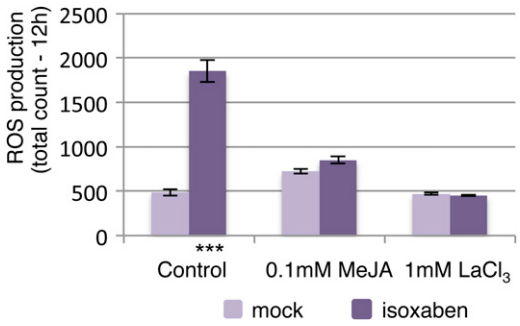

Figure 5. Effects of MeJA and $LaCl_3$ on ROS production in Col-0 seedlings. Results of ROS measurements in Col-0 seedlings mock treated, treated with MeJA, with the calcium antagonist $LaCl_3$ individually (light gray) or in conjunction with isoxaben (dark gray). Significance: $***P < 0.0001$. The others are not significantly different from Col-0 mock. [See online article for color version of this figure.]

production upon addition since the lignin deposition observed after 12 h resembled the lignin detected after 6 h rather than 12 h of treatment (Fig. 6B). To summarize, delayed addition of DPI and Ca^{2+} inhibitors allows CWD-induced JA production, whereas the Ca^{2+} inhibitors prevent ROS generation regardless of when they are being added. All three substances stopped lignin production upon addition.

DPI Treatment and Mutations in RBOHDF Have Different Effects on Expression of CWD Response Genes

To determine if DPI addition and mutations in RBOHDF have different effects on the early transcriptional response to CWD, time-course experiments were performed and the expression of CWD-induced genes analyzed (Fig. 8). Col-0 seedlings were treated with isoxaben, isoxaben + DPI, or isoxaben with DPI addition delayed by 2 h relative to isoxaben. In parallel, *rbohDF* seedlings were treated with isoxaben. To assess the impact of the treatments/mutations on the expression of CWD response genes, *AOS*, *OPCL1* (both implicated in JA biosynthesis), and *RBOHD* were chosen as representative indicators. Isoxaben treatment induced *OPCL1* expression (blue graphs) in an apparent biphasic manner, while cotreatment with DPI from the start reduced this induction. Delayed addition of DPI mimicked the effect of mutations in *RBOHDF*.

AOS expression (orange graphs) was induced after 4 and 7 h in isoxaben-treated Col-0 seedlings. Gene expression appeared reduced both in DPI- and delayed DPI-treated Col-0 seedlings with the absolute induction after 7 h not being as strong as in Col-0 seedlings treated with isoxaben alone. *AOS* expression was enhanced after 4 h in *rbohDF* seedlings (which can be correlated with enhanced JA production in the mutant). *RBOHD* expression (purple graphs) was induced in Col-0 seedlings by isoxaben treatment with a first peak after 1 h and a second induction after 7 h, which is similar to *OPCL1*. Expression of *RBOHD* was inhibited completely by both DPI treatments, suggesting that a DPI-sensitive process is required for isoxaben-induced *RBOHD* expression.

To summarize, our results suggest that the CWD response may include an early and a late stage. During the early stage, a DPI-sensitive enzyme is required for signaling that subsequently leads to induction of *RBOHD*, *OPCL1*, and *AOS* expression and JA production. The effect of the *rbohDF* mutations on *AOS* expression is different from the impact of the early DPI treatment of Col-0 seedlings, suggesting that RBOHDF may have a specific function in the regulation of *AOS* expression. JA and ROS production exhibit different sensitivities and dynamics in response to antagonist treatments. This indicates that Ca^{2+}-based signaling processes are important for both ROS and JA induction and that separate processes may regulate ROS and JA generation during the late stage.

DISCUSSION

Evidence is accumulating that a cell wall integrity sensing and maintenance mechanism exists in plants that coordinates the response to CWD (Seifert and Blaukopf, 2010). Previous research has implicated JA, ethylene, and ROS-based signaling cascades as well as the receptor-like kinase THE1 in mediating the response to CWD (Ellis et al., 2002; Caño-Delgado et al., 2003; Hématy et al., 2007). Here, we combined the phenotypic characterization of mutants involved in CWD perception and JA and ROS signaling with treatments affecting Ca^{2+}, ROS, and JA signaling cascades. This enabled us to perform a systematic analysis

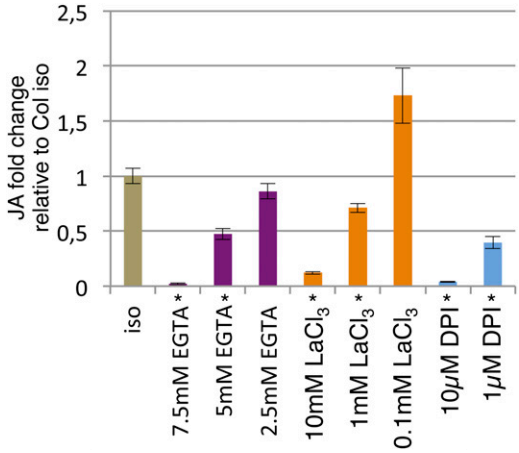

Figure 6. JA production in Col-0 seedlings treated with isoxaben and DPI or Ca^{2+} inhibitors. JA concentration in Col-0 seedlings treated with isoxaben and different ROS and Ca^{2+} signaling antagonists for 7 h normalized to Col-0 isoxaben treated for 7 h (beige), EGTA (violet), $LaCl_3$ (orange), and DPI (blue). Significance: $*P < 0.05$. The others are not significantly different from Col-0 isoxaben-treated seedlings.

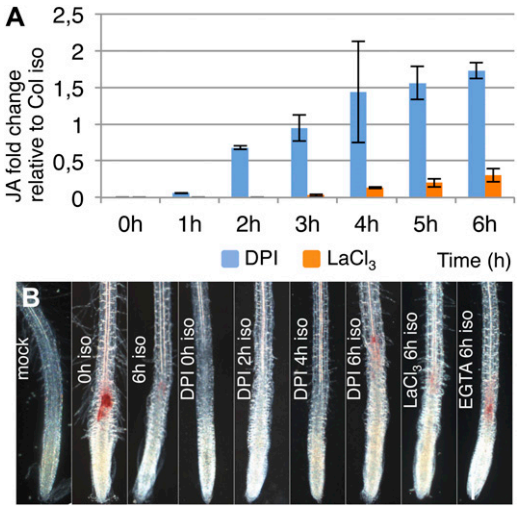

Figure 7. Phenotypic effects of delayed addition of different inhibitors. A, Effect of delaying addition of DPI (10 μM; light blue) or $LaCl_3$ (10 mM; orange) on JA accumulation in Col-0 seedlings treated with isoxaben. The *x* axis shows length of delay relative to start of isoxaben treatment in hours. Data for isoxaben/DPI- or $LaCL_3$-treated Col-0 seedlings are shown as fold change after normalization to JA concentrations observed in Col-0 seedlings after 7 h of isoxaben treatment. B, Dark-field images of primary root tips from Col-0 seedlings stained for lignin deposition after 12 h of mock or isoxaben treatment combined with DPI (10 μM), $LaCl_3$ (10 mM), or EGTA (7.5 mM). Hours on pictures show time points when inhibitors were added. Isoxaben was added at 0 h unless otherwise stated. Bar = 200 μm.

dissecting the response network regulating CWD-induced lignin biosynthesis.

The model depicted in Figure 9 summarizes our current understanding of the mode of action for the CWD response network in Arabidopsis. The initial stimulus is generated by CWD, and the downstream response analyzed here is lignin deposition. Intact ROS-based signaling processes are essential for CWD-induced lignin deposition since *oxi1*, *rbohD*, and *the1* affect ROS production, and all exhibit impaired lignin deposition. Lignification appears to be inhibited by JA-Ile, since in *jar1* seedlings (lacking JA-Ile), both JA concentration and lignin deposition are enhanced. This conclusion is supported by *aos* (not having any detectable JA) and *coi1* (impaired in JA-Ile perception) seedlings, which all exhibit enhanced lignin deposition (Fig. 1). In accordance with the mutant phenotypes observed, MeJA treatment prevents isoxaben-induced lignification in Col-0 seedlings in a concentration-dependent manner. JA production at this stage is in turn inhibited by RBOHD(F)-derived ROS, as shown by the JA increase and ROS decrease in *rbohD* and *rbohDF* seedlings. A similar effect can be observed in the wild-type seedlings exposed to delayed addition of DPI (Fig. 7A). The results from the quantitative reverse transcription (RT)-PCR-based expression analysis support this conclusion further and suggest the activity of AOS is transcriptionally regulated. In *the1* seedlings, JA production is increased and ROS decreased, independently supporting an inverse relationship between JA and ROS. *oxi1* seedlings exhibit increased ROS but normal JA production. These effects suggest that OXI1 formally acts as a repressor of ROS production and translator of the ROS signal to downstream targets (JA production) possibly via MAP kinase cascades (Rentel et al., 2004). Accordingly, loss of OXI1 would allow an increase in ROS production but prevent the increased ROS from inhibiting JA production since the signal is not translated to downstream targets. While MeJA treatment represses ROS production, neither *aos* nor *jar1* seedlings exhibited enhanced ROS production. These observations suggest additional regulatory elements mediating ROS-JA interactions. However, our data support the concept of a negative feedback loop mechanism coordinating JA and ROS production (Fig. 6). This feedback loop would then in turn regulate lignin production initiated in response to CWD.

Early additions of DPI and Ca^{2+} inhibitors prevented later production of JA and ROS. Induction of *OPCL1* and *AOS* was reduced by early DPI addition, while delayed addition mimicked the effect of the *rbohDF* mutations on gene expression. Transcriptional activation of *RBOHD* seems to depend on a DPI-sensitive process. CWD-induced JA and ROS production exhibit different levels of sensitivity to Ca^{2+} antagonist treatment. While delayed addition of ROS and Ca^{2+} antagonists allows late stage JA production, delaying addition of Ca^{2+} antagonists still prevents ROS production. Interestingly, DPI treatment of wild-type seedlings affects JA production in a different way from loss of *RBOHD*. These observations suggest that the CWD response process can be divided into early and late stages (Fig. 9, early and late stage) and that distinct Ca^{2+}-dependent signaling cascades regulate late stage ROS and JA production (Fig. 9, Ca^{2+}, blue). Furthermore, loss of RBOHD and RBOHF activity can be compensated for during the early ROS signaling step. The two-stage process resembles the classic biphasic ROS production observed during plant-pathogen interactions, highlighting possible similarities between the response to CWD and microbial challenge (Lamb and Dixon, 1997).

The NADPH oxidase RBOHD, which seems to be responsible for CWD-induced ROS production, is synergistically activated by changes in cytoplasmic Ca^{2+} concentration and phosphorylation (Benschop et al., 2007; Nühse et al., 2007; Ogasawara et al., 2008). MCA1 can enhance Ca^{2+} influx into the cytoplasm, which could occur upon CWD, thus representing a possible way to regulate RBOHD activity (Nakagawa et al., 2007). However, although *mca1* seedlings exhibit reduced lignin deposition in response to isoxaben treatment, ROS and JA production are normal in the *mca1* background. This observation suggests either a more complex role for MCA1 in the CWD response or that its paralog MCA2 may partially compensate for loss of MCA1 (Yamanaka et al., 2010). The less pronounced effects of Ca^{2+} antagonists on JA production (compared to ROS generation) could result from them affecting

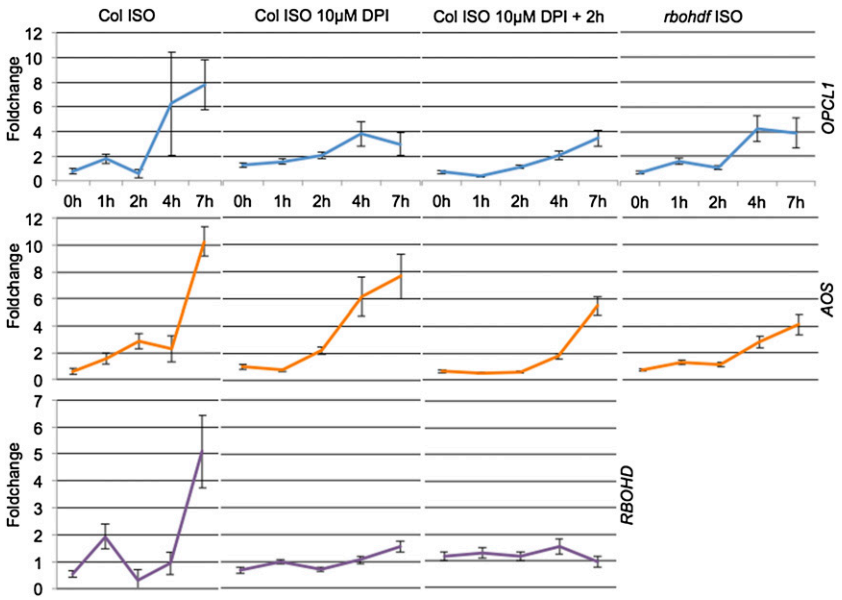

Figure 8. Quantitative RT-PCR characterization of selected genes in Col-0 and rbohD seedlings. Expression data for *OPCL1* (blue), *AOS* (orange), and *RBOHD* (purple) were standardized using *UBIQUITIN10* and normalized to the corresponding mock treatment (without isoxaben). Values and error bars (STDEV) are based on three biological replicates. ISO, Isoxaben; DPI + 2h, addition of DPI was delayed by 2 h relative to addition of isoxaben.

only the Ca^{2+}-dependent positive feedback loop enhancing JA biosynthesis and not the process initially inducing JA production (Bonaventure et al., 2007; Beyhl et al., 2009). The *TWO-PORE CHANNEL1* (*TPC1*) protein could be involved since its gain-of-function allele *fatty acid oxygenation upregulated2* causes enhanced JA production (Bonaventure et al., 2007). *TPC1* encodes a Ca^{2+}-sensitive and voltage-dependent channel that seems to mediate vacuole-derived Ca^{2+} signals (Peiter et al., 2005; Beyhl et al., 2009).

Pathogen-induced ROS production in the apoplast is generated by NADPH oxidases and peroxidases (Grant et al., 2000a, 2000b; Torres et al., 2002; Bindschedler et al., 2006; Choi et al., 2007). RBOHD and F have been implicated in this process (Torres et al., 2002; Kwak et al., 2003; Zhang et al., 2007). The modification of the isoxaben-induced phenotypic and transcriptional responses observed in the *rbohD*(*F*) mutant seedlings and DPI-treated wild-type seedlings suggest that an early DPI-sensitive process (possibly involving another NADPH oxidase) is required to initiate the CWD response. In the *rbohD*(*F*) mutant seedlings, another DPI-sensitive enzyme could compensate for the loss of RBOHD(F) activity, which would not be possible in DPI-treated seedlings.

In *the1* seedlings, ROS production is reduced, while JA induction is enhanced. This suggests that CWD is still being detected and JA production induced but not repressed, as in isoxaben-treated wild-type plants. Different scenarios could explain this situation. It could be due to redundancy, either because several cell wall integrity sensors exist in Arabidopsis or due to the action of other members of the *THE1* family (Hématy and Höfte, 2008). Wall-associated kinases are possible alternative cell wall integrity sensor candidates since they have been shown to bind to a cell wall carbohydrate (He et al., 1996; Brutus et al., 2010). Similarly, the THE1 paralogs FERONIA (FER) and HERKULES could theoretically play a similar role (Escobar-Restrepo et al., 2007; Guo et al., 2009). FER appears to be enriched in detergent-resistant membranes after elicitation of cell suspension cultures with the bacterial pathogen-associated molecular pattern (PAMP) flg22 (Keinath et al., 2010). In addition, *fer* seedlings exhibited an enhanced ROS burst upon stimulation with flg22, suggesting that FER negatively regulates PAMP-induced ROS burst (Keinath et al., 2010). In parallel, Deslauriers and Larsen (2010) found evidence suggesting that FER is required for the interaction between brassinosteroid- and ethylene-mediated processes during cell elongation. These observations suggest that FER is involved in different signaling processes; therefore, it may function as an exchangeable signaling module. Alternatively, THE1 could be acting downstream of the

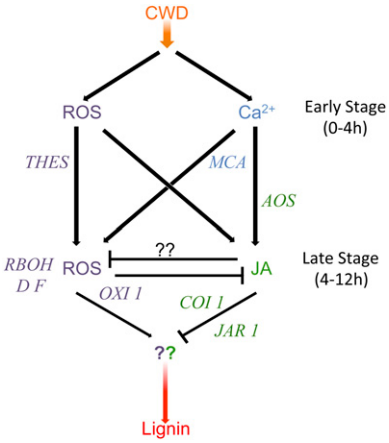

Figure 9. Model depicting the two-stage process mediating the response to CWD. Orange, Initial stimulus generated by CWD; blue, putative calcium signaling genes; purple, genes involved in ROS signaling in purple; green, JA-related genes; red,lignin production.

early stage and upstream of the later stage RBOHD(F)-mediated processes. The latter possibility would mean that THE1 acts as a connecting element between the early and late stage of the CWD response and not as a cell wall integrity sensor per se.

The CWD-induced ROS production and the network regulating it exhibit most of the characteristics described for plant responses to environmental stress and PAMPs (Galletti et al., 2008). It is becoming increasingly apparent that perception of damage-associated molecular patterns (DAMPs), such as cell wall fragments, induce similar responses as PAMPs (Boller and Felix, 2009; Zipfel, 2009). The mechanism by which the very specific effect of isoxaben on cellulose synthesis is detected and activates the responses is not known. One possibility is that it is similar to the yeast system where sensors monitor the physical state of the wall, turgor pressure, and/or membrane stretch to initiate appropriate responses. Alternatively, the disruption of the cell wall may release DAMPs, such as oligogalacturonides, from the wall matrix that may act as elicitors of lignification. The latter hypothesis is in agreement with the observation that most of the genes implicated in the response to CWD (*OXI1*, *RBOHD*, *RBOHF*, *COI1*, *AOS*, and *JAR1*) have been previously implicated in the responses to biotic stress (Rentel et al., 2004; Chico et al., 2008; Suza and Staswick, 2008; Boller and Felix, 2009). Whether it is elicited through an integrity sensor system or the release of DAMPs, we propose that the response to CWD may be a core element of all stress responses, a unifying factor that has simply not been recognized before.

MATERIALS AND METHODS

Plant Material

Arabidopsis (*Arabidopsis thaliana*; Col) tissue was generated using a liquid culture technique. Forty milligrams of seeds was sterilized with 70% ethanol for 5 min and 50% bleach for 5 min and then rinsed three times with sterile Milli-Q water. Seeds were then added to 125-mL flasks containing growth media. Growth media was composed of 2.1 g/L Murashige and Skoog salts (Murashige and Skoog basal medium with vitamin; PhytoTechnology Laboratories), 0.5 g/L MES salts (Acros Organics), and 1% Suc at pH 5.7. Flasks of tissue were then grown under a long-day cycle (16:8) at 23°C and 120 μmol m^{-2} s^{-1} light rotating at a constant speed of 130 rotations per minute. To generate CWD, isoxaben, a specific cellulose biosynthesis inhibitor was used (Scheible et al., 2001). Mutant lines were ordered either from the Nottingham Arabidopsis Stock Centre (http://arabidopsis.info/) or from the labs where they were originally isolated (*the 1-1*, Hofte Lab; *oxi1-1*, SALK_135617, Knight lab). Chemicals were ordered from Sigma-Aldrich unless stated otherwise.

Phytohormone Measurements

JA, ABA, and salicylic acid were measured as described by Forcat et al. (2008). Each data point in the manuscript is based on at least three biological replicates, and the error bars are based on SD. The experiments were repeated at least three times independently. Statistical significance was assessed using the Student's *t* test.

Microscopy

Phenotypic characterization of seedlings was performed using a Leica dissecting scope (Leica Microsystems) or a Zeiss Axioskop 2 Plus. Pictures were taken using a Leica DC 500 digital or Zeiss Axiocam camera. Adobe Photoshop and Illustrator CS 3 were used for data processing (Adobe). Lignin stains were performed as described by Caño-Delgado et al. (2003). For each treatment or genotype analyzed, at least 50 seedlings were characterized in a minimum of three independent experiments.

ROS Measurements

Arabidopsis seedlings were grown in 96-well plates on Gamborg 5 medium supplemented with 1% Suc for 6 d at 23°C under a long-day cycle (16:8). Detection of ROS production was monitored by a luminol-based assay adapted from Keppler et al. (1989). Before the measurement, the medium was removed and 100 μL of assay solution (17 mM luminol, 1 μM horseradish peroxidase, and 600 nM isoxaben) was added to the wells. For spatial imaging of ROS production L-012 solution was used at a concentration 0.5 μM (Wako Chemicals). Luminescence was measured using a Photek camera system and acquired over time. Each measurement is based on monitoring at least 12 individual seedlings. Statistical significance was assessed by comparison to control (Col-0 or mock) using one-way ANOVA followed by Dunnett's test: $^{*}P < 0.05$, $^{**}P < 0.001$, and $^{***}P < 0.0001$.

Evans Blue Stain

The Evans blue staining technique was adapted for use with 6-d-old Arabidopsis seedlings to determine if isoxaben or any of the chemicals employed causes seedling lethality (Sanevas and Sza, 2007). Positive controls were generated by treating seedlings of the same age with either 100% dimethyl sulfoxide or a saturated (6.15 M) NaCl solution for 30 min before washing with sterile water. After 12 h of treatment with the chemicals to be tested, 20 individual seedlings representing one biological replicate were isolated and treated with 0.25% (w/v) Evans blue for 10 min (20 biological replicates were performed per substance to be tested). They were washed thoroughly to remove all traces of excess dye on seedlings using sterile Milli-Q water. Seedlings representing one biological replicate were placed in 1 mL of formamide for 24 h. After 24 h, the formamide was removed and absorption at 600 nM measured. Average and SD were determined for 20 biological replicates per treatment and after subtracting the average mock absorption level, plotted as a bar graph. Error bars are based on SD.

Quantitative RT-PCR Analysis

For isolation of total RNA for quantitative RT-PCR experiments, an RNeasy mini kit was used as described by the manufacturer (Qiagen). For reverse transcription, the Qiagen QuantiTect Reverse transcription kit was used according to manufacturer's instructions. The Qiagen QuantiTect SYBR Green PCR kit was used for quantitative RT-PCR expression analysis. The gene-specific primers employed are as follows: *AOS* (5′-TTTGAGGCATGTGTTGTGGT-3′; 5′-CTTACCGGCGCATTGTTTAT-3′); *RBOHD* (5′-CTGCTCCGTGCTTTCAGAT-3′; 5′-AATCCTTGTGGCTTCGTCAT-3′); *OPCL1* (5′-ACGCTTCCACAGGTCAAAAC-3′; 5′-GAGACAAACGACGGGGAATA-3′); and *UBQ10* (5′-TCTCTCTACCGTGATCAAGATG-3′; 5′-CAAACCCAGAAATCGTCTTCA-3′). The quantitative PCR reactions were performed on a Corbett Rotorgene 3000 machine and an Applied Biosystems 7500 PCR system according to the manufacturer's instructions. The Relative Expression Software Tool 384, version 2, was used for quantification of gene expression and statistical analysis (Pfaffl et al., 2002). Each data point is based on three biological replicates.

Supplemental Data

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

Supplemental Figure S1. Lignin deposition in Col-0 and mutant Arabidopsis seedlings illustrating the variability observed.

Supplemental Figure S2. Quantification of jasmonic acid in mock- or isoxaben-treated Col-0 and mutant seedlings or treated with chemicals affecting ROS and Ca^{2+} signaling.

Supplemental Figure S3. Evans blue stain of Col-0 seedlings treated with different chemicals.

Supplemental Figure S4. ROS detection in Col-0 and *rbohd* seedlings.

Supplemental Figure S5. ROS Quantification in Col-0 seedlings treated with substances affecting ROS, JA, and calcium signaling.

Supplemental Figure S6. ROS quantification in Col-0 seedlings treated with isoxaben and calcium antagonists.

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