RBOH-mediated ROS production facilitates lateral root emergence in Arabidopsis

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ABSTRACT

Lateral root (LR) emergence is a highly coordinated process in which the plant hormone auxin plays a central role. Reactive oxygen species (ROS) have been proposed to function as important signals during auxin-regulated LR formation; however, their mode of action is poorly understood. Here, we report that Arabidopsis roots exposed to ROS show increased LR numbers due to the activation of LR pre-branch sites and LR primordia (LRP). Strikingly, ROS treatment can also restore LR formation in aux1 lax3 mutant lines in which auxin-mediated cell wall accommodation and remodeling in cells overlying the sites of LR formation is disrupted. Specifically, ROS are deposited in the apoplast of these cells during LR emergence, following a spatiotemporal pattern that overlaps the combined expression domains of extracellular ROS donors of the RESPIRATORY BURST OXIDASE HOMOLOGS (RBOH). We also show that disrupting (or enhancing) expression of RBOH in LR and/or overlying root tissues decelerates (or accelerates) the development and emergence of LRs. We conclude that RBOH-mediated ROS production facilitates LR outgrowth by promoting cell wall remodeling of overlying parental tissues.

KEY WORDS: Lateral root emergence, Reactive oxygen species, Auxin, Respiratory burst oxidase homologs, Auxin-mediated cell wall remodeling

INTRODUCTION

Root branching plays a crucial role enhancing the ability of the root system to explore and take up water and nutrients from the soil environment. In the model plant Arabidopsis, lateral roots (LRs) are derived from pairs of xylem pole pericycle cells located deep within the primary root (Dubrovsky et al., 2006; Himanen et al., 2002; Jansen et al., 2013; Malamy and Benfey, 1997). The hormone auxin plays a key role during early developmental stages of LRP (Casimiro et al., 2001). Increased auxin levels mediated by auxin influx and efflux transporters (Benkova et al., 2003; Marchant et al., 2002; Marhavy et al., 2013) are perceived by TIR1 and AFB receptors and trigger degradation of different AUX/IAA repressors of auxin response transcription factors (ARFs), releasing the expression of auxin-responsive genes (De Smet, 2011; Lavenus et al., 2013).

Early auxin-response modules controlling LR formation, namely ARF7 and ARF19 (Okushima et al., 2007), SLR (also known as IAA14) (Fukaki et al., 2002), IAA28 (Rogg et al., 2001) and SHY2 (IAA2) (Goh et al., 2012; Hosmani et al., 2013; Tian and Reed, 1999; Vermeer et al., 2014), operate within the LRP and in the tissues of the parental root that overlie the LRP to coordinate its initiation and emergence (Swarup et al., 2008). It is now clear that auxin-mediated modifications of cell wall properties represent an essential step during LR development. In the endodermis, the SHY2 signaling module triggers changes in cell volume and wall properties termed ‘spatial accommodation’, thereby facilitating the passage of LR (Vermeer et al., 2014). In the cortex and the epidermal cells overlying the expanding LRP, cell wall remodeling enzymes are induced to facilitate LR emergence (Gonzalez-Carranza et al., 2007; Lewis et al., 2013; Neuteboom et al., 1999; Swarup et al., 2008). The activity of the auxin influx carrier LIKE AUX1 3 (LAX3) localizes the auxin-induced expression of these cell wall remodeling genes that degrade the pectin-rich middle lamellae. In agreement with this, LR emergence through the cortex and epidermis is hampered in lax3 mutants (Swarup et al., 2008) and defects in genes involved in cell wall formation increase the rate of LR emergence, as shown recently with mutants with impaired cell wall biosynthesis (Roycewicz and Malamy, 2014) and abscission (Kumpf et al., 2013).

In addition to hormones like auxin, there is compelling evidence that ROS also function as signaling molecules during plant development, as shown for several signal transduction pathways (D’Haeze et al., 2003; Ishibashi et al., 2012; Joo et al., 2001; Mori et al., 2001) and developmental events such as xylem differentiation (Ros Barcelo, 2005), root gravitropism (Joo et al., 2001), adventitious root formation (Liao et al., 2012) and root-to-shoot coordination (Passaia et al., 2013). Recent evidence also suggests that ROS act during LR formation (Correa-Aragunde et al., 2013; Li and Jia, 2013; Manzano et al., 2014) in relation to auxin response (Correa-Aragunde et al., 2013; Ma et al., 2014), but the mechanistic basis of this crosstalk remains unclear. Among ROS, O2− and H2O2 were shown to be involved in cell wall modifications during several plant developmental processes (Carol et al., 2005; Foreman et al., 2003; Monshausen et al., 2007; Ros Barcelo, 2005). The production of ROS in extracellular spaces depends on several classes of enzymes, including respiratory burst oxidase homologs (RBOH) and class III peroxidases (Sagi and Flühr, 2006; Shapiguzov et al., 2014).
2012). Interestingly, the latter enzymes appear to regulate root branching in an auxin-independent manner (Manzano et al., 2014). To date, it has not been determined whether RBOH are involved in the auxin-mediated signaling leading to cell wall remodeling during LR formation.

In this study, we exploit gene expression datasets to highlight the existence of interplay between ROS and auxin signaling pathways during early steps of LR formation and we show that exogenous application of ROS can rescue LR-less mutants that are defective in auxin signaling in tissues overlying new LRP. Using high-resolution imaging, we reveal that ROS accumulate in the middle lamella of these cells. In addition, spatial expression analysis of several auxin-inducible RBOH genes during LR formation suggests that their activity cause the production of extracellular ROS during this developmental process. Finally, functional studies employing RBOH mutants and the tissue-specific overexpression of RBOH∆ validate the importance of this gene family in facilitating LRP emergence.

RESULTS
An interplay between auxin and ROS signaling during LR formation

We initially analyzed datasets from published microarray experiments (Affymetrix ATH1 arrays) that relate to auxin-mediated LR formation or ROS responses. The experiments involving auxin employed the LR inducible system (LRIS; Himanen et al., 2002; Jansen et al., 2013) and allowed us to pinpoint genes potentially involved in rapid transcriptional response to auxin and most likely involved in LR formation. In the LRIS system, seedlings are grown for 3 days on the auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA) and then treated for 2 h with synthetic auxin-related signaling molecules 1-naphthaleneacetic acid (NAA) or auxin to trigger synchronous LR formation in root pericycle cells (De Rybel et al., 2012; Vanneste et al., 2005). For experiments relating to ROS, 5-day-old seedlings were treated for 1 h with 20 mM H2O2 (Davletova et al., 2005) or 2-week-old seedlings were sprayed for 3 h with 20 mM H2O2 (Ng et al., 2013). A list of 108 overlapping genes (out of 489 genes from the two auxin experiments and 414 genes from at least one of the two H2O2 experiments) were selected employing cut-offs of an absolute fold change ≥2 and a P-value ≤0.05 (Table S1). Of these 108 genes, 90 genes were simultaneously induced in auxin and H2O2 datasets but only two were repressed in both. Furthermore, 13 of the genes were induced during LR formation and were found to relate to redox activity, and 24 were linked to stress response, suggesting that fine-regulation of redox balance is necessary during auxin-mediated LR formation. Consistent with this model, exogenous auxin application increased ROS levels in root tissues (Fig. S1A,B). Hence, our results suggest a link between ROS and auxin-mediated LR formation.

ROS application activates LR pre-branch sites

Seedlings exposed to H2O2 have been previously reported to exhibit an increase in LR number compared with control seedlings (Manzano et al., 2014). We validated this by exposing root segments to H2O2, which increased LR density and length in the exposed segments, whereas root growth rate decreased in a dose-dependent manner after onset of the treatment (Fig. 1A-E). The effect of H2O2 on primary root growth is unlikely to be caused by toxicity as it was reversed completely (for 1 mM H2O2) or partially (1.5 mM H2O2) within 2 days of transfer back on control medium (Fig. S1C). A permanent arrest of the primary root growth was only observed at 3 mM of H2O2.

To investigate further how H2O2 application impacts LR development, 5-day-old seedlings were exposed to H2O2 for 2 days (Fig. 1F). Upon H2O2 treatment, the number of emerged LRs increased, whereas the number of early stage LR primordia decreased. Arabidopsis seedlings produce an excess of LR pre-branch sites, but only a subset will be used for LR production (Van Norman et al., 2014). We used a modified LR inducible system (Himanen et al., 2002; Jansen et al., 2013) to explore the possibility that H2O2 treatment promotes the developmental progression of LRs from these unused precursor sites rather than inducing de novo LR formation. LR formation was synchronized by germinating pDR5:GUS transgenic seedlings for 3 days in the presence of 10 μM NPA followed by transfer onto control media or media supplemented with H2O2 (1.5 mM), the ROS scavenger potassium iodide (KI; 0.01 mM), both H2O2 and KI (1.5 mM and 0.01 mM, respectively), or NAA (10 μM; positive control). Samples were collected at 6 h, 12 h and 18 h after transfer and histochemically stained for GUS activity. In control conditions and upon KI treatment, GUS-positive foci, representing LR founder cells and initiation sites, appeared within 12 h, whereas in 86% of seedlings grown in the presence of H2O2, GUS-positive foci were already observed within 6 h. (Fig. S2A,B). Interestingly, the latter appeared in similar locations compared with control conditions, unlike upon NAA treatment, where synchronous LR formation was induced equally along the root. Hence, our results indicate that ROS facilitates early developmental events leading to LR formation but does not induce de novo LR initiation.

To uncover the effect of ROS on the kinetics of LR development, we employed the root bending assay (Fig. 1G), in which roots are given a 90° gravistimulus to synchronize LR initiation and emergence in the resulting root bend (Peret et al., 2012b) and LR stages are counted 20 and 44 h after gravistimulation (hag) according to the methods of Malamy and Benfey (1997). In parallel to the plate rotation, seedlings were treated with H2O2 (1.5 mM), the O2− donor methyl viologen dichloride hydrate (paraquat; 0.1 μM) or the ROS scavenger KI (0.1 mM). At 20 hag, control roots accumulated mainly stage I LRP. Seedlings treated with ROS donors exhibited a higher percentage of stage II and III in comparison with the control, whereas KI-treated seedlings showed a decrease in stage I LRP. At 44 hag, control plants accumulated mainly stage V, VI and VII LRP. Seedlings treated with ROS donors were more advanced than control seedlings and showed stage VII LRP and emerged LRs, whereas KI-treated seedlings showed a delay in LR emergence in which stages IV to VII were noted.

ROS treatment bypasses the requirement for auxin influx carrier activity during LR initiation and emergence

To assess the capacity of H2O2 to promote LR formation, we investigated whether ROS treatment could rescue mutations disrupting early steps of LR development. AUX1 and LAX3 encode members of a family of auxin influx carriers that are required for LR initiation and emergence, respectively (Lavenus et al., 2013). The combined loss of both genes results in a lateral rootless mutant phenotype (Fig. 2A; Swarup et al., 2008). Strikingly, H2O2 treatment (1.5 mM) of 5-day-old seedlings of the double aux1 lax3 mutant for 7 days resulted in the appearance of emerged lateral roots (Fig. 2A). We found that LR densities were 3.7±0.4 for aux1 lax3 seedlings (n=36) exposed to H2O2 and 3.0±0.2 (n=35) and 5.1±0.5 (n=37), respectively, for wild-type
seedlings in control conditions and exposed to H$_2$O$_2$ (LR/cm, mean ±c.i.). Next, evaluating sensitivity to H$_2$O$_2$ with respect to primary root growth showed that aux1 lax3 is equally sensitive to H$_2$O$_2$ as the control wild-type seedlings. In control conditions, primary root growth rate of 5-day-old wild-type plants transferred to a new control medium for 3 days is similar to aux1 lax3 (7.2±1.3 and

![Fig. 1. The effect of ROS on root development.](image)

(A) Morphology of Col-0 grown in control conditions and upon treatment with increasing H$_2$O$_2$ concentrations. Five-day-old seedlings (transferred region) were exposed to H$_2$O$_2$ for 7 days. White arrowheads indicate the root tip region at the moment of transfer. Scale bar: 1 cm. (B) Primary root (PR) growth rates upon treatment with increasing H$_2$O$_2$ concentrations. Five-day-old seedlings were transferred onto media supplemented with increasing concentrations of H$_2$O$_2$. The root tips of the seedlings were marked each day. After 7 days, the distances between each mark were measured and the average root growth for each time point (technical replicates, n=15 per sample) is shown in the graph. (C,D) Average emerged LR density (C) and LR length (D) in transferred region after 7 days of H$_2$O$_2$ treatment (in three biological replicates, n=30). Owing to a strong effect of H$_2$O$_2$ treatment on primary root growth rates, LR density and length were calculated only for the transferred regions of the root. (E) Average PR length after 7 days of H$_2$O$_2$ treatment (in three biological replicates, n=30). (F) Effect of ROS on LRP density after 2 days of different concentrations of H$_2$O$_2$. (C-F) The difference between groups denoted by different lowercase letters is statistically significant (P<0.005 according to Tukey’s HSD test after ANOVA). (G) Effect of ROS and ROS scavengers on LR emergence phenotype. Five-day-old seedlings were transferred onto media supplemented with various compounds, as indicated above each graph and gravistimulated by turning the plates 90° to achieve synchronization of LR formation. LRP stages according to Dubrovsky et al. (2006), Himanen et al. (2002), Jansen et al. (2013) and Malamy and Benfey (1997), starting from stage I to an emerged LR (E), are shown on x-axis. Data points represent mean±c.i. (in two biological replicates, n=20).
suggest that H$_2$O$_2$ treatment does not influence shootward auxin transport driven by AUX1, which is required for gravitropism, but rather overcomes the absence of the auxin gradient that has been shown to induce the expression of cell wall remodeling genes in the overlying cell layers, which is needed for LR emergence (Swarup et al., 2008).

Auxin efflux carrier activity is also important for LR development (Benkova et al., 2003; Casimiro et al., 2001). The $gnom^{R5}$ mutation (in an ARF GDP/GTP exchange factor involved in polar localization of the auxin efflux regulator PIN1) represents a weak allele and produces an embryonic root devoid of emerged LRs (Geldner et al., 2004). H$_2$O$_2$ treatment of $gnom^{R5}$ seedlings did not overcome the LR phenotype (Fig. S2C) and no massive proliferation of pericycle cells was observed after tissue clearing, indicating that its promoting effect is at least in part dependent on correct GNOM- and PIN1-mediated auxin transport.

To validate our genetic results, we also tested the effects of H$_2$O$_2$ when co-treating roots with inhibitors of auxin influx [1-naphthoxyacetic acid (1-NOA; 10 μM)] and efflux [NPA (1 μM) and 2,3,5-triiodobenzoic acid (TIBA; 10 μM)], which are known to disrupt early steps of LR formation (Casimiro et al., 2001; Peret et al., 2013). We observed that H$_2$O$_2$ treatment bypassed only the inhibitory effects of 1-NOA on LR formation (Fig. S2D,E). We conclude that ROS can bypass impaired influx-dependent auxin accumulation but not defects in auxin efflux carrier transport, as corroborated by the $gnom^{R5}$ data.

**ROS contributes to cell wall remodeling during LRP development**

The auxin influx carrier LAX3 facilitates the accumulation of auxin in cortical and epidermal cells directly overlying new LR primordia, resulting in the induction of cell wall remodeling enzymes to facilitate organ emergence (Swarup et al., 2008). As H$_2$O$_2$ treatment can overcome impaired cell wall remodeling in cortex and epidermis in the lax3 background, we tested whether this observation holds also true for plants with disrupted auxin-dependent endodermal cell wall remodeling. Transgenic lines expressing $pCASP1$:shy2-2 are specifically disrupted in their endodermal auxin response, resulting in an LR-less phenotype (Goh et al., 2012; Hosmani et al., 2013; Vermeer et al., 2014). Strikingly, treatment with 1.5 mM H$_2$O$_2$ rescued LR development in the $pCASP1$:shy2-2 gain-of-function mutants (Fig. 2B). By contrast, neither LRP nor LRs could be induced in mutants in which LR formation is compromised due to defects in pericycle auxin signaling, such as $iaa28$ (Rogg et al., 2001), $arf7$ $arf19$ (Okushima et al., 2007) and $slr$ (Fukaki et al., 2002), suggesting that H$_2$O$_2$ plays a specific role during auxin-mediated wall remodeling in cells overlying new LR primordia (Fig. S3A).

Localized root cell wall remodeling has been reported to be associated with changes in extracellular pH (Bibikova et al., 1998; Monshausen et al., 2007; Vermeer et al., 2014), prompting us to investigate whether H$_2$O$_2$ modifies extracellular pH during LRP development. Using the apo-pHusion reporter line (Gjetting et al., 2012), we observed significant apoplast acidification in the parental ground tissue after 1 day of H$_2$O$_2$ treatment (Fig. S3B). We therefore hypothesize that exposure to H$_2$O$_2$ triggers wall acidification in cells overlying LR primordia to facilitate cell wall remodeling and organ emergence.

**ROS are detected in the middle lamellae of cells overlying developing LRs**

Localization of ROS during LRP development has recently been reported employing a whole-mount staining assay in *Arabidopsis*...
Manzano et al., 2014) and maize (Fig. S3C). We corroborated these observations at a cellular level of resolution using confocal microscopy combined with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, 50 μM) to detect ROS (Aranda et al., 2013). Confocal imaging indicated strong DCFH-DA fluorescence surrounding cortex cells that overlay LRP (Fig. S3D), consistent with ROS playing a role during cell wall remodeling.

To resolve the subcellular localization of the most stable ROS species during LRP development, we employed transmission electron microscopy (TEM) to detect black cerium precipitates, which indicate the presence of H2O2. Our TEM approach detected H2O2 accumulation in the middle lamellae of cell walls, a pectin-based layer that cements the walls of adjacent cells together (Fig. S3D; Table S2). H2O2 accumulation was observed in the middle lamellae of cortical and endodermal cells overlying new LRP. The fine layer of H2O2 covering the entire LRP clearly separated the LRP from parental tissues (Fig. 3B,C). In addition, cerium precipitates were detected inside LRP, particularly within the middle lamellae of cells at their flanks (Fig. 3D). Hence, H2O2 is deposited in the middle lamellae of cells in contact with, and also flanking, LRP during organ emergence.

An auxin-inducible family of RBOH NADP oxidases produces extracellular ROS to facilitate LR development

Given the importance of extracellular ROS deposition during LR development, we investigated the spatial expression of several RBOH genes known to contribute to ROS production. The Arabidopsis
genome contains ten RBOH genes, named RBOHA to RBOHJ (accession numbers: RBOHA, At5g07390; RBOHB, At1g09090; RBOHC, At5g51060; RBOHD, At5g47910; RBOHE, At1g19230; RBOHF, At1g64060; RBOHG, At4g25090; RBOHH, At5g60010; RBOHI, At4g11230; and RBOHJ, At3g45810), expression of which in various organs has been related to different developmental processes (Boisson-Dernier et al., 2013; Foreman et al., 2003; Kwak et al., 2003; Lee et al., 2013; Muller et al., 2009; Torres et al., 2002). During LRP formation, the spatial expression patterns of GUS transgenes driven by various RBOH promoters largely overlap with H2O2 localization in the peripheral cells of the LRP (Fig. 4A). RBOHE was also strongly expressed in endodermis, cortex and epidermis cells overlying LRP (Fig. 4A; Fig. S4A). Interestingly, RBOHA, RBOHC and RBOHE were also expressed in the basal meristem (Fig. S4B), where LR priming occurs (De Smet et al., 2007) and expression of RBOHE is independent of AUX1 and LAX3 (Fig. S4C). Similarly, H2O2 treatment did not affect AUX1 or LAX3 promoter activities (Fig. S4D). Taken together, the expression pattern of RBOH genes inside the developing LRP and the overlying endodermis, cortex and epidermis cells are consistent with NADPH oxidase family members providing the extracellular ROS observed during LR development.

To overcome a potential genetic redundancy within RBOH family members, we employed treatments with the inhibitors of intra- and extracellular enzymes in parallel to the root bending assay. We used the RBOH inhibitor diphenyleneiodonium chloride (DPI, 1 μM) and diethyldithiocarbamate (DDC, 1 mM), which is known to affect the conversion of O2− into H2O2 (Fig. 4B). Whereas control roots at 20 hag accumulated mainly stage I LRP, very few LRP were noticed in inhibitor-treated seedlings. At 44 hag, control plants accumulated mainly stage V, VI and VII LRP. Although no remarkable differences from the control were observed upon treatment with DDC, mostly stage II was detected in DPI-treated seedlings. To determine whether ROS produced by specific RBOH enzymes contribute to LR development, we analyzed root phenotypes of mutant lines lacking selected individual or
combinations of RBOH genes. LR phenotyping of several RBOH mutants revealed a delay in the rate of organ emergence for selected lines (Fig. 4C,D). In particular, higher-order mutants lacking family members RBOHE and/or RBOHD were observed to have the strongest phenotype, consistent with both genes exhibiting the strongest and most widespread expression in overlying tissues (Fig. 4A) in the root bending assay (Fig. 4E). In summary, our genetic and pharmacological studies indicate that extracellular ROS donors contribute to LRP development.

We next investigated the possibility that expression of RBOH genes is auxin inducible. For this purpose, we employed qRT-PCR analysis and focused on RBOH transcript levels in root tissue of young seedlings. Upon treatment with NAA for a given duration, all RBOH transcripts detected in root tissue were upregulated by auxin already within 6 h of treatment (Fig. 5), in agreement with several published transcriptome datasets (Table S1). Hence, auxin was able to induce a strong upregulation of all members of the RBOH gene family expressed in roots.

Tissue-specific overexpression of RBOH promotes LR emergence

In our experimental conditions, seedlings of the 35S:RBOHD line showed many different developmental phenotypes making it impossible to distinguish between the effect of the constitutive expression on LR emergence from secondary effects on plant development (Fig. S4E), probably due to an overall increase in extracellular ROS levels (Mersmann et al., 2010). To determine which specific cell types were most sensitive to ROS accumulation during LR emergence, we targeted RBOH expression to selected root tissue(s) by crossing a homozygous UAS:RBOHD line with various GAL4-GFP enhancer trap lines. These included lines expressed in pericycle (J2661), endodermis and cortex (J3611), epidermis (J0634), simultaneously in LRP and overlying tissues (J0192) or in LRP alone (J1103).

The phenotypic effect of targeted RBOHD overexpression on LR emergence was analyzed using the root bending assay (Peret et al., 2012a) and stages of synchronized LRP development were counted at 44 hag. The control Col-0, C24, UAS:RBOHD and Col-0×C24 seedlings accumulated mainly stage V LRP (Fig. 6A). The activation of UAS:RBOHD construct in the root pericycle and LRP alone had no effect on LR emergence compared with controls, where LRP accumulated mainly at stage V. By contrast, LR emergence was accelerated when UAS:RBOHD overexpression was targeted to the LRP and overlying tissues or only to the overlying root tissues, where LRP accumulated mainly at stage VI and VII (Fig. 6B). Similarly, when we observed the emerged LR density in 10-day-old seedlings, we observed an increased emerged LR density when RBOHD expression was transactivated in LRP and/or overlying root tissues (Fig. 6C,D). In summary, targeted RBOHD overexpression in LRP and/or overlying root tissues promotes organ emergence, in agreement with (sub)cellular distribution of H$_2$O$_2$ (Fig. 3).

DISCUSSION

ROS act downstream of auxin

Multiple auxin response modules are sequentially activated during successive developmental steps leading to the formation of LRs (Lavenus et al., 2013). We report here that auxin is able to induce expression of several RBOH genes and that changes in expression of ROS-related genes are associated with early steps of auxin-induced LR formation. This corroborates previous reports that demonstrated ROS production to occur downstream of auxin-mediated signal transduction pathways (Correa-Aragunde et al., 2013; Ivanchenko et al., 2013; Joo et al., 2001; Ma et al., 2014). In line with their potentially harmful effects, the production of ROS compounds in the apoplast is targeted to restricted spatial and temporal domains within plant organs (Bibikova et al., 1998; Monshausen et al., 2007; Vermeer et al., 2014). In response to unfavorable environmental conditions, such as salinity and drought, LR development is inhibited (De Smet et al., 2006; Duan et al., 2013). It is tempting to speculate that the activation of ROS scavenging machinery that probably occurs during exposure to abiotic stress (Caverzan et al., 2012) interferes with RBOH-mediated ROS production and/or removal from the apoplast, thereby affecting LR development.

Auxin signaling modules, which control LR development both in the LRP and in overlying tissues (Lavenus et al., 2013), are good candidates for the restriction of spatiotemporal ROS production to appropriate cell wall domains. Our additional observation that increased H$_2$O$_2$ levels (supplied externally or most likely by tissue-specific overexpression of RBOHD) accelerate the early steps of LR emergence.
formation further suggests that the LRP and/or the overlying tissues are, at some point, receptive to a signal arising downstream of ROS. Taken together, we propose that auxin triggers RBOH-mediated ROS production where needed, thereby initiating the subsequent steps of LR formation.

**ROS action on cell wall remodeling**

Auxin-regulated changes in wall properties of cells overlying LRP are indispensable for successful LR formation (Swarup et al., 2008; Vermeer et al., 2014). In this study, we demonstrated that ROS treatment can bypass the suppression of expression of genes involved in cell wall remodeling in aux1 lax3 and pCASP1:shy2-22 backgrounds. We also observed that the tissue zone in which H$_2$O$_2$ was recorded in the middle lamellae during LRP development largely corresponds to the expression patterns of several RBOH enzymes known to produce extracellular O$_2$ (Sagi and Fluhr, 2006). Given the relevance of peroxidases producing H$_2$O$_2$ from O$_2$ (Manzano et al., 2014), RBOH enzymes probably serve as O$_2$ donors for peroxidases during this developmental process in defined locations. However, we cannot exclude the possibility that RBOH and peroxidases are acting independently, as conversion of O$_2$ to H$_2$O$_2$ can also occur spontaneously, without any enzymatic support. Pharmacological inhibition of every RBOH enzyme severely impeded LRP development, suggesting that several RBOH enzymes are likely to be involved. Among the members of the RBOH gene family, the auxin-inducible RBOHE was expressed inside the LRP and in overlying cells of the endodermis, cortex and epidermis. These results support the hypothesis that extracellular ROS are directly involved in the modification and/or degradation of the middle lamellae in front of LRP.

**Role of ROS in overlying tissues in LR emergence**

A major displacement in cell position occurs as the expanding LRP traverses the cortex and epidermis layers. In cortical and endodermal cells, LAX3 activity promotes auxin-dependent induction of cell wall remodeling enzymes such as SUBTILISIN-LIKE PROTEASE (AIR3), PEPTACE LYTASE (PLA2) and XYLOGLUCAN ENDOTRANSGLYCOSYLASE (XTR6) (Swarup et al., 2008). The degradation of the middle lamellae by ROS is likely to be a part of the machinery allowing slipping of the cell wall at the boundary between the outer layer cells of the LRP and the neighboring endodermis, cortex and epidermis cells as the LRP expands. Previous studies reported that ROS treatment increases LR number (Correa-
Our findings that RBOH function contributes to LR emergence, which requires cell wall remodeling and accommodation, reveal a key role for RBOH in the control of apoplastic ROS production targeted to restricted spatial and temporal domains during organ outgrowth (Fig. 7). The restriction of RBOH expression to the peripheral cells of the LRP and to the cell files overlying it suggests that auxin signaling pathways control their expression pattern and potentially their activity and subsequent generation of ROS in the middle lamellae. We do not yet know whether induction of RBOHE expression in LR-overflowing cells is auxin regulated, perhaps in parallel with LAX3 in the LBD29/LAX3 signaling module (Porco et al., 2016). Hence, such precise ROS deposition suggests an intimate relationship between ROS and auxin-controlled changes in cell wall biomechanics during LR emergence.

**MATERIALS AND METHODS**

**Plant material and growth conditions**

All *Arabidopsis* lines used in this study have been previously described: AUX1:GUS (Swarup et al., 2004), LAX3:GUS (Swarup et al., 2008), pmRBOH:nlsGFP:GUS (Lee et al., 2013), pCASP1:SHY2, pCASP1:shy2-22 (Vermeer et al., 2014), iaas28-1 (Rogg et al., 2001), slr (Fukaki et al., 2002), aux7 arf19 (Okushima et al., 2007), aux1 lax3 (Swarup and Peret, 2012). The crosses were generated from the SAIL/SALK lines rbohb (SAIL_749_B11), rboc (SALK_071801), rbod (SALK_070610), rbohe (SALK_064850) and rboph (SALK_059888) and were ordered from the Nottingham Arabidopsis Stock Centre.

The GAL4 enhancer trap lines were ordered from the Nottingham Arabidopsis Stock Centre and crosses with a homozygous UAS:RBOHD were generated to produce transactivating lines. The UAS:RBOHD construct was generated by cloning the RBOHD cDNA into plasmid pDONR221 and next into the destination plasmid pKm34Gw0 simultaneously with the pEN-L4-UAS-R1 promoter and pEN-R2-NOS-L3 terminator sequences using a Gateway (Invitrogen) cloning approach. Transgenic plants were generated by a standard floral dip method.

In all experiments with *Arabidopsis*, seeds were sterilized with chlorine gas and stratified at 4°C for 2 days in water. After cold treatment, seeds were sown over solid half-strength Murashige and Skoog (MS) growth medium (per liter: 2.15 g MS salts, 0.1 g myo-inositol, 0.5 g MES, 10 g sucrose, 8 g plant tissue culture agar; pH 5.7 with KOH) (hereafter termed ‘medium’) and grown vertically under continuous light (110 µE m⁻² s⁻¹) photosynthetically active radiation, supplied by cool-white fluorescent tungsten tubes, Osram) for 4-5 days. The scans of the plates were taken with a V700 scanner (Epson) or 3200 dpi scanner (Medion). Seedlings were analyzed in detail with a BX53 microscope (Olympus) equipped with DS-Fi1 camera (Nikon). Figures were arranged in Photoshop CS3 (Adobe Systems) and the brightness was increased equally across samples, without further modifications. To characterize GAL4 enhancer trap lines and transactivation lines, 5-day-old seedlings were imaged with an LSM5 (Axiovert, Zeiss) confocal microscope.

**Transmission electron microscopy**

Cerium hydroxide precipitates indicate H₂O₂ localization. Five-day-old seedlings were gravistimulated by 90° to achieve synchronization of LR formation. After 22 h and 44 h, 2-mm fragments that were expected to contain early and late LR were dissected under binoculars (n=50) and incubated for 1 h in 5 mM cerium chloride solution in 50 mM MOPS buffer [for 100 ml: 1.046 g of 3-(N-morpholino) propanesulfonic acid (MOPS; VWR Chemicals) in 90 ml of water and adjust the pH to 7.2 with 1.7 M Tris (VWR Chemicals)]. Tissue embedding and electron probe x-rays were performed as described (D’Haese et al., 2003).

**qRT-PCR analysis**

Col-0 seeds were sown on half-strength MS supplemented with 1% sucrose and grown for 7 days on a mesh. Seedlings were then transferred to 10 µM NAA for the indicated duration. RNA was extracted from dissected roots and grown vertically under continuous light (110 µE m⁻² s⁻¹) photosynthetically active radiation, supplied by cool-white fluorescent tungsten tubes, Osram) for 4-5 days. The scans of the plates were taken with a V700 scanner (Epson) or 3200 dpi scanner (Medion). Seedlings were analyzed in detail with a BX53 microscope (Olympus) equipped with DS-Fi1 camera (Nikon). Figures were arranged in Photoshop CS3 (Adobe Systems) and the brightness was increased equally across samples, without further modifications. To characterize GAL4 enhancer trap lines and transactivation lines, 5-day-old seedlings were imaged with an LSM5 (Axiovert, Zeiss) confocal microscope.

**LR phenotype analysis**

Five-day-old *Arabidopsis* Col-0 and/or mutant seedlings were transferred on fresh media (control) or on media supplemented with various compounds,
namely DPI (diphenyleuorodione chloride, Sigma-Aldrich), DDC (diethyldithiocarbamate, Alfa Aesar/VWR Chemicals), parquat (methyl viologen dichloride hydrate, Sigma-Aldrich), KI (potassium iodide, AppliChem Lifescience). After 1 h, seedlings were gravistimulated by 90° to achieve synchronization of LR formation. After 20 h and 44 h, seedlings were pre-fixed in 0.4% formaldehyde (Sigma-Aldrich) in 50 mM phosphate buffer (VWR Chemicals) pH 7 at 4°C under a gentle vacuum for 30 min. Subsequently, 2.5 g of chloral hydrate (VWR Chemicals) was dissolved per 1 ml of 30% glycerol (Sigma-Aldrich) and seedlings were left overnight in a cleaning solution. LRPs were observed with a BX53 dissecting microscope (Olympus) equipped with a DS-Fi1 (Nikon) camera and grouped according to developmental stages at 20 h and 44 h after the onset of gravistimulation.

Histological staining
For DAB (diaminobenzidine tetrahydrochloride; Applichem LifeSciences) and NBT (nitroblue tetrazolium chloride; Molekula/VWR Chemicals) staining in maize (B33 inbred line), the root segments were embedded in 6% agarose with 0.5% gelatine and 100-μm thick sections were cut with a vibratome. Sections were immediately transferred for 1 h to NBT staining solution (0.1% NBT in 10 mM potassium phosphate buffer, pH 7.8) according to the methods of Kawai-Yamada et al. (2004) or for 2-3 h to DAB staining solution [1 mg/mL DAB, TWEEN 20 (0.05%) v/v) and 10 mM NaH2PO4, pH=6.8] according to the methods of Daudi and O’Brien (2012). Upon signal development, sections were mounted with dialized water and immediately imaged with an AxioCam microscope (Zeiss).

For DCFH-DA (dichloro-dihydro-fluorescein diacetate; Sigma-Aldrich) staining in Arabidopsis, 5 day seedlings were stained for 15 min in DCFH staining solution (50 μM DCFH-DA in 50 mM phosphate buffer) in darkness according to the methods of Shin et al. (2005). Seedlings were washed briefly in phosphate buffer alone before imaging by confocal microscopy using an LSM5 microscope (Axiovert, Zeiss).

GUS staining
Seedlings were put overnight in 90% acetone, then transferred to a GUS-solution {1 mM X-Gluc, 0.5% (w/v) dimethylformamide (DMF), 0.5% (w/v) Triton X-100, 1 mM EDTA (pH 8), 0.5 mM potassium ferricyanide [K3Fe(CN)6], 0.5% potassium ferrocyanide [K4Fe(CN)6], 500 mM phosphate buffer (pH 7)} and incubated for 4 h at 37°C for GUS staining, and finally washed in 500 mM phosphate buffer (pH 7) for microscopic analysis, samples were cleared in chloral hydrate solution as described by Berleth and Jurgens (1993). Samples were analyzed by differential interference contrast microscopy with Primo Vert (Zeiss) equipped with Moticam 2300 (Motic).

Treatment with auxin inhibitors
Seedlings (5 dp) were transferred for 7 days to control growth media or to media supplemented with 10 μM 1-NOA (1-naphthoxyacetic acid; Alfa Aesar/VWR Chemicals), 10 μM TIBA (2,3,5-triiodobenzoic acid; Alfa Aesar/VWR Chemicals) and 1 μM NPA (N-1-naphthylphthalamic acid; Fluka/Sigma-Aldrich). The plates were scanned with a 3200 dpi scanner (Medion) and LR number was determined using a BX53 dissecting microscope (Olympus) equipped with DS-Fi1 (Nikon) camera.

Microarray data retrieval, normalization and treatment
The following microarray hybridization files were retrieved from the Gene Expression Omnibus database: GEO series GSE3350 (GSM75508, GSM75509, GSM75512, GSM75513, Vanneste et al., 2005), series GSM42896 (GSM1053030, GSM1053031, GSM1053032, GSM1053036, GSM1053037, GSM1053038, De Rybel et al., 2012), series GSE41136 (GSM1009032, GSM1009033, GSM1009034, GSM1009039, GSM1009030, GSM1009031; Ng et al., 2013) and series GSM5530 (GSM128757, GSM128758, GSM128759, GSM128760, GSM128761, GSM128762; Davletova et al., 2005). Each dataset was been normalized independently with the robust multi-array average method and the differential analysis performed using the moderated t-test using the vignette affy (Gautier et al., 2004) and limma (Smyth, 2005) within the R (www.r-project. org) bioconductor statistical package (www.bioconductor.org). Affymetrix probe sets to AGI ID assignment was performed using the affy_ATH1_array_elements-2010-12-20.txt file downloaded from TAIR (http://www.arabidopsis.org/download_files/Microarrays/Affymetrix/affy_ATH1_array_elements-2010-12-20.txt). A gene was considered as being differentially expressed if it fulfilled the following conditions: fold change ≥2 and P-value ≤0.05 in the two pairwise comparisons for the datasets related with NAA treatment, and at least in one of the two pairwise comparisons for the datasets related with H2O2 treatment. The number of probe sets that satisfied these criteria was 109, of which two were redundant, yielding a final list of 108 genes (Table S1). Gene ontologies were retrieved using Agrigo (http://bioinfo. cau.edu.cn/agriGO/) and TAIR (www.arabidopsis.org) databases.

Statistical analyses
All data analyses were performed with R software package, v. 2.15. Different letters in figures indicate significant differences according to Tukey’s HSD test after ANOVA unless stated otherwise.

Author contributions
B.O.-L., A.F., E.H. and R.d.R. performed the experiments; B.P. and B.O.-L. performed data analysis; M.J.B., T.B., X.D.O., F.V.B., C.P. and B.O.-L. developed concepts, interpreted the results and prepared the manuscript.

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Supplementary information
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