Full title: RBOH-mediated ROS production facilitates lateral root emergence in Arabidopsis.

Running title: ROS contribute to LR emergence

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Summary statement

Reactive oxygen species promote cell wall remodeling of cells overlying the sites of lateral root formation thereby contributing to lateral root emergence in Arabidopsis.

Abstract

Lateral root (LR) emergence represents 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 *pCASP1:shy2-2* and *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 spatio-temporal 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 LRP 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.

Introduction

Root branching plays a critical 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 LR primordia (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/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 LRP formation, namely *ARF7/ARF19* (Okushima et al., 2007)*, SLR/IAA14* (Fukaki et al., 2002)*, IAA28* (Rogg et al., 2001) and *SHY2/IAA3* (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 coordinates 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/IAA3* signalling module triggers changes in cell volume and wall properties termed ‘spatial accommodation’ thereby facilitating the passage of LRP (Vermeer et al., 2014). In the cortex and the epidermal cells overlying the expanding LRP, cell wall remodelling enzymes are induced to facilitate LRP emergence (Gonzalez-Carranza et al., 2007; Lewis et al., 2013; Neuteboom et al., 1999; Swarup et al., 2008). The activity of an auxin influx carrier LIKE-AUX1 (LAX3) localises the auxin-induced expression of these cell wall remodelling genes that degrade the pectin-rich middle lamellae. In agreement, LRP 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 LRP emergence, as shown recently with mutants impaired in 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 signalling 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 (Wei-Biao, 2012) and root-to-shoot coordination (Passaia et al., 2013). Recent evidence also suggests that ROS act during lateral root (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, O_2^- and H_2O_2 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 Fluhr, 2006; Shapiguzov et al., 2012). Interestingly, the latter enzymes appear to regulate root branching in an auxin-independent manner (Manzano et al., 2014). To date, whether the RBOHs are involved in the auxin-mediated signalling leading to cell wall remodelling during LR formation is not known.

In this study, we exploit gene expression datasets to highlight the existence of interplay between ROS and auxin signalling 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 signalling 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 *RBOHD* validate the importance of this gene family in facilitating LRP emergence.

Results

An interplay between auxin and ROS signalling during LR formation

We initially analysed 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 hours with synthetic auxin-related signalling molecules 1-naphthaleneacetic acid (NAA) or naxillin to trigger synchronous LR formation in root pericycle cells (De Rybel et al., 2012; Vanneste et al., 2005). For experiments relating to ROS, 5 days-old seedlings were treated for one hour with 20 mM H_20_2 (Davletova et al., 2005) or 2 weeks-old seedlings were sprayed for 3 hours with 20 mM H_20_2 (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 H_20_2 experiments) were selected employing 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 H_2O_2 datasets while only 2 were repressed in both. Furthermore, 13 of the genes were induced during LR formation and were found to relate to redox activity, whilst 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 aplication 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 H_2O_2 have been previously reported to exhibit an increase in LR number compared to control seedlings (Manzano et al., 2014). We validated this by exposing root segments to H_2O_2 , which increased LR density and length in the exposed segments, while root growth rate decreased in a dose-dependent manner after onset of the treatment (Fig. 1A through E). The effect of H_2O_2 on primary root growth was likely not the manifestation of toxicity as it was reversed completely (for 1 mM H_2O_2) or partially (1.5 mM H_2O_2) within two days after transfer back on control medium (Fig. S1C). A permanent arrest of the primary root growth was only observed at 3 mM of H_2O_2.

To further investigate how H_2O_2 application impacts LR development, 5 days-old seedlings were exposed to H_2O_2 for 2 days (Fig. 1F). Upon H_2O_2 treatment, the number of emerged LR 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 explored the possibility that H_2O_2

treatment promotes the developmental progression of LRs from these unused precursor sites and does not induce *de novo* LR formation using a modified LR inducible system (Himanen et al., 2002; Jansen et al., 2013). LR formation was synchronised by germinating *pDR5:GUS* transgenic seedlings for 3 days in the presence of 10 μM of NPA followed by a transfer on control media and on media supplemented with H_2O_2 (1.5 mM), ROS scavenger potassium iodide (KI, 0.01 mM), H_2O_2 (1.5 mM) + KI (0.01 mM) or NAA (10 μM) as a positive control. Samples were collected at 6 hours, 12 hours and 18 hours 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 hours, whereas in 86% of seedlings grown in the presence of H_2O_2, GUS-positive foci were already observed within 6 hours. (Fig. S2A,B). Interestingly, the latter appeared in similar locations as compared to 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 LRP formation but does not induce *de novo* LR initiation.

To uncover effect of ROS on the kinetics of LR development, we employed the root bending assay (Fig. 1G), where roots are given a 90^0 gravistimulus to synchronise LR initiation and emergence in the resulting root bend (Peret et al., 2012b) and LRP stages are counted 20 and 44 h after gravistimulation (hag) according to (Malamy and Benfey, 1997). In pararell to the plate rotation, seedlings were treated with H_2O_2 (1.5 mM), the O_2^- donor methyl viologen dichloride hydrate (paraquat, 0.1 μM) and with a 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 to the control, while 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, while 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 H_2O_2 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, H_2O_2 treatment (1.5 mM) of 5-days 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 H_2O_2 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_2O_2 (LR/cm, mean±c.i.). Next, evaluating sensitivity to H_2O_2 with respect to primary root growth showed that *aux1 lax3* is equally sensitive to H_2O_2 as the control wild-type seedlings. In control conditions, primary root growth rate of 5 days-old WT plants transferred to a new control medium for 3 days is similar to *aux1 lax3,* respectively 7,2±1,3 and 7,32±1,08 (mm/day, n=15). Similarly, upon treatment with 1.5 mM H_2O_2 for 3 days, root growth decreased equally in WT and in *aux1 lax3* genetic backgrounds, respectively 1,35±0,64 and 1,77±0,52 (mm/day, n=15). However, the *aux1 lax3* root gravitropic defect was not rescued (Fig. 2A). Our results suggest that H_2O_2 treatment does not influence shootward auxin transport driven by AUX1 that is required for gravitropism, but rather overcomes the absence of the auxin gradient that was shown to induce the expression of cell wall remodeling genes in the overlying cell layers, that 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 localisation of the auxin efflux regulator PIN1, represents a weak allele and produces an embryonic root devoid of emerged LRs (Geldner et al., 2004). H_2O_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-PIN1 mediated auxin transport.

To validate our genetic results, we also tested the effects of H_2O_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) known to disrupt early steps of LR formation (Casimiro et al., 2001; Peret et al., 2013). We observed that H_2O_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_2O_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 disrupted in auxin-dependent endodermal cell wall remodeling. Transgenic lines expressing *pCASP1:shy2-2* are specifically disrupted in their endodermal auxin response, resulting in a LR-less phenotype (Goh et al., 2012; Hosmani et al., 2013; Vermeer et al., 2014). Strikingly, treatment with 1.5 mM H_2O_2 rescued LR development in the *pCASP1:shy2-2* gain-of-function mutants (Fig. 2B). In contrast, no LRP nor LRs could be induced in mutants in which LR formation is compromised due to defects in pericycle auxin signalling such as *iaa28* (Rogg et al., 2001), *arf7arf19* (Okushima et al., 2007) and in *slr/iaa14* (Fukaki et al., 2002) suggesting that H_2O_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_2O_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 one day of H_2O_2 treatment (Fig. S3B). We therefore hypothesize that exposure to H_2O_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 Arabidopisis (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 overly LRP (Fig. S3D), consistent with ROS playing a role during cell wall remodeling.

To resolve the sub-cellular localization of the most stable ROS species during LRP development, we employed transmission electron microscopy (TEM) to detect black cerium precipitates that demark the presence of H_2O_2. Our TEM approach detected H_2O_2 within the middle lamellae of cell walls, a pectin-based layer that cements the walls of adjacent cells together (Fig. 3 and Table S2). H_2O_2 accumulation was observed in the middle lamellae of cortical and endodermal cells overlying new LRP. The fine layer of H_2O_2 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, H_2O_2 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 10 *RBOH* genes, named *RBOHA* to *RBOHJ,* whose expression 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 H_2O_2 localization in the peripheral cells of the LRP (Fig. 4A). *RBOHE* was also strongly expressed in endodermis, cortex and epidermis cells overlying LRP (Figs 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 from AUX1 and LAX3 (Fig. S4C). Similarly, H_2O_2 treatment did not affect *AUX1* nor *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) known to affect the conversion of O_2^- into H_2O_2 (Fig. 4B). While control roots at 20 hag accumulated mainly stage I LRP, nearly no LRP were noticed in inhibitors-treated seedlings. At 44 hag, control plants accumulated mainly stage V, VI and VII LRP. Although no remarkable differences with 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 analysed 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 *D* were observed to have the strongest phenotype, consistent with both genes exhibiting the strongest and most widespread expression in overlying tissues (Fig. 4A) in 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 time points, all *RBOH* transcripts detected in root tissue were up-regulated 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 up-regulation of all members of *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), likely due to an overal increase in extracellular ROS levels (Mersmann et al., 2010). To determine which specific cell types were most sensitive to ROS accumulation during lateral root 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 x 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 to controls, where LRP accumulated mainly at stage V. In 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 days-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_2O_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 harmfull effects, the production of ROS compounds in the apoplast is targetted 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 salnity and drought, LR development is inhibited (De Smet et al., 2006; Duan et al., 2013). It is tempting to speculate that activation of ROS scavenging machinery that likely occurs during exposure to abiotic stress (Caverzan et al., 2012),

interferes with RBOH-mediated ROS production and/or removal deposition from to the apoplast thereby affecting LR development.

Auxins signaling modules, which control LR development both in the LRP and in overlying tissues (Lavenus et al., 2013), are good candidates to regulate spatio-temporal ROS production to appropriate cell wall domains. Our additional observation that increased H_2O_2 levels (supplied externally or most likely by tissue-specific over expression of *RBOHD*) accelerate the early steps of LR 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 trigger RBOH-mediated ROS production where needed and release the subsequent steps of LR formation.

ROS action on cell wall remodelling

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_2O_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_2O_2 from O^{2-} and their promoting effect on LR formation (Manzano et al., 2014), RBOH enzymes probably serve as O^{2-} donors for peroxidases during this developmental process in defined locations. However, we can not exclude the possibility that RBOH and peroxidases are acting independently, as conversion of O^{2-} to H_2O_2 can also occur spontaneously, without any enzymatic support. Pharmacological inhibition of every RBOH enzymes 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*, *pectate lyase 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 neighbouring endodermis, cortex and epidermis cells as the LRP expands. Previous studies reported that ROS treatment increases LR number (Correa-Aragunde et al., 2013; Ma et al., 2014). However, we show here that exogenuous ROS treatment does not induce the formation of *de novo* LR initiation sites, but rather promotes the developmental progression of the existing LRP and LR pre-branch sites, leading eventually to increased emerged LR numbers. Restoring the LR formation capacity by ROS treatment of the *pCASP1:shy2-2* mutants further corroborates our hypothesis that ROS are mediating lateral root development through their action on cell wall mechanics because the incompetence of these mutants to form lateral roots is attributed to the lack of spatial accommodation. However, we cannot exclude the involvement of a downstream ROS signalling cascade in this process.

RBOH-mediated ROS production promotes LR emergence

Our findings that functioning of the RBOHs contribute to LR emergence, which requires cell wall remodeling and accomodation, reveal a key role for RBOH in the control of apoplastic ROS production targetted to restricted spatial and temporal domains during organ outgrowth (Fig. 7). The delimited RBOH expression to the peripheral cells of the LRP and to the cell files overlying it, suggests that auxin signalling 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 LRP overlying cells is auxin-regulated, perhaps in parallel to *LAX3* in *LBD29/LAX3* signalling module (Porco et al., 2016). Hence, such precise ROS deposition suggest an intimate relationship between ROS and auxin-controlled changes in cell wall biomechanics during LRP emergence.

Materials and Methods

Plant material and growth conditions

All Arabidopsis lines used in this study were previously described: *AUX1:GUS* (Swarup et al., 2004), *LAX3:GUS* (Swarup et al., 2008), *pRBOH:nlsGFP:GUS* (Lee et al., 2013), *pCASP1:SHY2, pCASP1:shy2-22* (Vermeer et al., 2014), *iaa28-1* (Rogg et al., 2001), *slr/iaa14* (Fukaki et al., 2002), *arf7arf19* (Okushima et al., 2007), *aux1lax3* (Swarup and Peret, 2012). The crosses were generated from the following SAIL/SALK lines: *rbohb* (SAIL_749_B11), r*bohc* (SALK_071801), *rbohd* (SALK_070610), *rbohe* (SALK_064850), *rbohf* (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 pKm34GW,0 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 MS growth medium (per litre: 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), called “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 V700 scanner (Epson) or 3200 dpi scanner (Medion). Seedlings were analyzed in details with BX53 microscope (Olympus) equipped with DS-Fi1 camera (Nicon). Figures were arranged in Photoshop CS3 (Adobe Systems Inc.) and the brightness was increased equally, without further modifications. To characterize *GAL4* enhancer trap lines and transactivation lines, 5 days-old seedlings were imaged with LSM5 (Axiovert, Zeiss) confocal microscope.

Transmission electron microscopy

Cerium-hydroxide precipitates indicate H_2O_2 localization. 5 daays-old seedlings were gravistimulated by 90 degrees to achieve synchronization of LR formation. After 22 h and 44 h, 2 mm fragments that were expected to contain early and late LRP were dissected under binocular (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 MOPS (3-(N-Morpholino) propanesulfonic Acid (VWR Chemicals; Louvain, Belgium) in 90 mL of water and adjust the pH to 7.2 with 1.7 M Tris (VWR Chemicals; Louvain, Belgium). Tissue embedding and electron probe x-rays were performed as described (D'Haeze et al., 2003).

qRT-PCR analysis

Col-0 seeds were sown on 1/2MS supplemented with 1% sucrose and grown for 7 days on a mesh. Seedlings were then transferred to 10 µM NAA for the indicated time points. RNA was extracted from dissected roots and 1µg of RNA was used for cDNA synthesis and qRT-PCR analysis as described before (Fernandez et al., 2013) with a given primer pairs (Table S3). Data were analysed with the "delta-delta method" (Pfaffl, 2001), taking primer efficiency into consideration, and normalized with *UBIQUITIN 10* as reference transcript. The sample with the maximum value for each gene was chosen as the calibrator (set to 1), the results of two biological replicates were averaged and the expression values are given in arbitrary units relative to t0. *RBOHJ* and *RBOHH* primers only amplified in a few samples of the second replicate consistent with the predicted expression pattern (not in roots) and are therefore not shown.

LR phenotype analysis

5 days-old Arabidopsis Col-0 and / or mutant seedlings were transferred on fresh media (Control) or on media supplemented with various compounds, namely DPI (Diphenyleneiodonium chloride, Sigma-Aldrich; Diegem, Belgium), DDC (diethyldithiocarbamate, Alfa Aesar/VWR; Leuven, Belgium), paraquat (Methyl viologen dichloride hydrate, Sigma-Aldrich; Diegem, Belgium), KI (potassium iodide, Applichem Lifescience; Lokeren, Belgium). After 1 h, seedlings were gravistimulated by 90 degrees to achieve synchronization of LR formation. After 20 h and 44 h seedlings were pre-fixed in 0.4 % formaldehyde (Sigma-Aldrich; Diegem, Belgium) in 50 mM phosphate buffer (VWR Chemicals; Leuven, Belgium) pH = 7 at 4 °C upon a gentle vaccum for 30 min. Subsequently, 2.5 grams of chloral hydrate (VWR Chemicals; Leuven, Belgium) was dissolved per 1 ml of 30% glycerol (Sigma-Aldrich; Diegem, Belgium) and seedlings were left overnight in a cleaning solution. LRP were observed with BX53 dissecting microscope (Olympus) equipped with DS-Fi1 (Nicon) camera and grouped according to developmental stages at 20 h and 44 h after the onset of gravistimulation.

426 **GUS staining**

427 Seedlings were put overnight in 90% acetone, then transferred to a GUS-solution [1 mM
428 X-Gluc, 0.5% (w/v) dimethylformamide (DMF), 0.5% (w/v) Triton X-100, 1 mM EDTA
429 (pH = 8), 0.5mM potassium ferricyanide ($K_3Fe(CN)_6$), 0.5% potassium ferrocyanide
430 ($K_4Fe(CN)_6$), 500mM phosphate buffer (pH = 7)] and incubated for 4 h at 37 °C for GUS
431 staining, and finally washed in 500mM phosphate buffer (pH = 7). For microscopic
432 analysis, samples were cleared in chloral hydrate solution as described in (Berleth and
433 Jurgens, 1993). Samples were analyzed by differential interference contrast microscopy
434 with Primo Vert (Zeiss) equiped with moticam 2300 (Motic)

435 **Microarray retrieving, normalization and treatment**

436 The following microarray hybridization files were retrieved from the Gene Expression
437 Omnibus database: GEO series GSE3350 (GSM75508, GSM75509, GSM75512,
438 GSM75513) for Vanneste et al. (Vanneste et al., 2005), series GSE42896 (GSM1053030,
439 GSM1053031, GSM1053032, GSM1053036, GSM1053037, GSM1053038) for De Rybel
440 et al., 2012 (Rybel et al., 2012), series GSE41136 (GSM1009032, GSM1009033,
441 GSM1009034, GSM1009029, GSM1009030, GSM1009031) for Ng et al. (Ng et al.,
442 2013) and series GSE5530 (GSM128757, GSM128758, GSM128759, GSM128760,
443 GSM128761, GSM128762) for Davletova et al., 2005 (Davletova et al., 2005). Each
444 datasets have been normalized independently with the robust multi-array average method
445 and the differential analysis performed using the moderated t-test using the vignettes affy
446 (Gautier et al., 2004) and limma (Smyth, 2004; Smyth, 2005) within the R (www.r-
447 project.org) bioconductor statistical package (www.bioconductor.org). Affymetrix
448 probesets to AGI ID assignment was done using the affy_ATH1_array_elements-2010-
449 12-20.txt file downloaded from TAIR (www.arabidopsis.org). A gene was considered as
450 being differentially expressed if it fulfilled the following conditions: fold change ≥2 and
451 p-value ≤0.05 in the two pairwise comparisons for the datasets related with NAA
452 treatment, and at least in one of the two pairwise comparisons for the datasets related with
453 H_20_2 treatment. 109 probesets satisfy this criteria, among which two are redundant,
454 yielding a final list of 108 genes (Table S1). Gene ontologies have been retrieved using
455 Agrigo (http://bioinfo.cau.edu.cn/agriGO/) and TAIR (www.arabidopsis.org) databases.

456 **Statistical analyses**

All data analyses were performed with R software package, v. 2.15.

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Competing interests

No competing interests declared.

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., F.V.B., C.P. and B.O.-L. developed concepts, interpreted the results and prepared the manuscript.

Founding

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Figure legends

Fig. 1.

The effect of ROS on root development. A, Morphology of Col-0 grown in control conditions and upon treatment with increasing H_2O_2 concentrations. 5 days-old seedlings (transferred region) were exposed to H_2O_2 for 7 days. White arrowheads indicate the root tip region at the moment of transfer. Bar = 1cm. **B,** Primary root (PR) growth rates upon treatment with increasing H_2O_2 concentrations. 5 days-old seedlings were transferred on media supplemented with increasing concentrations of H_2O_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,** Average emerged lateral root (LR) density and **D,** LR length in transferred region after 7 days of H_2O_2 treatment (in three biological repetitions, n = 30). Due to a strong effect of H_2O_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_2O_2 treatment (in three biological repetitions, n = 30). **F,** Effect of ROS on LRP density after 2 days upon different concentrations of H_2O_2. **G,** Effect of

ROS and ROS scavengers on LR emergence phenotype. 5 days-old seedlings were transferred on media supplemented with various compounds, as indicated above each graph and gravistimulated by turning the plates 90 degrees to achieve synchronization of LR formation. LRP stages according to (Dubrovsky et al., 2006; Himanen et al., 2002; Jansen et al., 2013; 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 repetitions, n = 20).

Fig. 2.

The effect of ROS on the LR phenotype of auxin mutants. A, Effect of exogenous H_2O_2 on LR formation in Col-0 (Control) and *aux1lax3* background. **B,** Effect of exogenous H_2O_2 on LR formation in *pCASP1:SHY2* (Control) and in *pCASP1:shy2-2* gain-of-function background. 5 days-old seedlings were exposed to H_2O_2 (1.5 mM) for 7-days. White arrowheads indicate the root tip region at the moment of transfer. Bar = 1 cm.

Fig. 3.

Representative transmission electron microscopic images of *Arabidopsis* LRP treated with cerium chloride to visualize localization of H_2O_2 by black cerium depositions. A through **D,** H_2O_2 localization during LR emergence in outer cells **B,** between LRP and endodermis **C,** and between flanking cells inside of LRP. **E,** H_2O_2 localization in LRP at stage II of development in middle lamellae between outer cell of LRP and endodermis, as LRP is passing through endodermis. **B** and **D** are magnified views of A. V indicates vasculature, LRP - Lateral Root Primordium, P - Pericycle, EN -Endodermis, CO - Cortex, EP – Epidermis; Numbers in (**C**) points to 1 – Cytoplasm, 2 - Cell wall of outer LR cell, 3 - Middle lamella, 4 - Periplasmatic space, 5 - Remnants of endodermis protoplast, 6 – Vacuole, 7 - Plasma membrane, 8 - Periplasmatic space, 9 – Endoplasmic reticulum, 10 – Tonoplast, 11 – Vacuole and 12 - Cell wall of endodermis cell. B and D are Bars, 20 um **(A)**, 6 um **(B)** and 2 um **(C-E)**; Magnifications, 1200 **(A)**, 4000 **(B)**, 12000 **(C** through **E);** White-filled arrowheads points to the cerium depositions, n = 15.

Fig. 4.

Expression pattern of *RBOH* genes during LR development and RBOH-mediated effect on LR emergence phenotype. A, Promoter activities of *RBOH* genes during LRP

development. 7 days-old seedlings of each *pRBOH:nlsGFP:GUS* line as indicated, were GUS-stained. Bar = 50 μm. **B,** Effect of superoxide dismutases blocker, diethyldithiocarbamate (DDC, 1 mM), and the RBOH inhibitor, diphenyleneiodonium chloride (DPI, 1 μM), on LRE phenotype, starting from stage I to an emerged LR (E) on X-axis. Data points represent mean±c.i. (in two biological repetitions, n = 20). **C** and **D,** LRE phenotype in WT and *rboh* single and higher order mutants, as indicated. Data points represent mean±c.i. (in two biological repetitions, n = 20). **E,** A synchronisation of LRP initiation (20 h) and emergence (44 h) is acheved by gravistimulation and occurs at the bending site.

Fig. 5.

Relative *RBOH* transcript levels in root tissue. *RBOH* transcript levels were measured by qRT-PCR. The data are shown for two independent biological replicates ± SE.

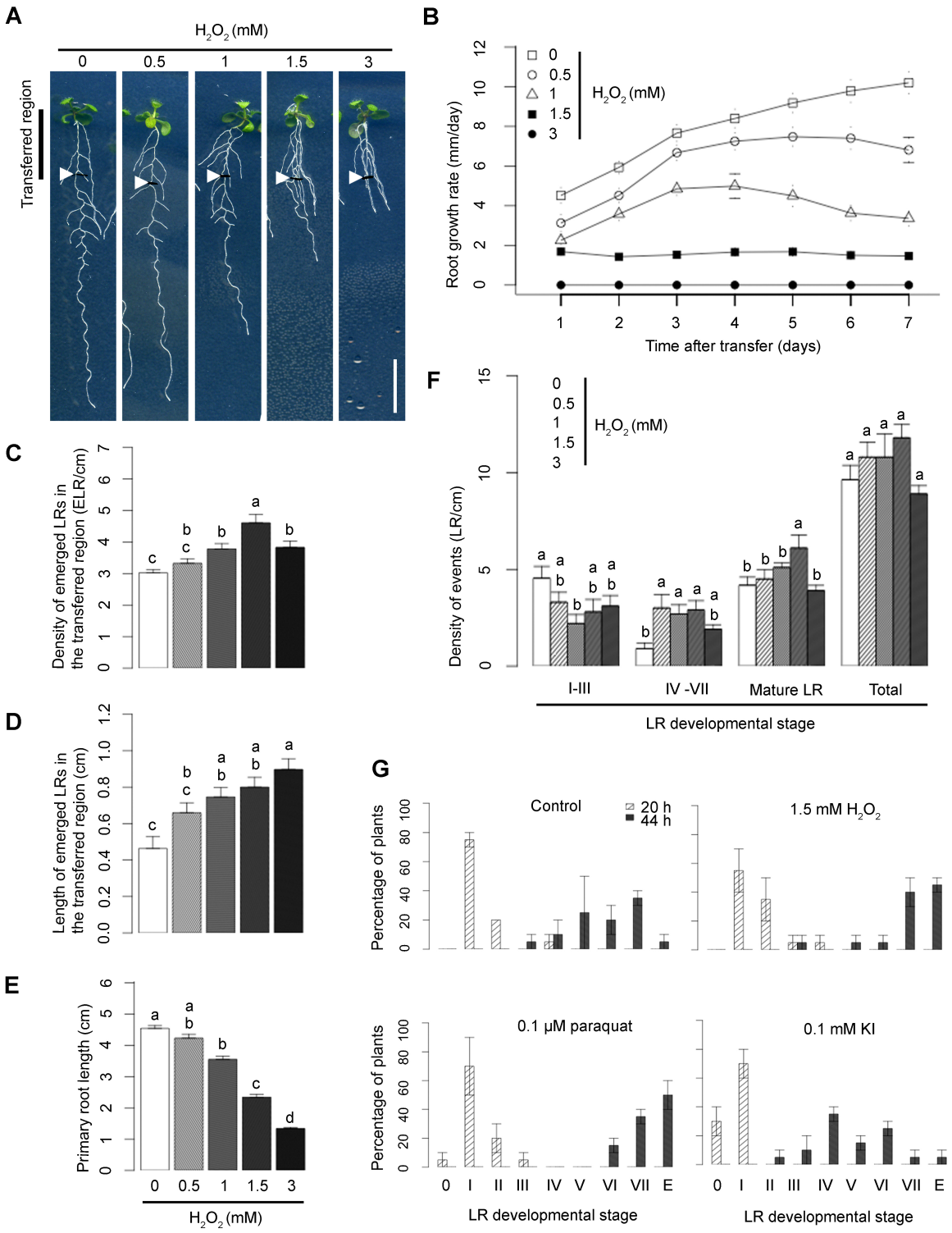
Fig. 6.

The effect of tissue-specific overexpression of *RBOHD* on LR development. A, LRE phenotype of control lines. 5 days-old seedlings were transferred on new media and gravistimulated by 90 degrees to achieve synchronization of LR formation. LRP were grouped according to developmental stages at 44 h after the onset of gravistimulation. Data points represent mean±c.i. (technical replicates). **B,** Expression pattern of GAL4 transactivation lines (upper panel) and LRE phenotype of *UAS:RBOHD* targeted to the corresponding GAL4 transactivation lines, as indicated. Bar = 50 μm. **C,** Representative root phenotypes of 10 days-old seedlings. **D,** Emerged LR number of control and tissue-specific transactivation lines of *UAS:RBOHD*. Data points represent mean±c.i. (technical replicates, n = 20). Means not sharing subscripts differ at $p < 0.001$ according to Tukey's HSD test after ANOVA.

Fig. 7.

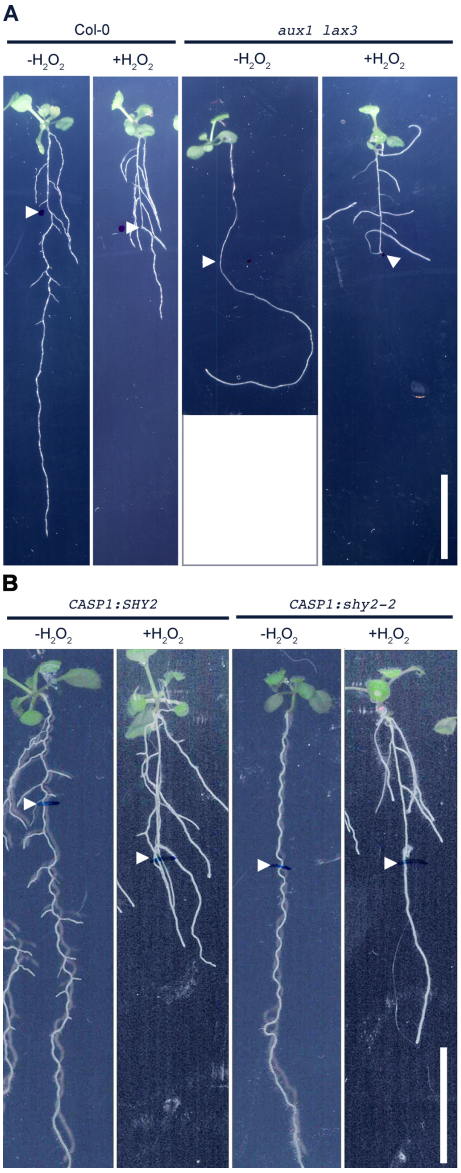
Linking RBOH-mediated ROS production to the current understanding of auxin-mediated LR formation. A, The expression patterns of *RBOH* genes overlap with ROS localization and promoter activities of auxin influx carriers during LR emergence (longitidnal section). *AUX1* is expressed inside LR primordia and in pericycle, whereas *LAX3* is expressed in cortex and epidermal cells in front of emerging LR primordia

(Swarup et al., 2008; Swarup and Peret, 2012). The promoters of *RBOH*s are active in peripheral cells of LRP and in cells surrounding the emerging LRP. ROS accumulates in middle lamella of peripheral cells of LRP and of cell files overlying LRP. The vascular localisations are omitted. Schematic representation is based on a EM tissue section from Fig. 3. **B,** Model of auxin and RBOH-mediated ROS action during LRP emergence. For a succesful LR initiation and emergence, localized cell wall remodeling in front of LRP is required and relies on an orchestrated operation of several auxin response modules (Swarup et al., 2008). Here, we propose that ROS deposited into the cell walls by the activity of auxin-inducible RBOH enzymes facilitate LRP emergence by promoting cell wall remodeling.



791

792 **Figure 1**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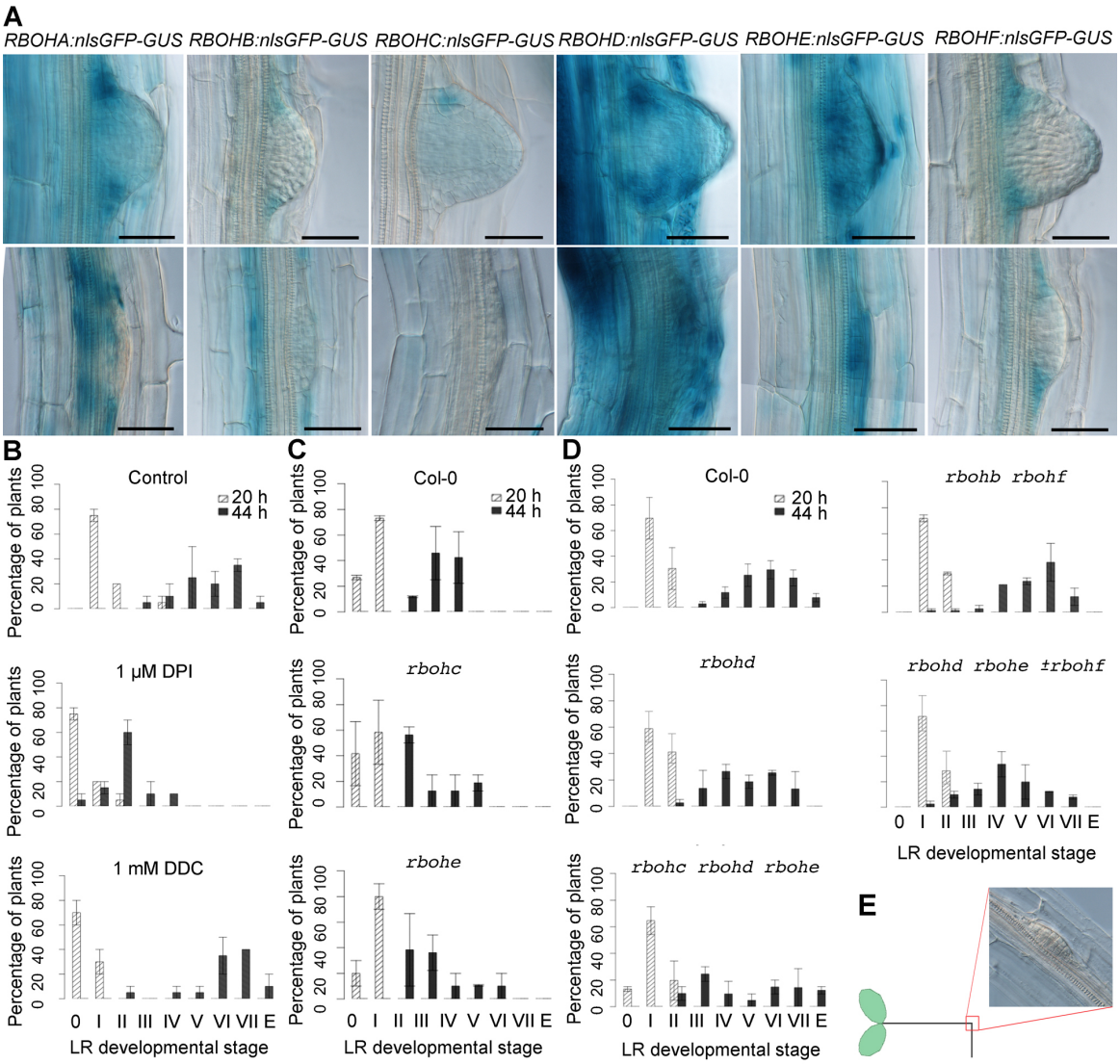
Col-0
aux1 lax3
-H2O2
+H2O2
-H2O2
+H2O2
B
CASP1:SHY2
CASP1:shy2-2
-H2O2
+H2O2
-H2O2
+H2O2

Figure 2



795

796 **Figure 4**

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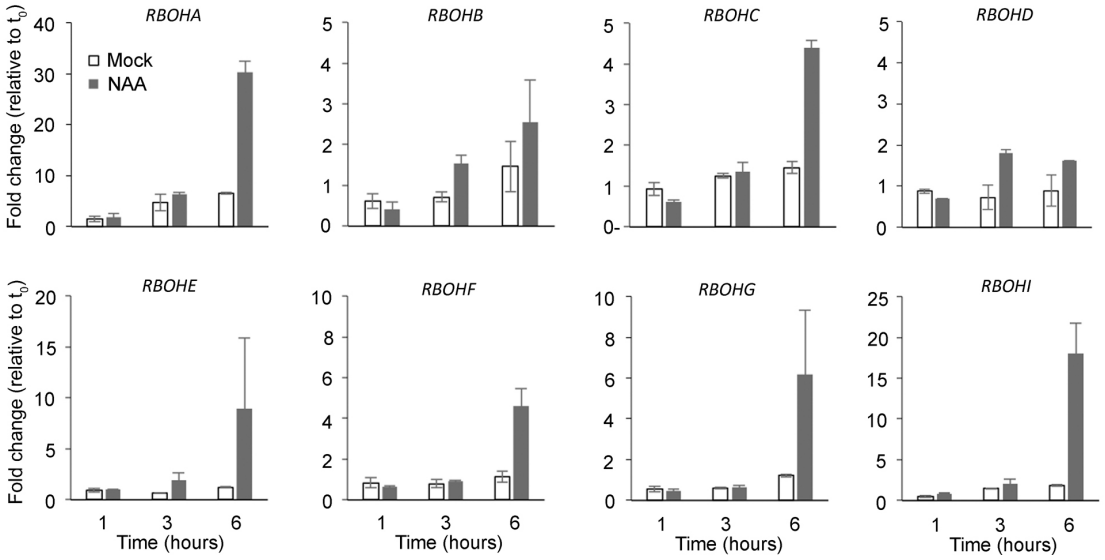

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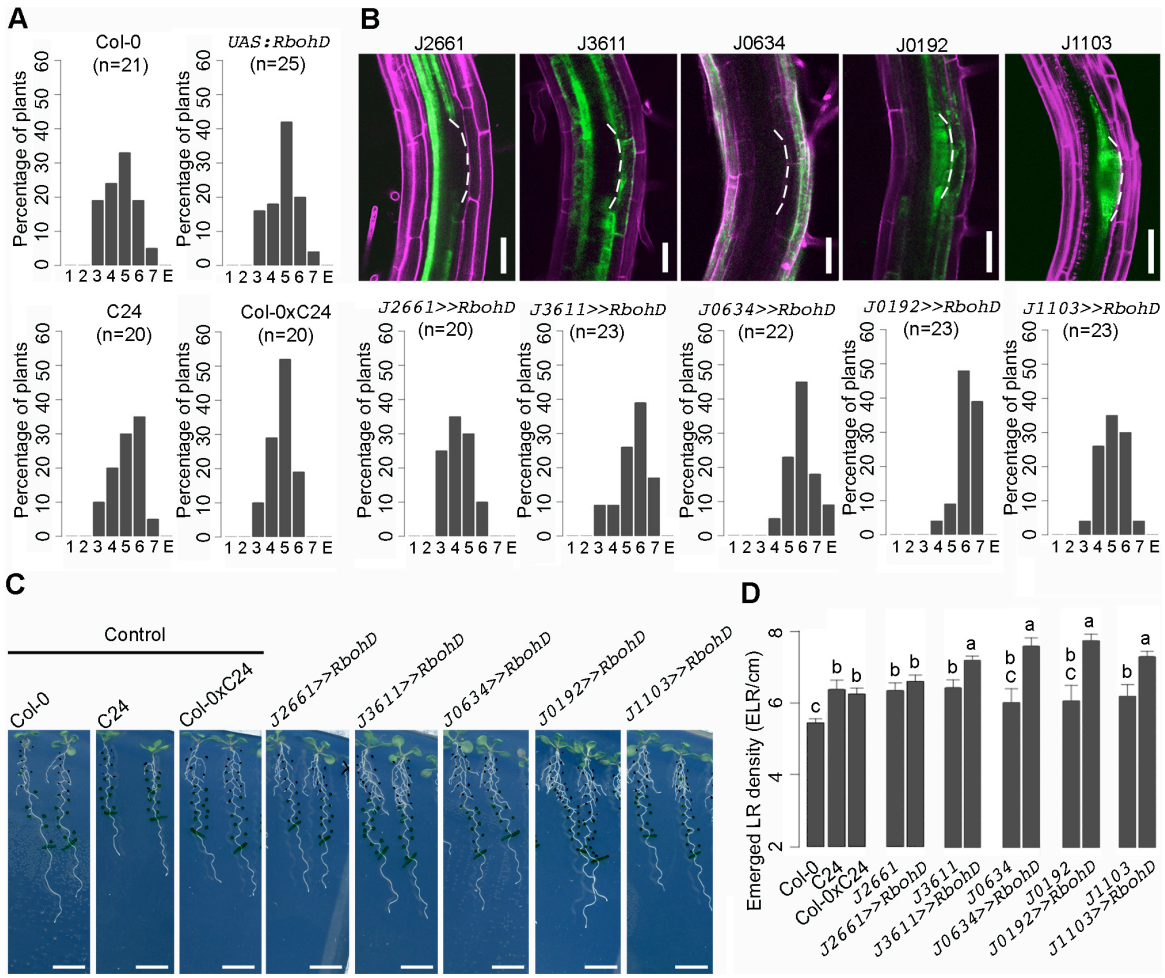
799 **Figure 5**



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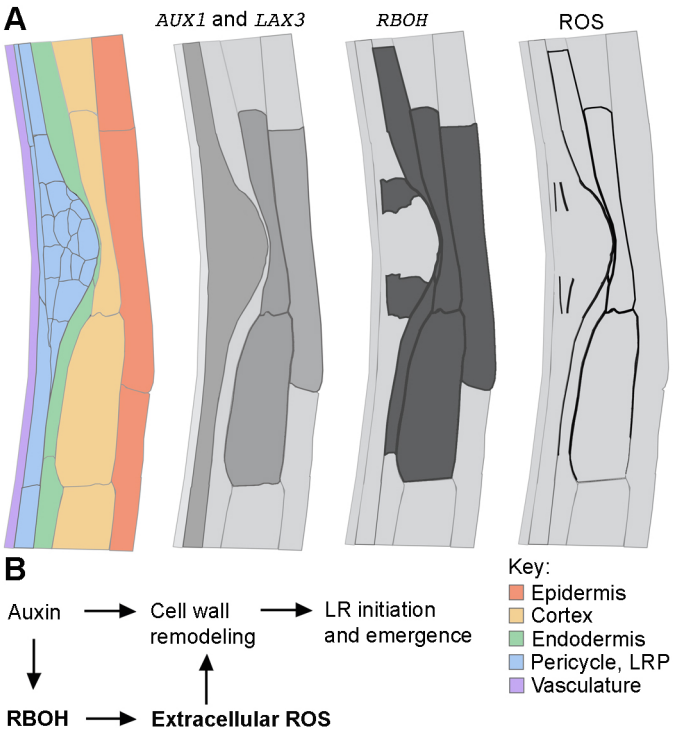
801 **Figure 6**



802

803 **Figure 7**