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A Mechanism for Localized Lignin Deposition in the Endodermis

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SUMMARY

The precise localization of extracellular matrix and cell wall components is of critical importance for multicellular organisms. Lignin is a major cell wall modification that often forms intricate subcellular patterns that are central to cellular function. Yet the mechanisms of lignin polymerization and the subcellular precision of its formation remain enigmatic. Here, we show that the Casparian strip, a lignin-based, paracellular diffusion barrier in plants, forms as a precise, median ring by the concerted action of a specific, localized NADPH oxidase, brought into proximity of localized peroxidases through the action of Casparian strip domain proteins (CASPs). Our findings in *Arabidopsis* provide a simple mechanistic model of how plant cells regulate lignin formation with subcellular precision. We speculate that scaffolding of NADPH oxidases to the downstream targets of the reactive oxygen species (ROS) that they produce might be a widespread mechanism to ensure specificity and subcellular precision of ROS action within the extracellular matrix.

INTRODUCTION

Localized deposition and modification of extracellular matrix are crucial for the correct development and function of multicellular organisms. In animals, the many-faceted roles of the extracellular matrix include providing cues for cellular migration, cell layer communication, or even controlling the spread and activity of morphogenic signals (Kim et al., 2011). Consequently, deregulation of matrix function can be intimately associated with tumor development. In plants, the highly turgescent cells undergo localized loosening, strengthening, or modification of the cell wall to drive morphogenesis of individual cells, as well as controlling the integrity of entire cell layers and organs (Cosgrove, 2005). Dramatic examples of highly localized loosening of cell walls are found in tip growing root hair cells and pollen tubes (Foreman et al., 2003; Monshausen et al., 2007; Swanson and Gilroy, 2010). Just as dramatic is the contact signal-dependent rupture of the pollen cell wall upon encounter of the female embryo sac and the explosive release of the sperm cells (Boisson-Dernier et al., 2009; Escobar-Restrepo et al., 2007; Miyazaki et al., 2009). The stomatal pores that regulate gas exchange across the leaf epidermis display a precisely localized separation of certain cell wall regions and thickening of others, both of which are crucial for the functionality of these pores (Bergmann and Sack, 2007). Xylem vessels result from an apoptotic program that leaves behind a strongly thickened but intricately structured set of interconnected hollow tubes that mediate nutrient and water flow within the plant (Oda and Fukuda, 2012b). Plant cells detect and respond to cell wall manipulations by pathogens with rapid deposition of altered and strengthened cell walls at sites of infection (Hématy et al., 2009). In all these examples, we are only beginning to identify the underlying molecular factors, and we are largely ignorant about how localized modifications of cell walls can be achieved.

Lignin deposition is a major cell wall modification that plants employ in many different cell types and in response to various environmental stresses. Added to the fundamental importance of lignin is a biotechnological incentive to manipulate lignin formation, as it remains a major obstacle for the utilization of cellulosic biofuels (Vanholme et al., 2010). Lignin formation has been studied extensively as part of xylem differentiation. However, the intricate subcellular patterns observed in this system are rather a result of the prior formation of localized secondary cellulosic walls (Oda and Fukuda, 2012a, 2012b). The formation of lignin per se was recently shown to be postapoptotic in differentiating xylem vessels. It is unclear to which degree polymerization of lignin itself is spatially controlled in these cells (Pesquet et al., 2010) and which factors would locally initiate or restrict lignin polymerization. Understanding localized lignin formation is also hindered by the many uncertainties concerning the general mechanisms of lignin polymerization in vivo (Liu, 2012). While it is well established that peroxidases and laccases catalyze oxidative coupling of monolignols in vitro, evidence for their role in planta has been more difficult to obtain. Recently, a laccase double mutant in *Arabidopsis* was shown to have significantly lower lignin content, providing in vivo evidence for laccases in lignin formation (Berthet et al., 2011). In the case of peroxidases, a high degree of genetic redundancy seems to have precluded identification of similarly strong lignin phenotypes, and current evidence is restricted to a number of RNA interference approaches that reported variable effects on lignin content (Fagerstedt et al., 2010; Liu, 2012). The source of

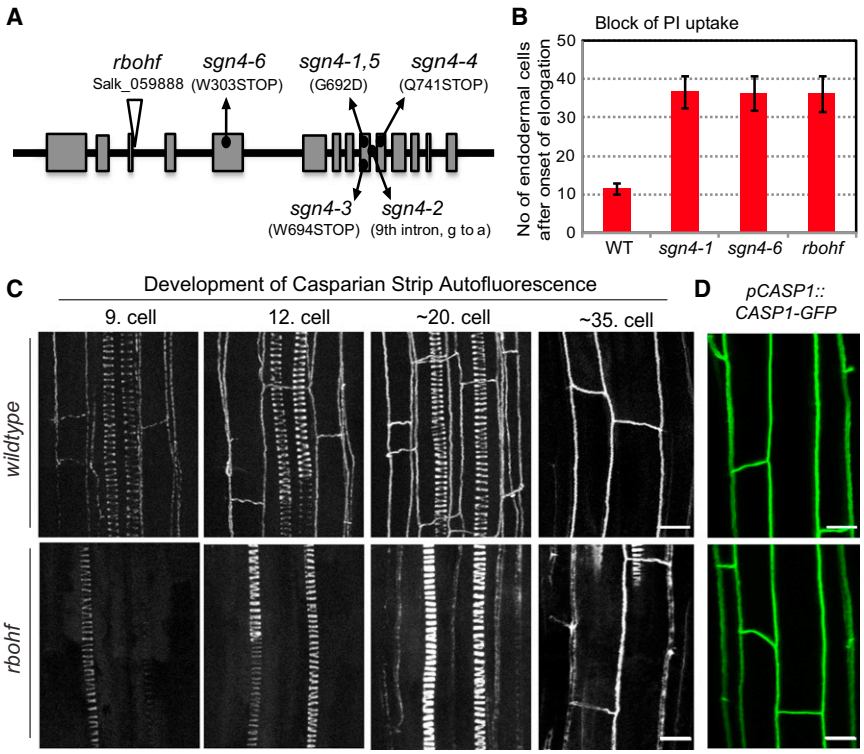

Figure 1. Casparian Strip Formation Is Delayed in *sgn4/rbohf* Mutants

(A) A diagram of RBOHF genomic region shows insertion site of the T-DNA and the positions of the different stop- and missense mutations identified in the six *sgn4* alleles (see also Tables S1 and S2).

(B) Apoplastic diffusion barrier was visualized by block of penetration of externally applied PI (15 μM) into the stele. Quantification was done by counting endodermal cells after "onset of elongation," defined when an endodermal cell in a median optical section was at least three times its width. From this point, cells in the file were counted until the PI signal was blocked at endodermal cells (5-day-old seedlings, mean ± SD, $21 < n < 31$).

(C) Casparian strip networks are visualized as autofluorescence after clearing in different developmental stages which are represented by counts of endodermal cells after "onset of elongation" (see above). Pictures are maximum projections of longitudinal, surface-to-median confocal image stacks, allowing visualization of the net-like nature of the Casparian strips. Note that xylem vessels are also observed as brightly fluorescent spiral structures.

(D) pCASP1::CASP1-GFP localizes at the net-like Casparian strip membrane domain (CSD) in wild-type and *rbohf*. Projections as in (C).

See also Figure S1. Scale bars, 10 μm.

hydrogen peroxide that would be required for peroxidase activity has also remained an open question. Plasma membrane localized NADPH oxidases have been implicated in production of reactive oxygen species (ROS) during lignification, but this has been entirely based on inhibitor studies, and no specific NADPH oxidase (called Respiratory burst oxidase homologs, RBOHs, in plants) has been shown to provide ROS for lignin formation (Barcelo, 1998; Kärkönen and Koutaniemi, 2010).

Recently, it has been demonstrated in *Arabidopsis* that Casparian strips in endodermal cells are lignin-based structures (Naseer et al., 2012). Casparian strips represent highly localized modifications of the primary cell walls that surround individual cells in a median position and confer paracellular (apoplastic) barrier properties to endodermal cells. The endodermis is a fundamental cell layer in the roots of vascular plants, which resembles polarized epithelia of animals, both in structure and function (Alassimone et al., 2010; Roppolo et al., 2011). A family of small transmembrane proteins has been identified, which predict the localized formation of Casparian strips and are necessary for its correct formation (Roppolo et al., 2011). These Casparian strip domain proteins (CASPs) were proposed to form an extensive, transmembrane polymeric platform and were speculated to guide the assembly and activity of lignin biosynthetic enzymes.

Here, we demonstrate that CASP1 is a determinant for the subcellular localization of a specific endodermal peroxidase. In addition, we reveal that mutations in one of the NADPH oxidase family members of *Arabidopsis* strongly delays formation of Casparian strips. This NADPH oxidase is recruited into the Casparian strip domain, and we show that a combination of specific N-terminal regulatory domain and subcellular localization determines its nonredundant activity. Based on our data, we can draw a model whereby subcellular precision of lignin polymerization is achieved by the combinatorial action of a locally restricted production of ROS substrate and localized peroxidase activity, brought together by the scaffolding activity of CASPs.

RESULTS

A Specific NADPH Oxidase Is Required for Casparian Strip Formation

In order to identify factors involved in Casparian strip formation, we undertook a forward genetic screen for mutants with an impaired endodermal barrier. We identified 11 mutants out of an EMS population of more than 20,000 lines (J.A., unpublished data). Six of these lines formed the single largest complementation group. We were able to rapidly determine the identity of this s*chengen4* (*sgn4*) complementation group, due to its phenotypic similarity with a T-DNA insertion line in the *Respiratory burst oxidase homolog F* (*RBOHF*) gene, investigated as part of a parallel, reverse genetic approach in our group. The mutants were termed "Schengen" after the treaty that established a borderless area between European member states. Noncomplementation with the *rbohf* T-DNA allele and identification of either stop or missense mutation in the *RBOHF* sequence of all six alleles demonstrated that *SGN4* is identical to *RBOHF* (Figure 1A; Tables S1 and S2 available online). *sgn4/rbohf* mutants show a very strong delay in the formation of an apoplastic diffusion barrier, as visualized by the penetration of externally applied propidium iodide (PI) into the stele, a convenient assay for presence

of a functional endodermal diffusion barrier (Alassimone et al., 2010; Naseer et al., 2012) (Figure 1B). The defective barrier is due to a complete absence of the Casparian strip in younger root parts, visualized by its autofluorescence after clearing (Figure 1C). Eventually, aberrantly structured Casparian strips form in older parts of the root, which eventually will succeed in effectively blocking penetration of PI into the stele. A similarly strong delay in the block of PI uptake (block at 36.3 ± 0.5 cells) can be observed in a *casp1 casp3* double mutant (P.S. Hosmani, T. Kamiya, J. Danku, S. Naseer, N.G., M.L. Guerinot, and D.E. Salt, unpublished data). In this case, however, an aberrant, patchy Casparian strip-like structure is formed (Roppolo et al., 2011). We tested whether SGN4/RBOHF is required for the expression, accumulation, or localization of the CASPs, which could cause the observed delay in Casparian strip formation. However, CASP1-GFP showed a normal accumulation and localization in the mutant (Figure 1D). Consistently, protein exclusion of generic plasma membrane markers and confinement of polarly localized proteins still occurs in *rbohf* (Figure S1), suggesting that the Casparian strip membrane domain (CSD) is present and functional. This places RBOHF downstream of the formation of the localized CASP platform, suggesting a role in the execution of the cell wall formation, guided by the CASPs.

NADPH Oxidase-Dependent Localized H_20_2 Production at Casparian Strips

Lignin formation requires oxidation of monolignols, which is thought to be catalyzed by peroxidases. Peroxidases require hydrogen peroxide (H_20_2) for this oxidation and NADPH oxidases have been implicated as major sources of regulated superoxide production, which can be enzymatically or nonenzymatically dismutated into H_20_2. Therefore, we considered it most straightforward that SGN4/RBOHF would be needed for the production of H_20_2 as a peroxidase cosubstrate. In this scenario, RBOHF has a very direct role, and even short-term inhibition of the NADPH oxidase should be able to block Casparian strip formation. The NADPH oxidase inhibitor diphenylene iodonium (DPI) allows us to precisely time the inhibition of NADPH oxidase activity, but its general effect on all NADPH oxidases severely inhibits root growth and causes cell death after prolonged treatment, making it difficult to analyze slow developmental processes (Figure S2). We therefore resorted to a recently developed short-term assay for Casparian strip formation (Naseer et al., 2012). For this, seedlings are grown for 24 hr on the monolignol biosynthesis inhibitor, piperonylic acid. Cells that have not formed Casparian strips are then induced to do so by externally supplying monolignols, leading to formation of Casparian strips within only 6 hr (Naseer et al., 2012) (Figure S3). This allowed us to investigate the immediate effect of NADPH oxidases on Casparian strip formation, uncoupled from their effect on general developmental processes. By coapplying DPI with monolignols, we observed a complete block in Casparian strip reconstitution after 6 hr, supporting a direct requirement of NADPH oxidases for lignin polymerization in the endodermis (Figure 2A). Continued PI uptake confirmed that the endodermal diffusion barrier had not formed and additionally showed that the DPI concentration and timescale used did not lead to any cell death, as this would lead to the appearance of strongly PI-stained nuclei (Figures 2A and S2).

We then tested whether we could observe production of apoplastic H_20_2 in the endodermis. To do so, we used the cerium chloride assay. Cerium chloride forms electron-dense precipitates in the presence of H_20_2 through formation of cerium perhydroxide, which can be observed with electron microscopy, providing us with the necessary resolution. With this assay, we observed strikingly localized cerium precipitations in endodermal cells, exclusively at the position of Casparian strips. While early Casparian strips displayed precipitates throughout their width, mature strips only showed signals at their outward-facing edge. The lack of signal at inward-facing edges is most probably due to the fact that more mature Casparian strips can function as an apoplastic barrier, not allowing uptake of cerium chloride beyond this point (Figure 2B). Sixty minutes of DPI treatment before cerium chloride application led to an almost complete absence of local H_20_2, indicating that NADPH oxidases are responsible for this highly localized production of H_20_2 (Figure 2B; Table S3). These findings strongly suggested that an NADPH oxidase is either localized or locally activated at the sites of Casparian strip formation.

Regulatory Input and Localization Determine Specificity of RBOHF Action

The specific role of RBOHF in Casparian strip formation was surprising, considering that at least one other NADPH oxidase, RBOHB, is strongly expressed in endodermal tissues (Figure S4). Indeed, insertion lines of RBOHB, or of four other root-expressed NADPH oxidases tested, did not display any delay in Casparian strip formation, nor did double mutants with RBOHF increase the severity of the RBOHF single mutant (Figure 3A). In addition, we recovered six alleles of RBOHF—but no other NADPH oxidase—in our large-scale forward genetic screen (Figure 1A). This strongly suggests that RBOHF plays a unique role in Casparian strip (CS) formation. A first explanation for its unique function was provided when we localized a complementing fusion construct of RBOHF (mCherry-RBOHF*gen*) and found that it displayed accumulation in the Casparian strip domain (Figures 3B–3D and S5A), while identical fusions of RBOHB and D were excluded from there (Figure 3E). The CSD localization of RBOHF was surprising, since all other plasma membrane proteins tested so far are excluded from this highly scaffolded domain that even blocks lateral diffusion of lipid tracers like FM4-64 (Alassimone et al., 2010; Roppolo et al., 2011). Therefore, RBOHF entry into this domain probably requires specific interactions between RBOHF and Casparian strip domain proteins like the CASPs. We conclude that differential localization is one element explaining the apparent specificity of RBOHF. We then generated chimerae between the regulatory and catalytic domains of RBOHB and RBOHF. Intriguingly, the catalytic domain of RBOHB (RBOHB*cat*) can partially rescue the *rbohf* phenotype when it is fused to the RBOHF regulatory domain (RBOHF*reg*). By contrast, a chimera of the RBOHB regulatory domain (RBOHB*reg*), fused to the RBOHF catalytic domain (RBOHF*cat*), was unable to rescue (Figure 3F). Thus, the N-terminal domain of RBOHF is able to receive a regulatory input that allows its activation for Casparian strip formation. This same input cannot be perceived by RBOHB present in the same cell. When localizing the different chimeric RBOH proteins, we noticed that neither

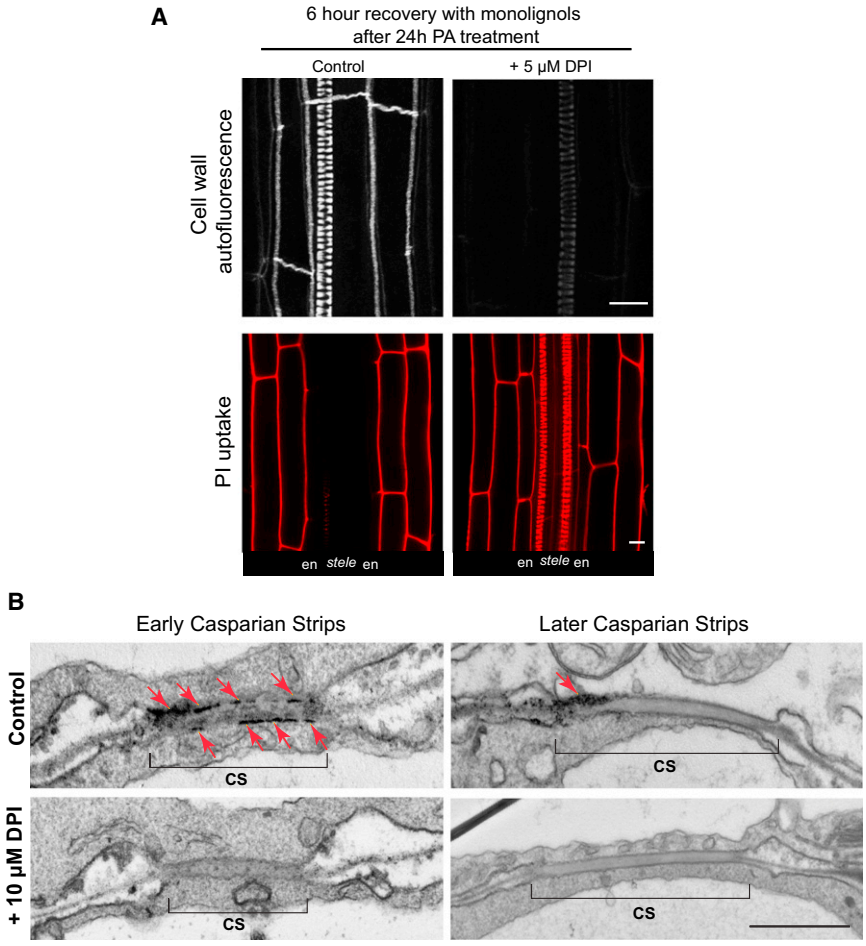

Figure 2. NADPH Oxidase Is Essential for Casparian Strip Formation and Localized ROS Production

(A) The NADPH oxidase inhibitor DPI abolishes complementation observed by monolignol addition in the newly grown root zone of 6-day-old seedlings. Roots were treated for 24 hr with lignin biosynthesis inhibitor PA. After this, seedling roots were incubated for 6 hr on plates with monolignols (20 µM coniferyl and sinapyl alcohol) with or without 5 µM DPI. Casparian strip networks (cell wall autofluorescence, upper panel) and diffusion barrier formation (PI tracer uptake, lower panel) were assessed (see also Figures S2 and S3). en, endodermis; stele, cell layers enclosed by endodermis.

(B) Localized H_2O_2 is detected in electron micrographs by its reaction with 10 mM cerium chloride ($CeCl_3$) to produce electron-dense deposits. DPI (10 µM) was pretreated for 1 hr. Arrows point to electron-dense deposits. Note the deposits are formed throughout CS region in developing strips, but are confined to outer edge of more established strips, probably reflecting block of cerium chloride uptake by the strip itself (see also Table S3).

Scale bars, 10 µm (A); 0.5 µm (B).

the F*reg*-B*cat* nor the B*reg*-F*cat* chimera accumulated detectably in the Casparian strip domain, demonstrating that both regulatory and catalytic domain of RBOHF are needed for correct localization and providing an explanation for the only partial rescue of the RBOHF*reg*-B*cat* chimera (Figure S5C). Therefore, the specific activity of RBOHF is apparently due to a concurrence of correct localization and ability to receive a specific stimulatory input. Eventually, aberrantly structured Casparian strip will be formed in *rbohf*, suggesting that, in late stages, additional sources of ROS can partially compensate for lack of RBOHF. One possibility is that one of the as-yet untested RBOH genes is able to account for this effect.

Peroxidases Use Localized Superoxide-Derived H_2O_2 for Casparian Strip Formation

Our model whereby localized RBOHF-mediated superoxide production determines local lignin polymerization leads to a number of falsifiable predictions (Figure 4A). First of all, the NADPH oxidase-produced superoxide must be dismutated into H_2O_2, often involving catalytic conversion through apoplastic dismutases (Karlsson et al., 2005; Karpinska et al., 2001). Indeed, applying diethyldithiocarbamic acid (DDC), an inhibitor of dismutases, led to a strong block in Casparian strip recovery in the short-term assay described above for DPI (Figure 4B). Eliminating the local H_2O_2 should then lead to a block in Casparian strip formation, and we indeed observed this by treating plants with potassium iodide (KI), a scavenger of H_2O_2. Finally, only peroxidases require H_2O_2, in contrast to the O_2-requiring laccases, also known to mediate lignin formation. Therefore, we applied salicylhydroxamic acid (SHAM), which interferes with the haem coenzyme of peroxidases. Again, we observed a strong block in Casparian strip and diffusion barrier formation (Figure 4C). Neither DDC nor SHAM is a very specific inhibitor of its respective enzyme group, and both can have very toxic side effects. Therefore, care was taken to determine a noncytotoxic concentration and to assess the effect in the shortest possible time window (Figure S2). It can be seen from the propidium iodide uptake experiments that, under our conditions, a block in Casparian strip formation is not simply due to cell death, since propidium iodide quickly penetrates into dead plant cells, leading to a very bright nuclear staining. The fact that three different manipulations, inhibition of dismutases, H_2O_2 scavenging, and inhibition of peroxidases, all block Casparian strip formation provides additional support to a model in which RBOHF-controlled superoxide production supplies H_2O_2 for a peroxidase-mediated polymerization of lignin, forming Casparian strips.

Specific, Endodermis-Expressed Peroxidases Are Required for Casparian Strip Formation

In order to obtain additional evidence for this model, we decided to focus on the last step of the process, the peroxidases. Indeed,

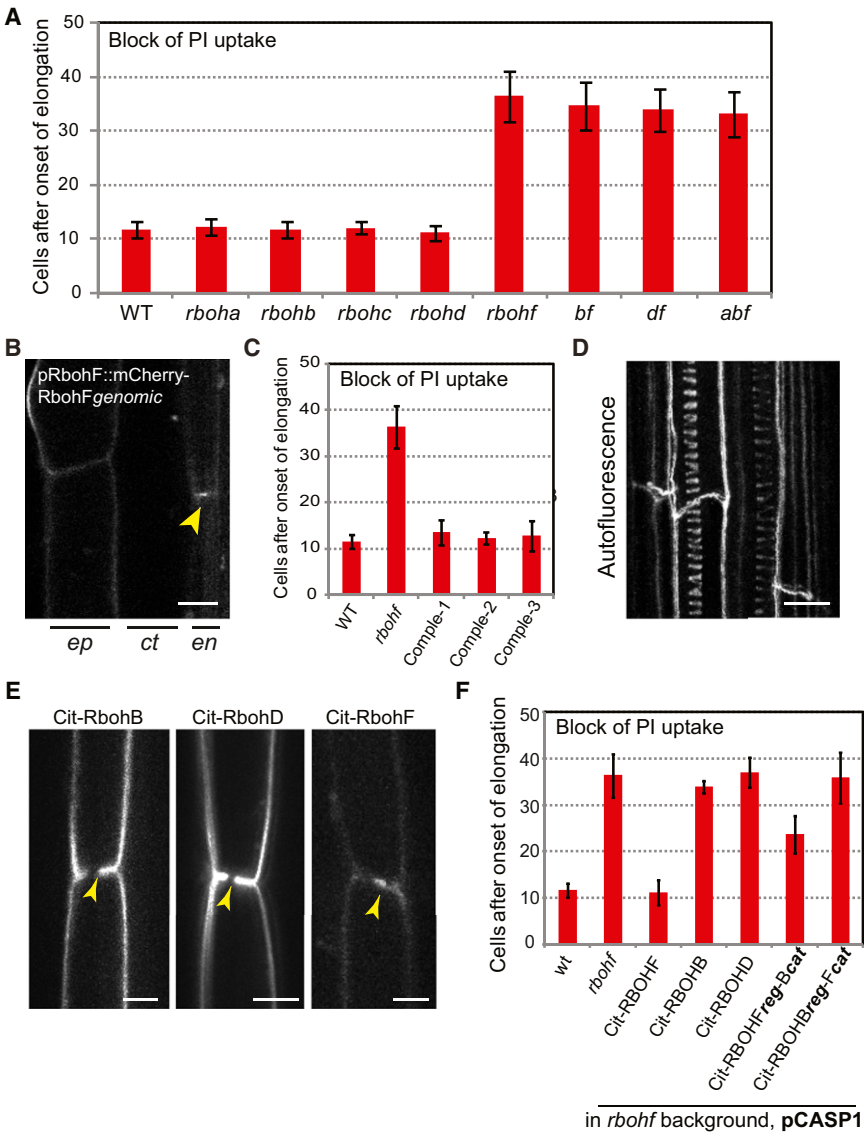

Figure 3. CSD Localization and Regulatory Input Determine Specificity of RBOHF Action in Casparian Strip Formation

(A) Establishment of a functional diffusion barrier is visualized by block of PI diffusion (mean ± SD, 13 < n < 31) (see also Figure S4 and Table S2).

(B) Line of mCherry-RBOHF genomic construct, driven by own promoter shows localization at the plasma membrane and expresses in every cell type of 5-day-old seedling roots, including endodermis where it accumulates at the CSD. Arrowhead points to CSD accumulation (see also Figure S5).

(C) Delayed PI block phenotype of *rbohf* is complemented in three independent complementation lines expressing pRBOHF::mCherry-RBOHF genomic construct (comple-1-3) (mean ± SD, 20 < n < 31).

(D) Rescued Casparian strip autofluorescence in a complementation line.

(E) Endodermal localization of mCitrine fused to RBOHB, D, and F under the CASP1 promoter. Only mCitrine-RBOHF localizes at the CSD. Arrowheads point to CSD (see also Table S5).

(F) Delayed PI block phenotype of *rbohf* is complemented by introduction of Cit-RBOHF CDS under the CASP1 promoter. When N-terminal regulatory domain of RBOHF is replaced by that of RbohB (Cit-RBOHB*reg*-F*cat*), no complementation is observed. By contrast, regulatory domain of RBOHF combined with catalytic domain of RbohB (Cit-RBOHF*reg*-B*cat*) shows partial complementation ($p < 0.01$, mean ± SD, 19 < n < 31) (see also Figure S5C).

Scale bars, 10 μm (B and D); 5 μm (E). ep, epidermis; ct, cortex; en, endodermis; st, stele.

cell-type specific microarrays show a good number of endodermis-enriched, secreted (type III) peroxidases, and coexpression analysis with CASP1 brings up a number of peroxidases at very good scores (Figure 5A). We confirmed endodermis-specific or enriched expression by generating promoter::NLS-GFP fusions of candidate peroxidase genes (Figure 5B; Table S4). Unfortunately, even when we combined four highly endodermis-enriched peroxidases into a quadruple mutant, we could not observe any delay in the formation of the diffusion barrier (Figure 5C; Table S2). However, one of the peroxidases that is strongly and specifically expressed in the endodermis, PER64, is not represented in available knockout collections. Therefore, we generated inducible, endodermis-specific artificial microRNA (amiRNA) knockdown lines of this peroxidase (Table S5). To our surprise, we observed a significant delay in diffusion barrier formation when PER64 amiRNA was induced in the wild-type background. This provides genetic evidence for a role of peroxidases in Casparian strip formation and indicates that PER64, probably in conjunction with other, endodermis-expressed peroxidases, is directly required for Casparian strip formation. A localized production of H_2O_2 at the Casparian strip domain, as a required cosubstrate for peroxidases, could, in principle, be sufficient for a localized lignin polymerization, even if the peroxidases themselves were nonlocalized within the endodermal cell wall space. However, this solution might not be optimal, because H_2O_2 diffusion is known to be rapid (Miller et al., 2009) and could thus easily lead to ectopic lignin formation, as would any ectopic, stress-related production of H_2O_2, provided lignin precursors are present. We therefore tested whether subcellular localization of PER64 and other endodermis-expressed peroxidases provide additional spatial specificity.

CASPs Determine Localization of an Endodermal Peroxidase to the Casparian Strips

When we localized a mCherry c-terminal fusion of PER64 in a CASP1-GFP background, we observed a stunning, essentially perfect, colocalization with CASP1-GFP (Figures 6A and 6B). This colocalization was not restricted to stages where a mature

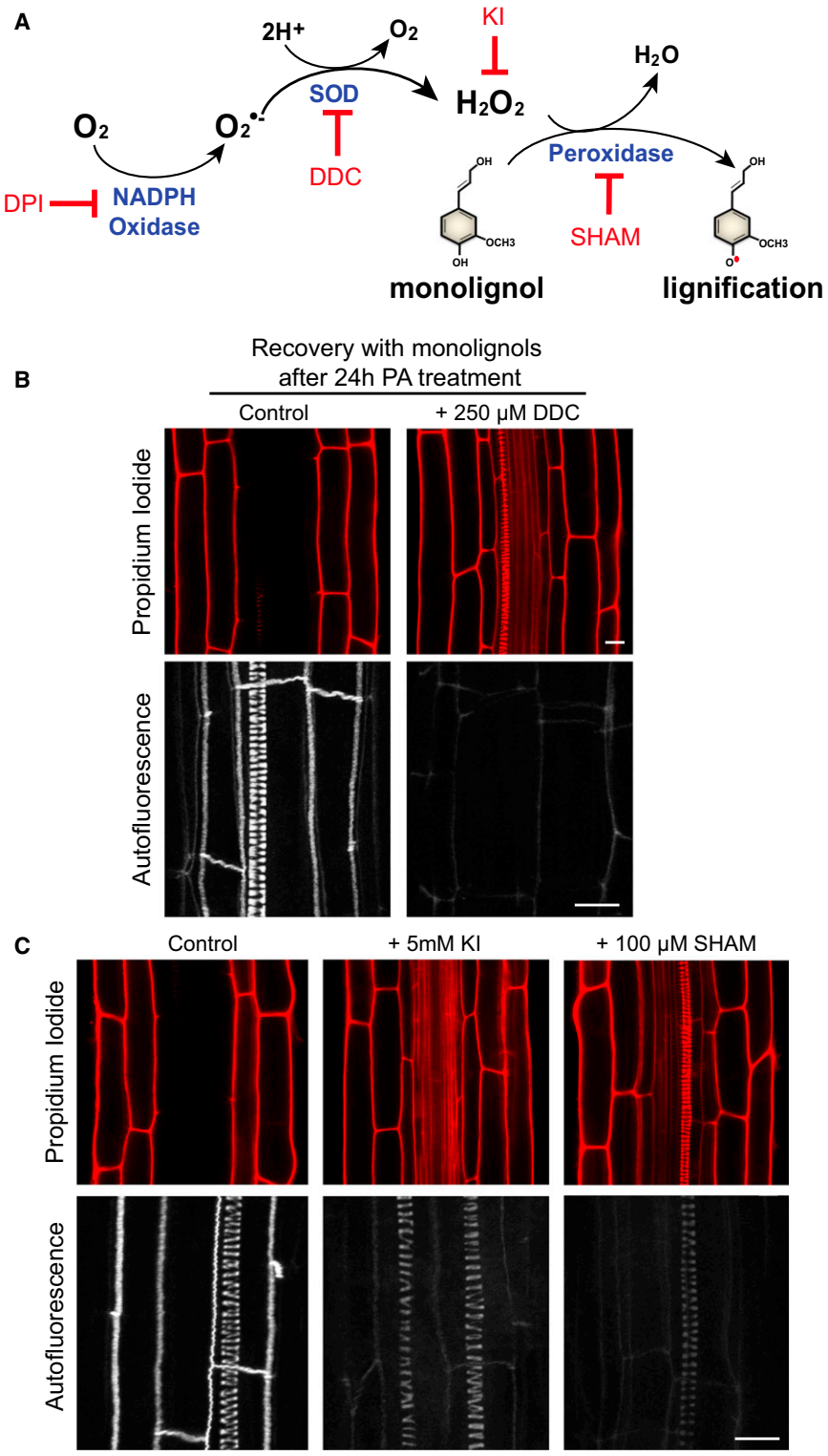

Figure 4. NADPH Oxidase Drives H_2O_2 Production for Peroxidase-Mediated Lignin Polymerization in Casparian Strips

(A) Schematic representation of the required steps between NADPH oxidase activity and lignin polymerization. Diphenyleneiodonium (DPI, NADPH oxidase inhibitor), diethyldithiocarbamic acid (DDC, superoxide dismutase inhibitor), potassium iodide (KI, H_2O_2 scavenger), salicylhydroxamic acid (SHAM, peroxidase inhibitor).

(B) When 250 µM DDC is applied together with monolignols after 24 hr of PA treatment, as in Figure 2A, Casparian strip reconstitution does not occur (see also Figure S2 and Table S6).

(C) When 5 mM KI and 100 µM SHAM is applied for 24 hr, root growth continues, but Casparian strip formation is inhibited in the newly grown root zone (see also Figure S2 and Table S6).

Scale bars, 10 µm.

CSD had been formed (Figure 6B), but could even be observed at very early stages, where CASP1-GFP is still present in a string of isolated microdomains at the plasma membrane (Roppolo et al., 2011) (Figure 6A). This suggests an intimate connection between CASP1 and PER64 subcellular targeting. Other endodermal peroxidases tested also showed an accumulation at the CSD, although none was as specific as PER64, and signals outside of the Casparian strip, such as in cell corners, vacuoles or other intracellular compartments could be observed (Figure S6A). We confirmed that accumulation in the Casparian strip is not a general feature for secreted proteins in the endodermis, by using the extracellular domain of FORMIN HOMOLOGY 1 (FH_{1-107}), which was previously shown to be efficiently secreted and to ubiquitously label the cell wall (Martinière et al., 2011). At first glance, the FH_{1-107}-mCherry appeared to be localized in a manner similar to the Casparian strips. The nonoverlapping distribution with CASP1-GFP, however, clearly showed that FH_{1-107}-mCherry simply accumulates preferentially in the outward-facing cell corners of the endodermis, possibly because this part contains by far the most cell wall material (Figure S6B). Whether the difference between PER64 and other peroxidases is due some special property of PER64, such as a better tolerance toward protein fusions remains to be determined. We then decided to ectopically coexpress CASP1-GFP and PER64-mCherry in epidermal cells by an inducible promoter in epidermal cells in order to see whether CASP1-GFP is sufficient for localizing PER64. Previously, we had shown that CASP misexpression in epidermal cells can lead to strong

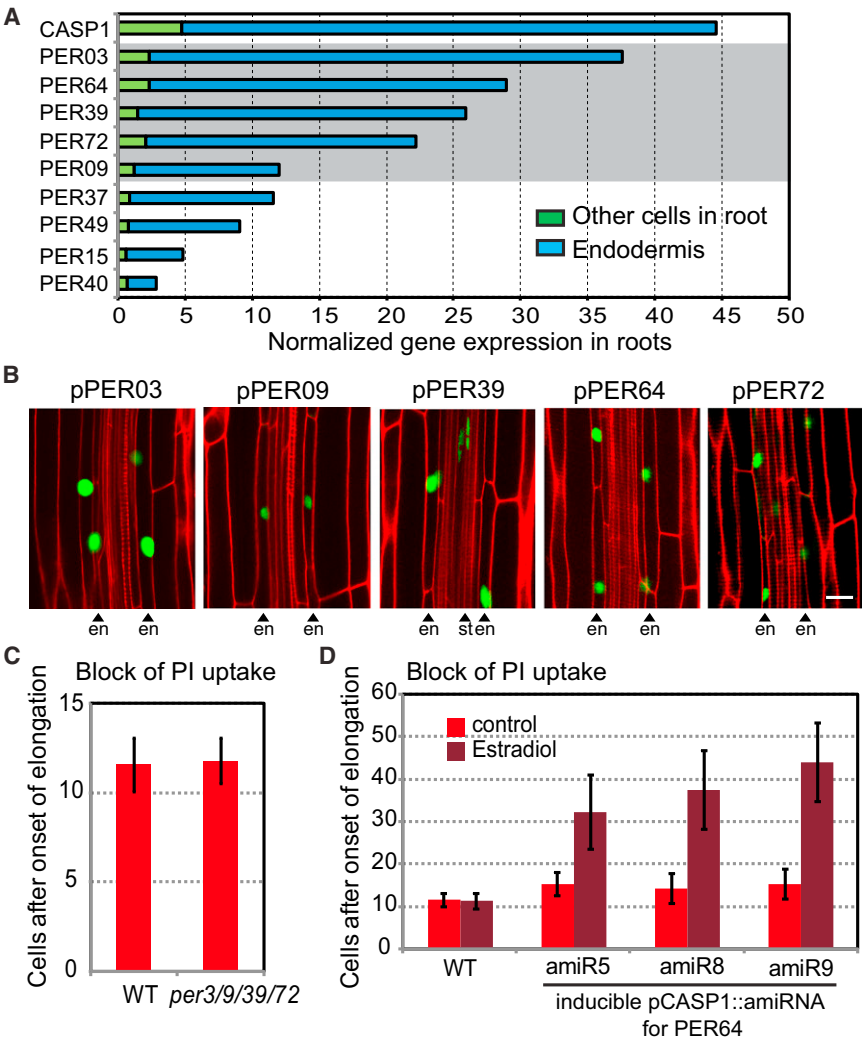

Figure 5. Peroxidases Are Involved in the Casparian Strip Formation

(A) Microarray data showing endodermis-enriched gene expression of CASP1 and peroxidases in roots (data from Birnbaum et al., 2003; Brady et al., 2007).

(B) Promoter gene expression analysis of peroxidases with nuclear-localized GFP-GUS as reporter confirms endodermis-specific/enriched gene expression in the endodermis. Arrowheads point to cell layers showing expression. en, endodermis; st, stele (see also Table S4). Scale bar, 20 μm.

(C) Establishment of a functional diffusion barrier, visualized by PI, is not affected in the quadruple mutant of *per3 per9 per39 per72* ($p > 0.5$, mean ± SD, $25 < n < 29$) (see also Table S2).

(D) Establishment of a functional diffusion barrier is delayed in mutants expressing inducible artificial microRNA for PER64 germinated in 1/2 MS plates including 10 μM estradiol ($p < 0.001$, mean ± SD, $16 < n < 27$) (see also Table S5).

accumulation of CASPs in structurally aberrant endoplasmic reticulum (ER) (Roppolo et al., 2011). Intriguingly, when PER64 was coexpressed with CASP1-GFP in the epidermis, both strongly accumulated in the aberrant ER, even though no other endodermis-specific factors are present (Figures 6C and S6C–S6E). Interestingly, CASP5-GFP, which also induced, and localized to, similar aberrant ER structures was not able to cause intracellular accumulation of PER64, which was secreted in a nonlocalized fashion to the cell walls of the epidermis (Figure 6D). Moreover, neither epidermal expressed CASP1-GFP nor CASP5-GFP was able to block secretion of the FH_{1-107}-mCherry control (Figure S6F). In our view, these data are suggestive of an interaction between CASP1 and PER64 and show that CASP1 is sufficient to determine the subcellular localization of PER64 in epidermal cells. In addition, we could demonstrate that CASPs are also necessary for correct PER64 localization in the endodermis. In a *casp1 casp3* double mutant, PER64-mCherry localization remained in interrupted bands at the location of the Casparian strip domain for a long period, eventually forming contiguous bands, but which appeared broader and more patchy than in the wild-type (Figure 6E). This localization of the putative lignin-forming PER64 nicely fits with the previously observed, aberrant formation of Casparian strips in this double mutant and additionally supports a role for PER64 in lignin formation (Figure 6F) (Roppolo et al., 2011).

The striking localization of many endodermis-enriched peroxidases to the Casparian strip strongly suggests that localized peroxidases contribute to the spatially confined formation in the endodermis, together with the localized RBOHF-dependent H_2O_2 production. The conjunction of the two localized activities might be necessary to ensure a tight spatial control of the essentially irreversible process of lignin polymerization.

DISCUSSION

Originally described as being responsible for the so-called respiratory burst in neutrophils, NADPH oxidases in animals are now known to control a vast array of functions. NADPH oxidase-produced ROS have been implicated in various stress signals, but also hormone production, generation of extracellular signaling gradients, or matrix formation (Bedard and Krause, 2007; Moribe et al., 2012; Niethammer et al., 2009). Plants have undergone an independent diversification of NADPH oxidases that mediate a similarly stunning array of functions (Sagi and Fluhr, 2006). *Arabidopsis* contains ten respiratory burst homolog (RBOH) genes. Initially described in the context of the oxidative burst during hypersensitive cell death (Torres et al., 2002), RBOHs became rapidly implicated in a more diverse array of signaling pathways, and RBOH-dependent ROS have now been established as an important intracellular second

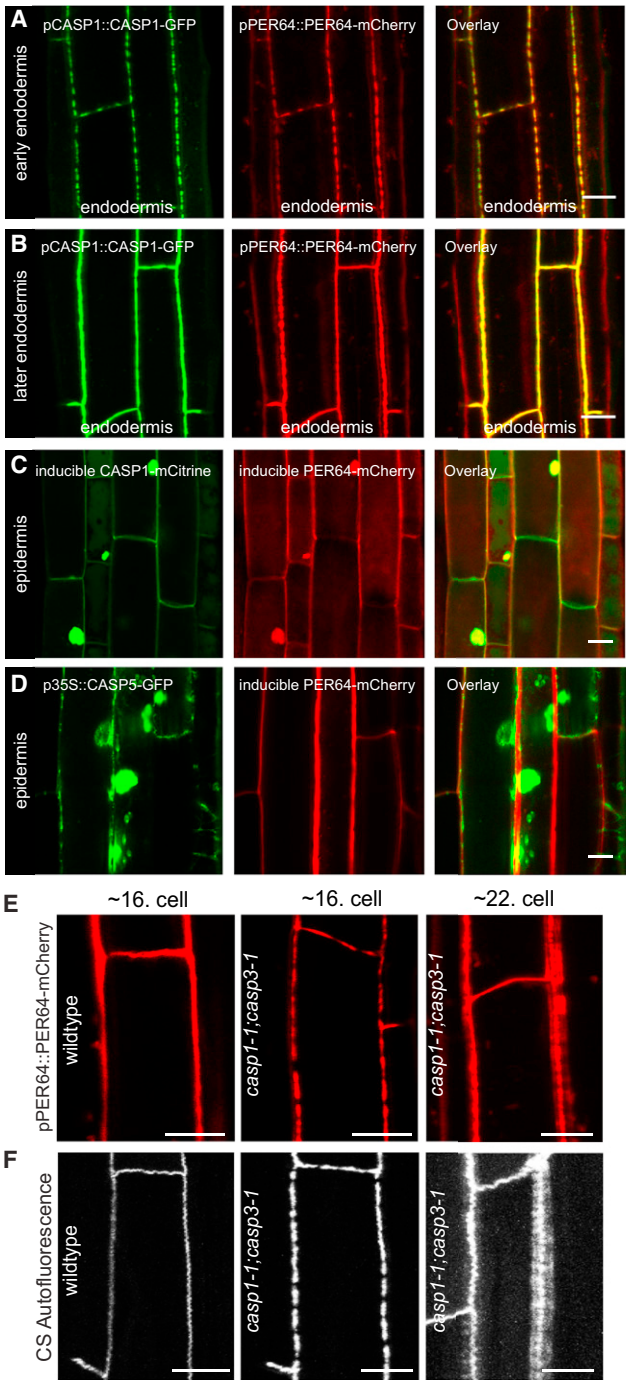

Figure 6. CASP1-GFP Determines PER64-mCherry Localization

(A and B) PER64-mCherry driven by own promoter colocalizes with CASP1-GFP in early endodermal cells (A) and later stage of endodermal cells (B) (see also Figures S6A and S6B and Table S5).

(C and D) Ectopically expressed PER64-mCherry, CASP1-mCitrine, and CASP5-GFP. PER64-mCherry colocalizes at the CASP1-mCitrine aberrant ER structures (C), but not at the CASP5-GFP aberrant ER structures (D) (see also Figures S6C–S6F).

(E) pPER64::PER64-mCherry in *casp1-1;casp3-1* mutant shows irregular localization of PER64, which is correlated with the pattern of CS autofluorescence in double mutant.

(F) Casparian strip formation defects of *casp1-1;casp3-1* mutant follows the defects of PER64-mCherry localization, with irregular, noncontiguous patches early (~16. cell) and more diffuse autofluorescence later (~22. cell).

Scale bars, 10 μm.

messenger that regulates expression of hundreds of genes in response to diverse stimuli, such as cellular damage, drought, salt stress, presence of elicitors, etc. However, the immediate ROS receptors that activate the diverse signaling pathways have remained elusive, and it is unclear how specificity of ROS signaling might be controlled (Møller and Sweetlove, 2010; Torres, 2010). While much recent work has focused on the role of NADPH-oxidase-produced ROS in signaling, initial reports described plasma-membrane-oxidase-produced ROS as an agent involved in cell wall crosslinking (Bradley et al., 1992). Root hair tip growth is one recent example in which a specific NADPH oxidase (RBOHC) was shown to produce ROS for cell wall modification. RBOHC was found to be mutated in the root-hair-defective 2 (rhd2) mutant and shown to localize to the growth zone of root hairs. The apoplastic ROS produced by RBOHC are thought to counteract pH-induced cell wall loosening in order to prevent root hair bursting. However, it is unclear which proteins or cell wall polymers are targeted by ROS in this context (Foreman et al., 2003; Monshausen et al., 2007; Takeda et al., 2008).

RBOHF Is a Versatile Signaling Module Involved in Diverse Cellular Responses

Here, we have provided evidence that RBOHF mediates a very different kind of cell wall modification, not associated with cellular growth. We show that RBOHF is strictly localized within the endodermal plasma membrane, due to its specific N-terminal regulatory domain and its ability to enter the CSD, a highly scaffolded membrane protein platform formed by the CASPs. Localized, extracellular H_20_2 is then used by CASP-recruited peroxidases to mediate monolignol oxidation and polymerization. Absence of RBOHF therefore leads to a very strong delay in the formation of Casparian strips and to an impaired apoplastic barrier. None of the characterized, root-expressed RBOHF homologs tested led to a similar phenotype or increased the *rbohf* phenotype in severity, nor did we identify another NADPH oxidase in our forward genetic screen (J.A., unpublished data). This specific, nonredundant activity contrasts with the many other, often partially redundant, activities described for RBOHF prior to our work. Initially, RBOHF, together with RBOHD, was shown to be responsible for the ROS burst associated with infection of avirulent bacteria, although RBOHF made only a minor contribution to the detectable ROS burst, while it had a more prominent role in the induction of cell death (Torres et al., 2002). A very different role for RBOHF in the abscisic-acid-induced ROS production in stomata, again in conjunction with RBOHD, was described a year later (Kwak et al., 2003). In this case, the H_20_2 produced is thought to act as a second messenger for activation of calcium channels and MAP kinases (Jammes et al., 2009). Very recently, RBOHF has been found as a salt-hypersensitive mutant, necessary for salt-induced ROS production in the stele, which contributes to retention of NaCl from the xylem sap (Jiang et al., 2012). In most reported RBOHF

functions, it remains unclear what the direct downstream targets of ROS are and how they relate to the observed phenotypes. Clearly, many reported phenotypes of *rbohf*, such as smaller size, altered cell death response or salt sensitivity could, in part, be explained as consequences of delayed Casparian strip formation and consequent pleiotropic alterations of nutrient homeostasis. In the future, it will be important to untangle the relationship between the different reported cellular functions and the overall phenotype. This could be done by organ- and cell-type-specific complementation assays of RBOHF in stele, root endodermis, and stomata, for example. Yet, it remains that RBOHF has multiple different functions in different cells. In stomata, RBOHF receives signals downstream of ABA signaling, and RBOHF-produced ROS serve as an intracellular second messenger for the activation of MAP kinases. This is very different role from the localized, apoplastic ROS production for lignin polymerization that we report here. We speculate that RBOHF, and its homologs, have evolved into versatile signaling modules that can coexist in the same cell, that can be specifically activated by diverse inputs, and whose outputs—superoxide, dismutated into H_20_2—will have different activities, depending on their respective subcellular location and the protein components that are assembled around the NADPH oxidase.

CASPs Are Organizers of Cell-Wall-Modifying Protein Activities

We have found that CASP1-GFP colocalizes very closely with PER64, as much in the mature Casparian strip domain as during its early assembly. Moreover, ectopically expressed CASP1 becomes retained in the ER, causing retention of coexpressed PER64 in the same ER structures. This does not occur upon ectopic expression of CASP5, which is equally retained in similar ER bodies, nor are other secreted substrates hindered in their secretion. This specific, induced colocalization of CASP1 and PER64 is a strong indication of an interaction between CASP1 and PER64. It is most plausible to assume a direct interaction, since indirect interactions would require that bridging factors are present in the epidermis and allow interaction of two endodermis-specific proteins. Unfortunately, in vivo pull-down experiments are rendered difficult by the polymeric nature of the Casparian strip domain and the tight interaction of both CASPs and peroxidases with the lignified cell wall. Therefore, we have not yet been able to detect interactions between CASPs and peroxidases in such assays (Y.L., unpublished data). PER64 is secreted to the apoplast in the epidermis in the absence of CASP1 and shows a broader, patchy distribution in the endodermis of a *casp1 casp3* mutant plant. This shows that PER64 does not require CASP1 for its secretion, but only for its localization to the Casparian strip domain. Therefore, mutating CASPs should not interfere with lignin polymerization, but rather lead to a delocalized formation of lignin in the endodermis, which is exactly what is observed in the *casp1 casp3* double mutant (Roppolo et al., 2011; this work). We therefore view the function of CASPs—and by extension the large family of CASP-like proteins, as plasma membrane platforms that serve to localize peroxidases—and possibly additional cell-wall-modifying enzymes—to the right subcellular location. Even in cases where a very restricted formation of lignin is not required, bringing NADPH oxidases into close proximity of peroxidases could provide channeling of the produced ROS toward peroxidases and increase the efficiency of lignin polymerization. It will be interesting to see whether assembly of lignin polymerizing complexes by CASPs or CASP-likes will turn out to be a general feature that applies to other lignifying cell types. A scaffolding and channeling of ROS by CASPs could explain how different activities of ROS can be kept separate, a major current problem for our understanding of ROS action (Møller and Sweetlove, 2010; Sagi and Fluhr, 2006).

NADPH Oxidase/Peroxidase Scaffolding: A Widespread Mechanism?

The mechanism that we describe of bringing together NADPH oxidases with the downstream ROS-dependent enzymes might also apply to processes in the animal kingdom. It was shown previously in *C. elegans* that DUOX, an animal-specific NADPH oxidase, and a peroxidase are involved in collagen crosslinking, necessary for cuticle formation (Edens et al., 2001). Recently, a tetraspanin was shown to be necessary for DUOX activity at the plasma membrane (Moribe et al., 2012). Tetraspanins bear no discernable homology to CASPs, but are also involved in organizing membrane microdomains (Charrin et al., 2009). Possibly, tetraspanins could act like CASPs bringing together NADPH oxidase and peroxidase and ensure the localized activation of the oxidase. This would be an intriguing case of convergent evolution that could be driven by the strong advantage in restricting ROS availability to a subset of selected ROS receptors or ROS-dependent enzymes within the cell.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions

Arabidopsis thaliana ecotype Columbia was used for most experiments. For detail of knockout mutants, see Table S1 and S2. The *rbohd rbohf* double-knockout seeds were obtained from M.A. Torres (Universidad Politécnica de Madrid, Madrid, Spain). Plants were germinated on 1/2 MS (Murashige and Skoog) agar plates after 2 days in dark at 4°C. Seedlings were grown vertically in Percival chambers at 22°C, under long days (16 hr light/8 hr dark) and were used at 5 days after shift to room temperature.

Chemicals

Details of chemicals used in present work are summarized in Table S6.

Transgenic Lines

For generation of expression constructs, Gateway Cloning Technology (Invitrogen) was used. For primer details, see Tables S4 and S5. Artificial microRNAs were designed using the Web microRNA Designer (http://wmd2.weigelworld.org) targeting PER64 nucleotides 555–575 of the CDS, and expressed under the estradiol-inducible *CASP1* promoter. Transgenic plants were generated by introduction of the plant expression constructs into an *Agrobacterium tumefaciens* strain GV3101 and transformation was done by floral dipping (Clough and Bent, 1998).

GUS Staining

For promoter::GUS analysis, 5-day-old seedling were incubated in 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) staining buffer solution (10 mM EDTA, 0.1% Triton X-100, 2 mM Fe2+CN, 2 mM Fe3+CN, 1 mg/ml X-Gluc) in 50 mM sodium phosphate buffer (pH 7.2) at 37°C for 5∼24 hr in darkness.

Microscopy and Quantitative Analysis

Confocal laser scanning microscopy was performed on a Zeiss LSM 700 confocal microscope. Excitation and detection windows were set as

follows: GFP 488 nm, 500–600 nm; mCherry 594 nm, 600–700 nm; propidium iodide 488 nm, 600–700 nm; mCitrine and mCherry 514 and 594, 520–560, and 600–700 nm. Casparian strips were visualized, as described (Alassimone et al., 2010; Naseer et al., 2012). For visualization of the apoplastic barrier, seedlings were incubated in the dark for 10 min in a fresh solution of 15 μM (10 μg/ml) PI and rinsed two times in water (Alassimone et al., 2010; Naseer et al., 2012). For quantification, "onset of elongation" was defined as the point where an endodermal cell in a median optical section was more than three times its width. From this point, cells in the file were counted until the PI signal was blocked in the endodermal cells.

Inhibitor Treatments

For the treatment of potassium iodide (H_2O_2 scavenger) and SHAM (peroxidase inhibitor), 5-day-old seedlings were transferred to the plates containing 5 mM KI or 100 μM SHAM and incubated for 24 hr in dark. For the DPI (NADPH oxidase inhibitor) and DDC (superoxide dismutase inhibitor) treatment, 5-day-old seedlings were preincubated in plates containing 10 μM piperonylic acid (PA, lignin inhibitor) for 24 hr in dark and transferred to 1/2 MS solution including 5 μM DPI or 250 μM DDC together with two monolignols: 20 μM of each coniferyl alcohol and sinapyl alcohol.

Transmission Electron Microscopy for Cerium-Chloride-Based H_2O_2 Detection

The histochemical method based on the generation of cerium perhydroxides as described (Bestwick et al., 1997) and was used for the location of H_2O_2 at the Casparian strips. Cerous ions (Ce^{3+}) react with H_2O_2 forming electron dense cerium perhydroxide precipitates, which are detected by electron microscopy.

Five-day-old seedlings were preincubated in 50 mM MOPS (3-[*N*-morpholino] propane sulphonic acid) (pH 7.2) alone or with 10 μM DPI for 1 hr, and then freshly prepared 10 mM cerium chloride ($CeCl_3$) was added. After 30 min incubation with $CeCl_3$, plants were washed twice in MOPS for 5 min and then fixed in glutaraldehyde solution (EMS, Hatfield, PA) 2.5% in phosphate buffer (PB 0.1 M [pH 7.4]) during 1 hr at room temperature (RT). Then, they were postfixed in a fresh mixture of glutaraldehyde 2.5% in osmium tetroxide 1% (EMS) with 1.5% of potassium ferrocyanide (Sigma, St. Louis, MO) in PB buffer for 1 hr at RT. The samples were then washed two times in distilled water and dehydrated in acetone solution (Sigma) at graded concentrations (30% 40 min; 50% 40 min; 70% 40 min; three times (100% 1 hr). This was followed by infiltration in Spurr resin (EMS) at graded concentrations (Spurr 33% in acetone, 12 hr; Spurr 66% in acetone, 12 hr; Spurr two times (100% 8 hr) and finally polymerized for 48 hr at 60°C in an oven. Ultrathin sections 60 nm thick were cut transversally at 2 mm from the root tip, on a Leica Ultracut (Leica Mikrosysteme GmbH, Vienna, Austria) and picked up on a nickel slot grid 2 × 1 mm (EMS) coated with a polystyrene film (Sigma). Sections were poststained with uranylacetate (Sigma) 4% in H_2O during 10 min, rinsed several times with H_2O followed by Coogeshall lead citrate 0.4% in H_2O (Sigma) during 10 min, and rinsed several times with H_2O. Micrographs of the Casparian stripes were taken with a transmission electron microscope FEI CM100 (FEI, Eindhoven, The Netherlands) at an acceleration voltage of 80 kV and 24,500× magnification (pixel size of 1.385 nm and horizontal field width of 5.55 μm) with a Morada SIS digital camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany) using the software SIS iTEM (Olympus Soft Imaging Solutions GmbH).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.02.045.

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