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Ogorek, Lucas León Peralta; Takahashi, Hirokazu; Nakazono, Mikio; Pedersen, Ole

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The barrier to radial oxygen loss protects roots against hydrogen sulphide intrusion and its toxic effect

Lucas León Peralta Ogorek1, Hirokazu Takahashi2, Mikio Nakazono2,3 and Ole Pedersen1,3

1The Freshwater Biological Laboratory, Department of Biology, University of Copenhagen, Universitetsparken 4, 3rd Floor, Copenhagen 2100, Denmark; 2Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa, Nagoya 464-8601, Japan; 3School of Agriculture and Environment, The University of Western Australia, Crawley, WA 6009, Australia

Summary

- The root barrier to radial O2 loss (ROL) is a key root trait preventing O2 loss from roots to anoxic soils, thereby enabling root growth into anoxic, flooded soils.
- We hypothesized that the ROL barrier can also prevent intrusion of hydrogen sulphide (H2S), a potent phytotoxin in flooded soils. Using H2S- and O2-sensitive microsensors, we measured the apparent permeance to H2S of rice roots, tested whether restricted H2S intrusion reduced its adverse effects on root respiration, and whether H2S could induce the formation of a ROL barrier.
- The ROL barrier reduced apparent permeance to H2S by almost 99%, greatly restricting H2S intrusion. The ROL barrier acted as a shield towards H2S; O2 consumption in roots with a ROL barrier remained unaffected at high H2S concentration (500 μM), compared to a 67% decline in roots without a barrier. Importantly, low H2S concentrations induced the formation of a ROL barrier.
- In conclusion, the ROL barrier plays a key role in protecting against H2S intrusion, and H2S can act as an environmental signalling molecule for the induction of the barrier. This study demonstrates the multiple functions of the suberized/lignified outer part of the rice root beyond that of restricting ROL.

Introduction

Plant growth in flooded, anoxic soils relies on several key root traits with the barrier to radial O2 loss (ROL) being one of the most important. The root barrier ROL consists of suberized and sometimes lignified (Ranathunge et al., 2011a) exodermal cells restricting O2 diffusion to the soil and retaining O2 within the root (Watanabe et al., 2013). O2 diffuses longitudinally towards the root tip following a concentration gradient and enables cell division and continued root extension that only takes place in the presence of molecular O2 (Armstrong & Webb, 1985; Colmer, 2003a). Here, we show that the ROL barrier also restricts radial H2S intrusion, thereby protecting the root tissue from this potent soil phytotoxin. We also show that low and non-lethal concentrations of H2S act as an environmental signal for the ROL barrier formation through upregulations of genes related to lignin rather than suberin biosynthesis.

Roots of wetland plants rely on longitudinal diffusion of O2 from the shoot to the root tips when growing in flooded soils. Flooded soils are generally anoxic since molecular diffusion in water is too slow to replenish O2 consumed by plant roots and soil microbes (Colmer & Voesenek, 2009), and roots cannot rely on radial supply of O2 from the rhizosphere. Instead, longitudinal diffusion of O2 is facilitated via gas-filled spaces (aerenchyma) providing a low resistance pathway for gas diffusion (Yamauchi et al., 2019). Roots of rice constitutively form aerenchyma (Shiono et al., 2011) in drained soils, helping the existing roots survive should soil flooding occur. Nevertheless, additional aerenchyma is induced as a response to soil flooding, further enhancing the capacity for internal aeration (Pedersen et al., 2020). While a large proportion of the O2 diffusing towards the root apex is radially lost into the anoxic rhizosphere, ROL can be significantly restricted by a barrier to ROL (Kotula et al., 2009), thereby extending the maximum root length achieved in flooded soils (Jiménez et al., 2020).

The formation of the ROL barrier can be induced by several environmental cues. Soil flooding in itself is able to induce a formation of the ROL barrier (Colmer, 2003b); although abscisic acid is involved in the ROL barrier formation (Shiono et al., 2022), the exact mechanisms behind it remain unclear. Neither low O2, high CO2 nor ethylene acts as signals for the ROL barrier formation in rice (Colmer et al., 2006), whereas several of the phytotoxins produced by soil microbes in flooded soils have been shown to induce ROL barrier formation, namely low-molecular weight carboxylic acids (Colmer et al., 2019) and Fe2+ (Mongon et al., 2014). Interestingly, high concentrations of sulphides can further enhance the tightness of an existing ROL barrier (Armstrong & Armstrong, 2005), but it is not known whether low and non-lethal concentrations of H2S can also act as an environmental cue for the ROL barrier.
Histochemically, the root ROL barrier consists of enhanced cell wall depositions of suberin and lignin in the exodermis. While the exodermis can also be lignified (Ranathunge et al., 2011b), it is hypothesized that the gas-tight feature of the exodermis is primarily due to the suberization (De Simone et al., 2003; Nishiuchi et al., 2012). In accordance with this hypothesis, upregulation of genes involved in suberin biosynthesis is observed when roots of rice are exposed to the relevant environmental signals (Shiono et al., 2014), whereas upregulation of lignin-related genes is seen as a stress response once the signalling compounds are supplied in high concentrations (Colmer et al., 2019).

A true jack of all trades, the root barrier to ROL not only restricts radial diffusion of molecular O2 but also diffusion of other gases and even movements of ions through the apoplast. The ROL barrier greatly restricts radial diffusion of H2 (Peralta Ogorek et al., 2021), even if molecular H2 is a much smaller molecule compared to O2 and, therefore, diffuses faster (Zhang & Cloud, 2006). Furthermore, radial diffusion of water is likewise restricted, sparking speculations that the ROL barrier is possibly of importance as a drought tolerance trait (Peralta Ogorek et al., 2021), and it has been shown that low water potentials can induce a ROL barrier in roots of rice (Song et al., 2023). Finally, radial movement of solutes via the apoplast is also restricted by the ROL barrier; the permeability to NaCl decreased by 60% compared to that in roots without a barrier (Ranathunge et al., 2011a). When supplied with reduced iron, Fe2+ mostly accumulated exteriorly to the exodermis in mature regions of the roots where a ROL barrier was formed, whereas Fe2+− was localized also in the cortex and endodermis in the root tip region where the barrier is not formed (Jimenez et al., 2021). Consequently, it has been suggested that the ROL barrier could also restrict H2S diffusion into the roots (Armstrong & Armstrong, 2005; Soukup et al., 2007; Nishiuchi et al., 2012), but although H2S can be an important phytotoxin in some soils, this hypothesis remained untested.

H2S is a potent phytotoxin present in anoxic, acidic flooded soils. H2S is produced by anaerobic sulphate-reducing bacteria using sulphate as electron acceptor (Lamers et al., 2013), and acts similarly to cyanide by inhibiting the cytochrome c oxidase at concentrations of 1–10 μM (Raven & Scrimgeour, 1997; Armstrong & Armstrong, 2005). H2S does not require any facilitator to diffuse through the lipid bilayer cell membrane and can diffuse as fast as O2 or CO2 despite the fact that H2S is a polar molecule that can form hydrogen bonds (Riahi & Rowley, 2014). Once H2S permeates inside the root tissue, it quickly diffuses through the aerenchyma (Pedersen et al., 2004), and even though H2S is spontaneously oxidized by O2, the oxidation rate is slow without the aid of H2S-oxidizing bacteria (Pedersen et al., 2004). Furthermore, H2S in the soil has been linked to a disease in rice called ‘Straighthead’ that reduces rice productivity (Joshi et al., 1975; Lamers et al., 2013), making this phytotoxin of great economic interest. This suggests that H2S could diffuse through the root aerenchyma, all the way to the shoot, and cause the disease.

We aimed at closing the long-standing knowledge gaps related to the role of H2S signalling for ROL barrier formation as well as the putative capacity of the ROL barrier to restrict H2S intrusion. We tested the hypothesis that the ROL barrier restricts H2S intrusion into roots of rice using H2S-specific microelectrodes to obtain the permeance to H2S of the outer part of the roots without or with a ROL barrier. Moreover, we measured H2S intrusion on intact roots while manipulating the O2 supply from the shoot to the roots since root O2 status was shown to be an important factor to prevent H2S intrusion into seagrass roots (Borum et al., 2005). We also tested the toxicity of H2S on respiration in roots with or without a ROL barrier and established a dose–response curve for tissue toxicity. Finally, we investigated the role of low and non-lethal concentrations of H2S as a signal for the ROL barrier formation and evaluated upregulated and downregulated key genes involved in suberin and lignin biosynthesis during H2S exposure.

Materials and methods

The following sections conceptually describe the experimental procedures (see Supporting Information Methods S1 for details).

Plant material

Seeds of rice (Oryza sativa L.) genotype ‘IR42’, a moderate drought and submergence tolerant cultivar (Ponnamperuma, 1979), were germinated and grown in hydroponics without (nutrient solution with forced aeration using air pumps) or with a ROL barrier (7 d in stagnant, deoxygenated nutrient solution) as described in Peralta Ogorek et al. (2021). Plant age during measurements ranged between 26-d to 36-d-old, except for plants with a weak barrier, which was induced by growing plants in aerated nutrient solution for 44–55 d.

O2 or H2S flux into root segments

Fluxes of O2 or H2S into root segments were used to assess the presence or absence of a ROL barrier (O2 intrusion) or to evaluate the capacity of the barrier to ROL to restrict H2S intrusion; see Peralta Ogorek et al. (2021; Fig. 1a) for details on the experimental setup. Segments from position 30–60 mm behind the root tip were prepared from target roots without or with a ROL barrier. The segments had their cut ends sealed with lanoline to prevent gas intrusion via the cut ends, and were fixed on a metal mesh using rubber bands. The mesh was placed inside an aquarium, where an O2 or H2S microsensor was inserted 150–250 μm into the root cortex. Solutions with specific composition of O2 or H2S (c. 60 kPa pO2; c. 0.04 or 0.22 kPa pH2S) were added to the aquarium and gas intrusion into the cortex was recorded. Measurements lasted a minimum of 15–20 min or until a quasi steady state was achieved (O2 or H2S readings in the cortex changed < 5% per 5 min). The intrusion rate of O2 was calculated from the linear slope when the cortex reached air equilibrium (20.6 kPa). For H2S, the intrusion rate was calculated when 50% of the quasi steady state of pH2S inside the root cortex was obtained.

The intrusion rates were used to calculate the apparent permeance (P, m s−1) to O2 or H2S following the equation described in Lendzian (2006):
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positioned on a metal mesh and had a H2S microsensor inserted 150 μm from the root tip were prepared from 10 to 14 cm long roots which had the root cortex 2–3 cm below the root–shoot junction. The root compartment was filled with a stagnant solution and then cortex O2 status was measured in the absence of H2S. Then, the root chamber was drained, and the liquid replaced with a stagnant solution now containing 0.18–0.22 kPa pH2S to follow H2S intrusion with the shoot either in air or submerged to facilitate or restrict O2 diffusion from the shoot to the root.

H2S toxicity measured as impact on respiration

We assessed the toxicity of H2S on respiration of root segments without or with a ROL barrier measured as O2 consumption. We also measured the respiration of root tips to obtain a toxicity curve and estimate the EC50 of H2S.

Target roots without or with a barrier were shortened to 50 mm segments, had the apical 30 mm removed, and their cut ends sealed with lanoline. They were then incubated in a solution without (control) or with 500 μM H2S for 4 h. Afterwards, the segments were rinsed, split open through the middle (Jiménez et al., 2020), left to acclimate for 15 min covered with moist paper towel, and finally placed inside glass vials with DI water at air equilibrium; see Results section for justification of the splitting approach. For root tip respiration measurements, target roots were incubated in various concentrations of H2S for 4 h after which the most apical 10–12 mm were excised from the roots, rinsed in DI water, and placed inside glass vials with DI water at air equilibrium. An O2 minioptode was inserted into the vials to measure the decline of O2 over time to calculate respiration rates expressed by unit of fresh mass.

The effect of splitting the roots was also tested by incubating target roots without or with a barrier to ROL for 1 h in DI water saturated with air. The procedure was as described earlier, except a group of segments were split before placing them inside the vials, or directly placed in the vials without splitting.

Root elongation rates in the presence or absence of H2S

We measured the elongation rates of roots without H2S (control) or with H2S in the nutrient solution. Plants without a barrier to ROL were exposed to 10–20 μM H2S (EC50 concentration of root tip – see the Results section) by purging the nutrient solution with a mixture of air and H2S. The H2S concentration in the nutrient solution was constantly monitored using H2S microsensors.

Target roots were tagged to follow their growth during 5 d of H2S exposure, and the elongation rate was calculated from daily measurements of the tagged roots. After 5 d, the purging of H2S was stopped, the nutrient solution renewed, and was only aerated with air. New roots were tagged, and their elongation followed to

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measure roots elongation in the recovery phase. Finally, elongation rates were also followed on new adventitious roots that formed from primordia during the recovery phase.

Induction of a ROL barrier by H$_2$S

Several environmental signals have been shown to trigger the ROL barrier formation in rice. Therefore, we tested whether low concentrations of H$_2$S could induce a ROL barrier. Plants without a barrier to ROH were moved into a nutrient solution purged with a mixture of air and H$_2$S (10–20 μM H$_2$S, see earlier). Another group of plants were transferred to stagnant nutrient solution to serve as a benchmark for the ROL barrier formation. Target roots were harvested to conduct O$_2$ intrusion measurements on segments to calculate $P_a$ to $P_s$ (see ‘O$_2$ or H$_2$S flux into root segments’ in the Materials and Methods section) to monitor the barrier formation with time.

Gene expression during ROL barrier formation by H$_2$S

Root segments from plants of the same age growing in aerated conditions and 24 h after being transferred into H$_2$S or stagnant nutrient solutions were harvested and fixed in 100% methanol. The segments were embedded in paraﬃn (Takahashi et al., 2010) and laser microdissection (LM) was used to obtain serial cross-sections of 12 μm thickness. Total RNA was extracted from the LM-isolated tissues, and the quality of the RNA was assessed as described by Takahashi et al. (2010). RNA sequencing was performed at MacroGen Inc. (www.macrogen.com) using NovaSeq6000. After constructing the gene libraries, STAR (Dobin et al., 2013) was used to map a single-end read to the reference rice genome, which was downloaded from the PHOTOTOME v.13 database (https://phototome-next.jgi.doe.gov). Differentially expressed genes were extracted using DESEQ2 (Love et al., 2014) between aerated and stagnant or H$_2$S conditions, selecting genes that showed greater than twofold change in read counts. Information on gene annotation in rice and homologous genes in Arabidopsis thaliana were downloaded from PHOTOTOME v.13. Finally, gene ontology (GO) enrichment analysis was performed with SHINYGO (http://bioinformatics.sdstate.edu/go/), selecting biological processes of GO terms with an enrichment false discovery rate (FDR) < 0.01.

Histochemical staining of lignin and suberin in cell walls

To visualize the modifications of the cells related to the ROL barrier, histochemical staining of lignin and suberin was conducted on root segments from aerated, stagnant or H$_2$S-exposed conditions. Lignin depositions were detected using phloroglucinol (Jensen, 1962) or the Maule reaction (Kutscha & Gray, 1972). Cinnamaldehyde and syringal groups stain red–pink and brown–orange–pink, respectively, under white light. Suberin depositions in the cell walls were detected using Fluorol Yellow 088 solution under UV light (Brundrett et al., 1991). To facilitate the interpretation of suberin patterns, we calculated the percentage of suberized cell and tested these with a one-way ANOVA.

Statistical analyses

GraphPad Prism software (v.8.4.3) was used to plot and analyze the data, except GO enrichment analysis that was conducted with SHINYGO (http://bioinformatics.sdstate.edu/go/). The statistical analyses conducted for each experiment are detailed in the corresponding figure captions, together with number of replicates and significance levels. $P_a$ to H$_2$S on root segments and percentage of suberized cells was log-transformed to improve data homoscedasticity and normality. For all figures, non-transformed data are shown.

Results

H$_2$S intrusion into root segments

In addition to O$_2$, the ROL barrier restricts radial diffusion of gaseous H$_2$ and H$_2$O (Peralta Ogorek et al., 2021). Therefore, we tested whether the ROL barrier prevents intrusion of H$_2$S into the root when exposed to 0.04 or 0.22 kPa pH$_2$S. Cortical H$_2$S of segments without a barrier surged and reached quasi steady state within a few minutes of exposure, indicating a very low resistance to radial H$_2$S diffusion (Fig. 1a). By contrast, segments with a barrier showed high resistance to radial diffusion of H$_2$S. For the first c. 50 min at 0.04 kPa, there was no detectable intrusion, whereas cortical H$_2$S slowly increased but rarely exceeded 0.01 kPa after 90 min of exposure. Similarly, with pH$_2$S of 0.22 kPa, there was a long lag phase with cortical H$_2$S below the detection limit (c. 20 min), after which H$_2$S intrusion occurred faster than when exposed to 0.04 kPa (Fig. 1a).

Rates of H$_2$S intrusion were converted into apparent permeance ($P_a$) to H$_2$S to correct for differences in intrusion rates due to variations in root thickness and concentration gradients. Mean $P_a$ to H$_2$S for roots without a barrier was $2.50 \times 10^{-6}$ m$^2$ s$^{-1}$, being 75-fold higher than for roots with a barrier, which had a mean $P_a$ of $3.34 \times 10^{-8}$ m$^2$ s$^{-1}$ (Fig. 1b). These results show that the ROL barrier in the outer part of the root cannot entirely prevent H$_2$S intrusion but greatly restricts radial intrusion.

H$_2$S intrusion into intact roots

We mimicked a natural situation where roots have a constant supply of O$_2$ diffusing from the aerial parts via the aerenchyma to the root tip. Here, the internal molecular O$_2$ has the potential to oxidize H$_2$S. Moreover, the root tip represents a window without a ROL barrier where H$_2$S may enter and thereby bypass the ROL barrier present further up the root axis. H$_2$S and O$_2$ micro-sensors were inserted simultaneously into the cortex 3 cm below the root shoot junction (Fig. 2a). First, the O$_2$ status was assessed with the roots submerged into a stagnant (deoxygenated) solution without H$_2$S, and the cortical pO$_2$ remained constant at c. 15 kPa. Next, the root medium was replaced with a stagnant solution containing 0.18–0.22 kPa pH$_2$S. The intrusion of H$_2$S into intact roots without a barrier was fast and reached quasi steady state within few minutes (Fig. 2b), and cortical O$_2$ was not affected by H$_2$S. The cortical pH$_2$S remained almost constant.
throughout the measurements, even when the shoot was submerged to restrict O₂ supply to the roots. After the shoot was de-submerged and O₂ supply to the roots was re-established, pH₂S in the cortex remained unaffected showing that there was little spontaneous oxidation of H₂S inside the cortex.

Intrusion of H₂S into roots with a ROL barrier showed a different pattern (Fig. 2c). There was a long lag phase where H₂S was below detection limit, whereafter H₂S increased at a slower pace than for isolated root segments with similar external pH₂S. When the shoot was submerged, there was a small increase in H₂S intrusion, and when the shoot was de-submerged, pH₂S again declined slightly (Fig. 2c). Direct comparison of pH₂S and pH₂S at the end of each measurement (orange arrowhead) were extracted and compared as a bar plot (d; mean, bar; error lines, SE), where n = 3 for without or with barrier. Statistical comparisons were conducted using a two-way ANOVA followed by a Tukey test (**, P ≤ 0.01; ns, non-significant). 20.6 kPa pO₂ = 293 μM at 23°C and 0.27 kPa pH₂S = 293 μM at 23°C.

Impact of H₂S in root respiration and growth

H₂S impacts mitochondrial respiration in a similar way to cyanide (Raven & Scrimgeour, 1997), and we therefore tested whether the slower intrusion rates observed in roots with a barrier to ROL would also show reduced effects on root respiration. Root segments without or with a barrier were incubated in 500 μM H₂S, and after exposure to H₂S the segments were cut longitudinally to expose respiring tissue since the barrier would prevent radial O₂ diffusion and, thus, respiration. The possible effect on root respiration caused by splitting the roots was tested, but with no significant effect on tissue splitting (Fig. S1). As hypothesized, H₂S greatly reduced O₂ respiration in segments without a ROL barrier and respiration was threefold higher in controls than segments exposed to H₂S (Fig. 3a). Importantly, respiration of segments with a barrier was not at all affected by exposure to 500 μM H₂S, which is 50- to 500-fold the established level for impact at the cytochrome c level.

Root tips do not form a ROL barrier, and it is therefore relevant to determine the toxicity of H₂S on root tips since these hold the root meristem. We measured respiration of detached root tips after exposure to H₂S concentrations ranging from 0 to 500 μM (Fig. 3b). The respiration rate of root tips showed a typical dose–response relationship and the EC₅₀ was estimated to be 15 μM. The respiration continued to decline with increasing...
Fig. 3 Hydrogen sulphide toxicity in root segments of rice (Oryza sativa) is reduced by the barrier to radial oxygen loss (ROL). Panel (a) shows the respiration rate of root segments from 26- to 36-d-old plants without (aerated nutrient solution) or with (stagnant, deoxygenated nutrient solutions) a barrier to ROL as a box whisker plot (mean; +; median; horizontal line; 50% quartiles; box; minimum and maximum values; whiskers), where \( n = 4 \) for without or with barrier. Roots 10–14 cm had the most apical 3 cm removed and shortened to 8 cm segments, whereas the cut ends were sealed with lanolin and incubated in 500 \( \mu \)M H\(_2\)S for 4 h. The segments were then further shortened to 32 mm and split open through the middle with a razor blade before starting the respiration measurements. The respiration rates were compared using a two-way ANOVA followed by a Tukey test (**, \( P \leq 0.001; \) ns, non-significant). Panel (b) shows the respiration rates of root tips (black dots, mean; vertical lines, SD; horizontal lines, concentration range; black line, non-linear curve fit), where \( n = 4 \) for all concentrations. Roots 10–14 cm long were severed and incubated in the different H\(_2\)S concentrations for 4 h. Afterwards, the most apical 10–12 cm were cut and used for respiration measurements. All respiration measurements were conducted in a constant temperature bath at 25°C. EC\(_{50} = 15 \mu \)M or 0.013 kPa \( \rho \)H\(_2\)S and 500 \( \mu \)M H\(_2\)S = 0.46 kPa \( \rho \)H\(_2\)S. Panel (c) shows the elongation rate of roots without a barrier growing in aerated conditions (aerated controls) and aerated conditions with constant H\(_2\)S purge (10–20 \( \mu \)M; H\(_2\)S-exposed) as a box whisker plot (mean; +; median; horizontal line; 50% quartiles; box; minimum and maximum values; whiskers), where \( n = 5 \) for all conditions. Elongation was measured every day for 4–5 d on roots with a starting length of 3–5 cm. The elongation rates were compared using a one-way ANOVA (**, \( P \leq 0.01; \) ns, non-significant).
Beyond 48 h, $P_a$ to $O_2$ of H$_2$S-exposed roots did not decline, indicating that the barrier was not further strengthened.

**Gene expression during ROL barrier formation**

Since the ROL barrier was formed in response to both H$_2$S and stagnant treatments, a shared gene network between the two conditions likely exists. Therefore, we performed RNA-seq analysis on tissues from the outer part of the root using LM on roots grown in the aerated nutrient solution without or with H$_2$S, or in stagnant solution. Compared to aerated conditions, we found that 1694 and 114 < 7 genes were upregulated (Fig. 5a; Tables S1, S2), while 2084 and 1271 genes (Fig. 5b; Tables S3, S4) were downregulated for H$_2$S and stagnant treatments, respectively. This clearly reveals a more extensive response to H$_2$S-exposed compared to stagnant conditions. From these, 276 genes were commonly upregulated and 385 genes downregulated between H$_2$S and stagnant treatments (Tables S5, S6, respectively).

We also conducted a GO term enrichment analysis using genes in *Arabidopsis*, which are homologous to 276 rice genes (Fig. 6a), selecting GO terms with an enrichment FDR < 0.01. We found that gene terms related to lignin biosynthesis (e.g. ‘regulation of lignin biosynthetic process’, ‘lignin biosynthetic process’, ‘lignin metabolic process’) or related to both suberin and lignin biosynthesis (e.g. ‘phenylpropanoid biosynthetic process’ and ‘phenylpropanoid metabolic process’) were significantly enriched in commonly upregulated genes under both H$_2$S and stagnant treatments. Moreover, 11 genes were commonly upregulated in both H$_2$S and stagnant treatments and classified into phenylpropanoid and/or lignin metabolic processes (Table 1). Similarly, GO terms...
of several catabolic processes like ‘leucine catabolic process’ were enriched among the downregulated genes under both H2S and stagnant treatments (Fig. 6b).

Suberin might be a key component of the ROL barrier, but we only found patchy depositions in the cell walls (see later). Interestingly, LOC_Os08g44360 encoding fatty acid reductase (FAR) and LOC_Os11g31090, a homolog gene of REDUCED LEVELS OF WALL-BOUND PHENOLICS 1 (RWP1) in Arabidopsis, were upregulated in both H2S and stagnant conditions (Table 1). Moreover, we found that different copies of suberin biosynthesis genes were upregulated under H2S and stagnant conditions. One copy of 3-ketoacyl-CoA synthases (KCS), involved in fatty acid elongation, was significantly upregulated under H2S conditions, and other four copies were upregulated under stagnant conditions (Table S7). Similarly, one copy of glycerol-3-phosphate acyltransferase (GPAT) was significantly upregulated under H2S conditions, and other two copies were upregulated under stagnant conditions (Table S7). Finally, one copy of CYP86A, involved in fatty acid oxidation, was upregulated under stagnant conditions (Table S7).

Table 1 Commonly upregulated genes of rice (Oryza sativa) roots from 26- to 36-d-old plants grown in aerated conditions with hydrogen sulphide or in stagnant, deoxygenated conditions, classified into phenylpropanoid and/or lignin metabolic process.

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Genes were obtained from RNA-seq performed on the outer parts of the root using laser microdissection. Roots were grown in aerated conditions with H2S or in stagnant, deoxygenated conditions for 24 h.

Fig. 6 Gene ontology (GO) term enrichment analysis of commonly upregulated or downregulated genes between roots of rice (Oryza sativa) grown in aerated conditions with hydrogen sulphide or in stagnant, deoxygenated conditions revealed predominant enrichment of lignin-related terms. Panels (a, b) show the GO terms enriched in the upregulated or downregulated expressed genes, respectively, shared between the outer parts of the root from 26- to 36-d-old plants grown in aerated conditions with H2S or in stagnant, deoxygenated conditions for 24 h. Fisher’s exact test was applied to identify the most enriched GO terms. Larger circles in the hierarchical clustering trees represent more genes annotated to the respective GO terms. The right-hand colour bars indicate P values (< 0.05); n = 4 for all.
Histochemical staining of lignin and suberin

We characterized the ROL barrier using histochemical staining, detecting lignin and suberin deposits in the cell walls (Fig. 7). Lignin staining revealed cinnamaldehyde and syringil depositions in aerated controls throughout the sclerenchyma layer (Fig. 7a,d). However, roots exposed to H$_2$S showed a distinct pattern: cinnamaldehyde and syringil lignin depositions intruded between the exodermal cells above the sclerenchyma (Fig. 7b,e). Moreover, the coloration of lignin staining appeared to be slightly stronger in roots exposed to H$_2$S in the part of the sclerenchyma immediately inside of the exodermal cells compared to the part facing the cortex. Cinnamaldehyde depositions were also detected enveloping some exodermal cells (Fig. 7b). For stagnant-grown roots, both lignin groups were evenly stained in the sclerenchyma, with slightly less lignin intrusion between the exodermal cells compared with that of roots exposed to H$_2$S (Fig. 7c,f).

Suberin staining was also detected in roots of all three growth conditions showing a 'patchy' deposition pattern as some cells were suberized, whereas others were not (Fig. 7g–i). Accordingly, quantitative comparison of suberin depositions under the three growth conditions showed no significant differences in the percentage of suberized cells in the exodermis ( aerated, 34.5 ± 0.07%; aerated+H$_2$S, 31.7 ± 0.04%; stagnant, 53.0 ± 0.16%; mean ± SE; n = 4).

Discussion

Hydrogen sulphide is known as a potent phytotoxin and it has long been debated whether the root barrier to ROL can restrict radial diffusion of H$_2$S from flooded soils into the root tissue, but this hypothesis had never been tested. We found that the ROL barrier greatly restricts H$_2$S intrusion, and thereby root respiration remained unaffected even at very high external H$_2$S concentrations. We also showed that low concentrations of H$_2$S act as a signal for the ROL barrier formation. Below, we discuss these findings in detail, including the genes involved in the formation of the ROL barrier when exposed to H$_2$S or stagnant conditions.

The ROL barrier reduced intrusion of H$_2$S

We evaluated whether the ROL barrier would restrict H$_2$S intrusion from the rhizosphere. We found that the barrier decreased apparent permeance ($P_a$) to H$_2$S by almost 99% (Fig. 1b), clearly demonstrating the protective role of the ROL barrier.

Fig. 7 Lignin and suberin staining shows that low concentrations of hydrogen sulphide triggered histochemical modifications in the outer parts of rice (Oryza sativa) roots. Histochemical staining of cinnamaldehyde groups of lignin was detected using phloroglucinol across the sclerenchyma and exodermal cell layers in root cross sections (position 30–55 mm behind the root tip) from 26- to 36-d-old plants growing in aerated conditions (a), after 3 d in aerated conditions with a constant H$_2$S purge (b, 15 μmol H$_2$S), and after 3 d in stagnant, deoxygenated conditions (c). Cinnamaldehyde groups of lignin were detected in all growth conditions (white arrowheads). In Aerated + H$_2$S (b), lignin staining appears to be slightly stronger in the sclerenchyma layer facing the exodermis, compared with the sclerenchyma layer facing the cortex. Moreover, red/pink coloration was detected between exodermal cells (black arrow). Panels (d–f) illustrate staining of syringil lignin groups using the Maule reaction (red/orange/brown colour) at the same position and treatments as used in the phloroglucinol method. Syringil lignin groups were detected in all growth conditions (black arrowheads). Aerated + H$_2$S (e) and stagnant conditions (f) show lignin ‘intrusion’ between the cells in the exodermis above the sclerenchyma (white arrows). Panels (g–i) show suberin staining using Fluorol Yellow O88 in aerated controls, aerated + H$_2$S and stagnant conditions, respectively (plant age and position similar to lignin staining). Examples of cells with suberin depositions are indicated with yellow arrowheads and cells without suberin deposition are indicated with white arrows. Comparison of percentage of suberized cells shows no significant difference between the growth conditions ( aerated, 34.5 ± 0.07%; aerated + H$_2$S, 31.7 ± 0.04%; stagnant, 53.0 ± 0.16%; mean ± SE; one-way ANOVA on log-transformed data). Ep, epidermal cells; Ex, exodermal cells; Scl, sclerenchyma cells; n = 4. Bar, 25 μm.
comparison, a tight barrier virtually blocks O\textsubscript{2} intrusion, whereas the reduction in P\textsubscript{r} to H\textsubscript{2} or water vapour by the barrier is comparatively lower (reduced by 74\% and 94\%, respectively, Peralta Ogorek et al. (2021)). We therefore propose that the evolutionary role of the barrier is not only restricted to prevent ROL, but also serves a protective role against soil phytoxins intrusion, now shown for H\textsubscript{2}S and also for reduced Fe (Jimenez et al., 2021).

Interestingly, shoot O\textsubscript{2} supply and internal aeration had little influence on H\textsubscript{2}S intrusion in intact roots (Fig. 2b,c). This is different to seagrasses, where a reduction in O\textsubscript{2} supply to the shoot accelerated H\textsubscript{2}S intrusion into the belowground tissues (Pedersen et al., 2004; Borum et al., 2005; Brodersen et al., 2017). However, roots growing in hydroponics are unlikely to form a stable biofilm hosting H\textsubscript{2}S-oxidizing bacteria, and such bacteria greatly increase H\textsubscript{2}S oxidation in the presence of O\textsubscript{2} (Pedersen et al., 2004); these biofilms would likely establish in paddy soils just like in seagrass sediments (Brodersen et al., 2015). Importantly, a radial flux of O\textsubscript{2} to the rhizosphere can be beneficial and act as a means of detoxification when H\textsubscript{2}S is oxidized to SO\textsubscript{4}\textsuperscript{2-}, a process that also reduces the formation of Fe\textsubscript{S} plaques; such plaques hamper nutrient uptake in wild rice (LaFond-Hudson et al., 2018). Regardless, during flood events where the entire shoot becomes submerged, reduced O\textsubscript{2} supply to the roots occurs during nighttime (Pedersen et al., 2009; Winkel et al., 2013), and this could lead to critically high tissue H\textsubscript{2}S concentrations since we do observe a slight acceleration in H\textsubscript{2}S intrusion when shoot O\textsubscript{2} supply is restricted, even in our short-term experiments.

Toxic effects of H\textsubscript{2}S on root respiration and elongation

The presence of the ROL barrier significantly reduced the toxic effect of H\textsubscript{2}S. We used a new approach enabling root respiration measurements in the presence of a ROL barrier (Jimenez et al., 2020) and found that the respiration rates of segments without a barrier were reduced by 67\% at 500 \textmu M of H\textsubscript{2}S. In comparison, 380–480 \textmu M (undissociated) of four different types of low molecular mass organic acids reduced O\textsubscript{2} consumption by only 22\% (Colmer et al., 2019) demonstrating the high toxicity of H\textsubscript{2}S (Raven & Scrimgeour, 1997). Importantly, a tight barrier restricted H\textsubscript{2}S intrusion so that even at 500 \textmu M external concentration, the respiration remained unaffected, whereas a weak barrier resulted in some decline in tissue respiration (Fig. S3).

Root tips do not form a ROL barrier and root tip respiration is essential for root elongation (Armstrong & Beckett, 1987). Our dose–response experiment revealed an EC\textsubscript{50} of root tip respiration of 15 \textmu M H\textsubscript{2}S (Fig. 3b), which is substantially lower than that of organic acids, being in the mM range (Colmer et al., 2019). A low EC\textsubscript{50} would be expected considering that mitochondrial function is impacted already at concentrations between 1 and 10 \textmu M (Raven & Scrimgeour, 1997). Physiological function is also impacted as evidenced by a reduction of 80\% in phosphorous uptake for four rice cultivars when exposed to 15 \textmu M of H\textsubscript{2}S (Joshi et al., 1975). There was some residual O\textsubscript{2} consumption even at the highest concentration used, so we tested the effect of incubation time and found that the residual respiration declined significantly from 2 to 4 h (the latter was used in Fig. 3b). Therefore, we cannot rule out that the residual respiration would decline further with even longer incubation times.

The physiological impact of H\textsubscript{2}S on root tips was clearly demonstrated in this study by the reduction in root growth. The reduction in root elongation (Fig. 3c) corresponded closely to the EC\textsubscript{50} a 50\% reduction in O\textsubscript{2} consumption also resulted in halving of the root elongation rate. A previous study showed that much higher H\textsubscript{2}S concentrations (174 \textmu M) completely stunned root growth (Armstrong & Armstrong, 2005). This past study also showed that root growth resumed when H\textsubscript{2}S was removed from the nutrient solution. In this study, there was a tendency of recovery of the H\textsubscript{2}S-exposed roots within 4 d (Fig. 3c) suggesting that the damage at the mitochondrial level is severe close to EC\textsubscript{50} but new adventitious roots emerged after the H\textsubscript{2}S exposure and grew at similar rates to that of the control plants (Fig. S2), demonstrating that the root primordia had not been damaged. However, we did not test whether the significant restriction in H\textsubscript{2}S intrusion caused by the presence of the ROL barrier was beneficial at the whole plant level (root elongation or shoot growth rates).

Low concentrations of H\textsubscript{2}S trigger the ROL barrier formation

We found that low H\textsubscript{2}S concentrations act as an environmental signal for the ROL barrier formation. This is the first observation where H\textsubscript{2}S induced the formation of a ROL barrier in roots without a barrier exposed to concentration levels near EC\textsubscript{50} (10–20 \textmu M), whereas a pioneer study showed that the presence of high H\textsubscript{2}S concentrations, in combination with stagnant conditions, further enhanced the barrier strength (Armstrong & Armstrong, 2005). Concentrations near EC\textsubscript{50} are indeed found in some rice soils (Allam, 1971) and H\textsubscript{2}S is therefore likely to act in concert with other signalling molecules for barrier formation under natural growth conditions. Consequently, we add another well-known by-product from anaerobic bacterial activity acting as an environmental signal for the barrier formation, for example reduced Fe (Mongon et al., 2014), low-molecular weight carboxylic acids (Armstrong & Armstrong, 2001; Colmer et al., 2019) and now H\textsubscript{2}S. Interestingly, the H\textsubscript{2}S-induced barrier tightness was similar to that induced in stagnant conditions (Fig. 4b), suggesting that regardless of the environmental signal, the net results of the histochemical modifications of the cell walls led to the same tightness to molecular O\textsubscript{2} even if the cell walls showed contrasting staining to lignin and suberin (Fig. 7).

We found that a weak barrier was present after 48 h (Fig. 4b) of exposure to H\textsubscript{2}S or to stagnant conditions. This time response differs to that of a previous study where ROL was significantly reduced already after 6–12 h of exposure to stagnant conditions (Shiono et al., 2011), possibly because measurements were conducted at a different root position, that is closer to the root–shoot junction. On the other hand, high concentrations of H\textsubscript{2}S (174 \textmu M) enhanced barrier strength 30 mm behind the root tip within 24 h when H\textsubscript{2}S was added into the stagnant medium.
Gene regulation in ROL barrier formation

It has been suggested that phenylpropanoids, such as suberin or lignin, are the major components of the ROL barrier, although it is not clear which one has a more significant role (Kotula et al., 2009). Some genes were commonly upregulated under H₂S and stagnant conditions, and different copies of suberin biosynthesis genes like KCS, GPAT, CYP86A4 were upregulated in both conditions (Table S7), and two GPAT genes (LOC_Os02g02340 and LOC_05g38350) have been shown to be specifically expressed in the exodermis (Nishiuchi et al., 2021). Previous studies have identified a number of genes related to suberin biosynthesis responding to stagnant conditions (Kulichikhin et al., 2014; Shiono et al., 2014). Compared to Shiono et al. (2014), only 15 genes overlapped with this study even under stagnant conditions. The little overlap is possibly due to the former study using the basal part of the root as compared with this study focusing on the apical region where the ROL barrier is being formed. By contrast, Kulichikhin et al. (2014) also focused on the apical region and showed significantly higher overlap with 459 genes commonly upregulated in the two studies. These findings suggest that the regulation during ROL barrier formation differs between the apical and basal parts of roots.

Moreover, several lignin biosynthesis-related genes were commonly upregulated in both H₂S and stagnant conditions (Table 1), and six of these overlapped with Kulichikhin et al. (2014). LOC_Os02g1680, LOC_Os04g43760, and LOC_Os05g35290 all encode phenylalanine ammonia-lyase, LOC_Os09g04050 encodes cinnamoyl COA reductase, and LOC_Os10g36840 encodes coniferaldehyde 5-hydroxylase. These enzymes are involved in phenylpropanoid biosynthesis and contribute to lignin biosynthesis (Yao et al., 2021). Additionally, LOC_Os09g23620 is a homolog gene of AtMYB43, which directly activates lignin biosynthesis genes (Geng et al., 2020). Altogether, these results suggest that the lignin biosynthesis pathway was activated in the outer part of root under H₂S and stagnant conditions, indicating that lignin is also a major component of the ROL barrier in rice.

Histochemical staining revealed a clear pattern of lignin depositions during H₂S exposure. The observed lignin staining with phloroglucinol and/or Maule reaction of roots growing in stagnant conditions (Fig. 7c,f) was similar to that of previous studies (e.g. Kotula et al., 2009; Ranathunge et al., 2011a). However, the pattern of H₂S-exposed roots was strikingly different: lignin staining revealed that depositions occurred strongly at the sclerenchyma layer facing the exodermal cells, but lignin was also deposited between the exodermal cells as well (Fig. 7b,e). This could be related to the shape of exodermal cells that changed from a rectangular to a circular shape (Fig. 7b,e) allowing for more lignin depositions in the new intercellular spaces. Interestingly, this pattern was not observed in rice when exposed to other phytotoxins such as organic acids (Colmer et al., 2019) or with comparatively high concentrations of H₂S in stagnant conditions (Armstrong & Armstrong, 2005).

Suberin was detected in all growth conditions, even in aerated controls. Similarly, low concentrations of organic acids induced a patchy layer of suberin (Colmer et al., 2019), whereas high concentrations of sulphides induced a more homogeneously developed suberin layer (Armstrong & Armstrong, 2005). This could be due to the higher sulphide concentration used, the fact that sulphide was added to roots growing in stagnant conditions, or different sampling times/positions behind the root tip. Regardless, staining for lignin was more extensive than suberin, correlating with the higher number of genes involved in lignin biosynthesis compared with suberin-related genes.

Conclusions

This study showed that the barrier to ROL effectively restricts radial H₂S intrusion from the environment. Thereby, the mature zone of rice roots with a ROL barrier are well protected from the toxic effect of H₂S. The EC₅₀ for H₂S is low and the toxic effects, once these occur, are severe as shown on root elongation. Moreover, H₂S concentrations in the range of EC₅₀ can also act as an environmental cue for the ROL barrier formation. The root ROL barrier can thus be seen as a jack of all trades as it not only conserves O₂ in the root tissue by preventing ROL, but also restricts (1) radial water loss (Peralta Ogorek et al., 2021), (2) radial intrusion of reduced Fe (Jimenez et al., 2021), (3) intrusion of sodium (Ranathunge et al., 2011a), and (4) radial intrusion of H₂S.

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Competing interests

None declared.

Author contributions

LLPO and OP planned and designed the study. LLPO performed all experiments and data analyses except for the gene expression and suberin staining conducted by MN and HT. LLPO and OP wrote the manuscript with input from MN and HT regarding gene expression methods, results, and discussion.
Data availability

RNA-seq are available from DRA in DDBJ (accession nos. DRR446089–DRR446100), and the remaining data are available at 10.5061/dryad.8w9ghx3rm.

References


**Supporting Information**

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Respiration rate of rice (*Oryza sativa*) root segments without or with a barrier to radial O₂ loss and without or with splitting of the segment.

**Fig. S2** Elongation rates of rice (*Oryza sativa*) roots during or after exposure to H₂S.

**Fig. S3** H₂S toxicity on rice (*Oryza sativa*) root segments respiration without, with a weak or tight barrier to radial O₂ loss.

**Methods S1** Detailed materials and methods.

**Table S1** Upregulated genes in rice (*Oryza sativa*) root segments grown in aerated conditions with H₂S for 24 h.

**Table S2** Upregulated genes in rice (*Oryza sativa*) root segments grown in stagnant, deoxygenated conditions for 24 h.

**Table S3** Downregulated genes in rice (*Oryza sativa*) root segments grown in aerated conditions with H₂S for 24 h.

**Table S4** Downregulated genes in rice (*Oryza sativa*) root segments grown in stagnant, deoxygenated conditions for 24 h.

**Table S5** Commonly upregulated genes in rice (*Oryza sativa*) root segments grown in aerated conditions with H₂S or in stagnant, deoxygenated conditions 24 h.

**Table S6** Commonly downregulated genes in rice (*Oryza sativa*) root segments grown in aerated conditions with H₂S or in stagnant, deoxygenated conditions 24 h.

**Table S7** Upregulated suberin biosynthesis genes in rice (*Oryza sativa*) root segments grown in aerated conditions with H₂S or in stagnant, deoxygenated conditions 24 h.

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