Formation of a barrier to radial oxygen loss in L-type lateral roots of rice

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Abstract: A barrier to restrict radial oxygen loss (ROLS) promotes the adaptation of plants to waterlogged soil conditions. A ROL barrier is formed in the basal parts of roots and contributes to the enhancement of the longitudinal diffusion of oxygen (O\(_2\)) via aerenchyma toward the root tips. The adventitious roots of rice (\(Oryza sativa\)) can form a ROL barrier in waterlogged soil; however, whether a ROL barrier can be formed in the lateral roots (LRs) of rice remains unclear. The rice possesses two types of LRs: L- and S-type. L-type LRs are generally long, thick, and capable of branching, whereas S-type LRs are short, thin, and incapable of branching. In this study, we examined whether the L- and S-type LRs of rice possess the ability to form ROL barriers. In L-type LRs, a ROL barrier was formed; the aerenchyma, which is constitutively formed under aerated conditions, was further developed under stagnant deoxygenated conditions, suggesting that these traits promote the supply of O\(_2\) to the root tips of LRs. However, neither a ROL barrier, nor aerenchyma was formed in S-type LRs, and thus ROL was observed mostly in the basal parts. Patterns of deposition of suberin, which is thought to be one of the components of the ROL barrier, were consistent with the patterns of ROL-barrier formation in L-type and S-type LRs. These results suggest that L- and S-type LRs play distinct roles in the growth of rice plants in waterlogged soils and in oxygenating the rhizosphere.

Keywords: aerenchyma, lateral roots, \(Oryza sativa\), radial oxygen loss barrier, rice

Abbreviation: ROL, radial oxygen loss; LR, lateral root

Introduction

Climate change has aggravated the severity of environmental conditions. Among the climate change effects, annual precipitation has increased, which causes soil flooding and hampers the growth of plants. The slow diffusion rate of gases in water compared with that in air is one of the negative effects of flooding on plants. Therefore, the uptake of O\(_2\) and CO\(_2\) is extremely impeded when plants become completely submerged (Voesenek et al. 2006). Rice (\(Oryza sativa\)) is generally cultivated in paddy fields, which are usually in anaerobic and chemically reduced conditions (Ponnamperuma 1984). Under these circumstances, internal transport of gases in the tissues is crucial for rice and other wetland species to maintain their growth in waterlogged soils. The roots of wetland plant species contain large volumes of aerenchyma (Clark and Harris 1981), which consist of longitudinally interconnected gas spaces that enable the rapid transport of gases such as O\(_2\) (Armstrong 1979). Moreover, many wetland plant species (including rice) form a barrier that impedes radial oxygen loss (ROLS) in the basal parts of their roots (Armstrong 1979, Colmer 2003). The ROL barrier prevents oxygen leakage radially from the aerenchyma to the soil and inhibits the entry of phytotoxins from the soil into the root tissues (Armstrong 1979, Ejiri et al. 2021). Therefore, the formation of aerenchyma and a ROL barrier enhances the longitudinal transport of gases in the tissues.
diffusion of O$_2$ from the shoots towards the root tips (Armstrong 1979).

When rice plants are grown in stagnant deoxygenated solutions [conditions that mimic the changes in gas composition (low O$_2$, elevated ethylene, and CO$_2$) typically found in waterlogged soils (Wiengweera et al. 1997)], an impervious barrier to ROL is formed in the basal parts of adventitious roots (Colmer 2003, Kotula and Steudle 2009, Shiono et al. 2011). Suberin lamellae, which are deposited in cell walls (Franke and Schreiber 2007), are often observed in the outer cell layers (e.g., the exodermis and sclerenchyma) in the basal parts of the adventitious roots of some ROL-barrier-forming wetland plant species [including rice (Kotula et al. 2009, Shiono et al. 2011), Zea nicaraguensis (a wild relative of maize (Z. mays subsp. mays); Abiko et al. 2012), wild Echinochloa species (Ejiri and Shiono 2019), and Oryza glumaeapatula (Ejiri et al. 2020)] under stagnant deoxygenated conditions. Therefore, suberin lamellae have been hypothesized to function as components of the ROL barrier (Ejiri et al. 2021). Indeed, many suberin biosynthesis-related genes were upregulated in the outer cell layers of adventitious roots in rice during ROL-barrier formation (Shiono et al. 2014).

Unlike studies on ROL-barrier formation in adventitious roots, ROL-barrier formation in lateral roots (LRs) has been poorly studied. In rice and Phragmites australis, there is no indication that a ROL barrier is formed in LRs under deoxygenated conditions, suggesting that LRs are crucial sites for oxygenation in the rhizosphere (Armstrong 1970, Armstrong et al. 1996). However, the application of sulfide to rice roots reduced ROL from the LRs (as well as adventitious roots), possibly because of sulfide-induced cell-wall suberization and thickening of the superficial layers—although substantial ROL was still observed in the LRs (Armstrong and Armstrong 2005). This result implies that rice LRs may have the potential to form a ROL barrier. Recently, Pedersen et al. (2021) reported that a ROL barrier was formed in the LRs of Z. nicaraguensis, which has high waterlogging tolerance (Illits and Benz 2000, Mano and Omori 2007). Interestingly, a maize introgression line (IL #468), which possesses a locus for ROL-barrier formation on the short arm of chromosome 3 of Z. nicaraguensis (Watanabe et al. 2017), formed a ROL barrier in the LRs as well as the adventitious roots. This result suggests that the gene on chromosome 3 controls ROL-barrier formation in both adventitious roots and LRs (Pedersen et al. 2021).

In rice, LRs are classified into two types, L-type and S-type, based on their morphological and anatomical characteristics (Kawata and Shibayama 1965, Kono et al. 1972, Yamauchi et al. 1987). L-type LRs are generally long and thick, often with secondary LR branching, whereas S-type LRs are short and thin, without branched secondary lateral roots. S-type LRs contribute more to the hydraulic conductivity of the root system, which represents its water uptake ability, than L-type LRs or adventitious roots (Watanabe et al. 2020). L-type LRs (but not S-type LRs) have sclerenchyma and multiple cortical cells. These characteristics are similar to those of adventitious roots, which can form a ROL barrier. This evidence raises the possibility that L-type LRs (but not S-type LRs) possess the potential to form ROL barriers. To date, ROL-barrier formation in rice LRs has not been evaluated by distinguishing L- and S-type LRs. We examined whether a ROL barrier was formed in L- and S-type LRs under stagnant deoxygenated conditions using two methods: methylene blue staining and measurement of ROL by root-sleeving cylindrical platinum oxygen electrodes. Moreover, we investigated aerenchyma formation and suberin accumulation in L- and S-type LRs. Based on these investigations, we discuss the roles of L- and S-type LRs in the growth of rice plants under stagnant deoxygenated conditions (i.e., waterlogged soil conditions).

Materials and Methods

Plant material and growth conditions

Seeds of rice (O. sativa ‘Nipponbare’) were sterilized with 10% (v/v) sodium hypochlorite solution and one drop of Tween 20® for 30 min and then washed several times with deionized water. The sterilized seeds were placed in Petri dishes with 25 mL deionized water for 1 d (4°C, 24 h dark). After 1 d of imbibition, the seeds were transferred to a mesh float with aerated quarter-strength nutrient solution (Colmer et al. 2006) under constant temperature and light conditions (28°C; lighting conditions: PAR of 250–300 µmol m$^{-2}$ s$^{-1}$, 24 h light) in a growth chamber (LH-411SP, Nippon Medical & Chemical Instruments Co. Ltd., Osaka, Japan) for 7 d. After that, the seedlings were transferred to 5-L pots (four plants per pot; 250 × 180 × 120 mm, H × L × W) containing the full-strength nutrient solution in either aerated or stagnant deoxygenated conditions; then the plants were grown at 28°C for 28 d. Every 7 d, the nutrient solutions were renewed. The stagnant deoxygenated nutrient solution contained 0.1% (w/v) dissolved O$_2$ < 0.5 mg L$^{-1}$) with N$_2$ gas flushing before use (Wiengweera et
al. 1997). We previously reported that a ROL barrier was formed in the adventitious roots of rice plants grown under the conditions used in this study (Kulichikhin et al. 2014). When the plants were 28-d old, an adventitious root (45–65 mm long) without LRs was selected; a thread was loosely tied around the selected adventitious root, and the plants were grown for a further 7 d (until 35 d old) under aerated or stagnant deoxygenated conditions. The newly elongated LRs of 35-d-old plants were used for the experiments.

**Qualitative assessment of ROL from roots using methylene blue staining**

Methylene blue is a redox indicator dye; it is blue under oxygenized conditions and colorless under deoxygenized conditions, and thus enables the qualitative assessment of ROL in roots (Armstrong et al. 1992, Shiono et al. 2011). A methylene blue solution was prepared with 13 mg L\(^{-1}\) methylene blue and 0.1% (w/v) agar; then, it was deoxygenized by adding 130 mg L\(^{-1}\) sodium dithionite (Na\(_2\)S\(_2\)O\(_4\)) and became colorless. Thirty-five-day-old plants with adventitious roots 130–150 mm long (all other roots were trimmed off) were selected and transferred to a transparent acrylic tank (300 × 220 × 35 mm, H × L × W) containing the methylene blue solution. The root-shoot junctions of the plants were positioned 30 mm below the surface of the solution, with the shoot in the air. The experiment was conducted at room temperature (25°C) and under light conditions. The staining patterns of the roots were evaluated after 30–45 min.

If the whole length of the root was stained blue, it was assumed that a ROL barrier did not form, whereas if the apical parts were stained blue, but the other parts (i.e., the middle and basal parts) showed very faint blue staining, it was assumed that a ROL barrier had formed. The number of L- and S-type LRs that formed and did not form ROL barriers was counted, using one adventitious root from each of the 10 plants grown under aerated or stagnant deoxygenated conditions. The newly elongated LRs of 35-d-old plants were used for the experiments.

**Measurement of ROL using a root-sleeving cylindrical platinum oxygen electrode**

ROL can be measured using a root-sleeving oxygen electrode. It enables ROL quantification at selected positions along roots in an oxygen-free medium (Armstrong and Wright 1975). The oxygen-free medium contained 0.1% (w/v) agar, 5 mM KCl, and 0.5 mM CaSO\(_4\) (Colmer et al. 1998). For deoxygenation, the solution was flushed with N\(_2\) gas. Plants were transferred to a 7 L gray pot (150 × 370 × 240 mm, H × L × W) containing an oxygen-free medium. The roots were immersed in a deoxygenated medium and the residual oxygen left in the medium was measured. A cylindrical platinum electrode (2.25 mm diameter, 5.0 mm high) fitted with root-centralizing models was arranged around a selected position of the LRs. A polarizing voltage applied to the electrode led to the reduction of oxygen in the water on the electrode surface. The voltage was adjusted using a potentiostat (HA-1010 mM1A, Hokuto Denko Co., Japan) to obtain a current adjacent to the peak of the current-voltage curve. The voltage and current outputs were displayed on a computer using custom-made software. The amount of reduced oxygen was measured as an electrical current in microamperes and converted to values of oxygen flux (ROL) from the root (Armstrong 1971). ROL was assessed along the root length with the center of the electrodes at positions 10, 20, 30, 40, and 50 mm from the root tips of longer L-type LRs (55–60 mm in length) or at positions 10, 20, and 30 mm from the root tips of shorter L-type LRs (35–40 mm in length). Five plants were selected, and for each plant, one L-type LR was selected for ROL measurements. The experiment was conducted at 25°C under light conditions. The diameter of the roots was measured at each position using a calibrated microscope equipped with a DP70 CCD camera (Olympus Optical Co. Ltd., Tokyo, Japan).

**Preparation of root cross sections for histochemical analysis**

Root cross sections were prepared from 5 mm long root segments cut from the 55–60 mm long L-type LRs or 20-mm long S-type LRs of rice plants grown under aerated or stagnant deoxygenated conditions. Root segments were prepared at distances from 5–55 mm (i.e., 5–15, 15–25, 25–35, 35–45, and 45–55 mm) from the root tips of L-type LRs or 5–15 mm from the root tips of S-type LRs. The segments were fixed in 4% (w/v) agar. Cross sections (75-80 µm) were prepared by cutting agar blocks containing root segment using a vibrating microtome (VT 1200S, Leica Biosystems Nussloch GmbH, Nussloch, Germany). The sections were incubated with clearing solution (2 g mL\(^{-1}\) chloral hydrate in glycerol:water, 1:3; 1 h; 70°C) to extract the agglutinant agar. After clearing, the cross sections were washed several times with deionized water. Transverse sections were mounted on glass slides in water drops and viewed with a DP70 CCD camera attached to a BX60 light microscope (Olympus Optical Co. Ltd., Japan).
Anatomical observations of root aerenchyma

Cross sections were mounted in water and viewed with bright field illumination using a fluorescence microscope (BX60, Olympus Optical Co. Ltd., Tokyo, Japan) linked to a computer. The percentage of aerenchyma was calculated from the area of aerenchyma and root cross sections using Image J software (Ver.1.39u; NIH, Bethesda, MD, USA; Schneider et al. 2012).

Histochemical staining of suberin

Suberin accumulation was evaluated by staining the root cross sections with Fluorol Yellow 088. The staining solution was prepared by adding Fluorol Yellow 088 to polyethylene glycol 400 (PEG-400) at 0.1% (w/v), heating at 90°C for 1 h, and then adding 90% glycerol at the same volume as PEG-400 (Brundrett et al. 1991, Lux et al. 2005). Root cross sections were stained (1 h, dark, 25°C) and then washed several times with warm deionized water. Suberin lamellae were observed as yellowish green fluorescence with a CCD camera (DP70, Olympus) upon excitation by UV light (U-RFL-T, Olympus) under a fluorescence microscope (BX60, Olympus) equipped with a U-MWU filter cube (band pass, 450-480 exciter filter; dichroic mirror, DM500; barrier filter, BA515).

Statistical analysis

Data are presented as mean ± standard deviation. Means were compared at the 95% confidence level using a paired t-test (response of position within one treatment) and two-sample t-test (response of treatment).

Results

Methylene blue staining to visualize the location of ROL in LRs

To determine whether a ROL barrier is formed in the L- and S-type LRs of rice, we performed methylene blue staining for the qualitative assessment of the spatial patterns of ROL. In rice plants grown in aerated nutrient solution, the whole of the L-type LRs and adventitious roots were stained blue (Fig. 1a and 1c). As the amount of O$_2$ that moves to the root tip may depend on the respiration activity in the root tip cells, the basal parts of S-type LRs may be stained blue when respiration in the root tip cells is active. Meanwhile, the whole root may show very faint blue staining when respiration in the root tip cells is much less active. In a few of the L- and S-type LRs, the root tips as well as the whole root or basal parts were stained blue under aerated conditions (Fig. 1a and 1c). If a ROL barrier was formed in these LRs, only the apical part would be stained blue. Thus, we believe that these LRs did not form ROL barriers. Therefore, these results suggest that a ROL barrier was not formed in L- and S-type LRs or adventitious roots under aerated conditions. Indeed, none of the 36 L-type LRs or 524 S-type LRs tested formed ROL barriers under aerated conditions. In contrast, in rice plants grown in stagnant deoxygenated nutrient solution, the apical parts in L-type LRs and adventitious roots showed strong blue staining,
Fig. 2. Radial oxygen loss (ROL) from L-type lateral roots of rice. Rates of ROL were measured using a rootsleeving cylindrical platinum electrode on 55–60 mm long (a, b) and 35–40 mm long (c, d) lateral roots grown under aerated conditions (a, c) or stagnant deoxygenated conditions (b, d). Shoots were in the air, and roots were in an oxygen-free medium. Data are mean ± SD ($n = 5$); replicates represent individual plants from which one adventitious root was used.

Fig. 3. Aerenchyma of 55–60 mm long L-type lateral roots (LRs; 10, 20, 30, 40, and 50 mm from the root tips) (a) and 20 mm long S-type LRs (10 mm from the root tips) (b) of 35-d-old rice plants grown under aerated or stagnant deoxygenated conditions. Bar = 50 µm. The percentage of aerenchyma of root cross-sectional area of 55–60 mm long L-type LRs of rice plants grown under aerated or stagnant deoxygenated conditions (c). Significance levels of $P \leq 0.05$ are denoted by asterisk (*) (two-sample $t$-test). Data are mean ± SD ($n = 9$); replicates represent individual plants from which three L-type LRs were used.
whereas the other parts (i.e., the middle and basal parts) of these roots showed very faint blue staining (Fig. 1b and 1d), suggesting that ROL barriers were formed in these roots. Among the 41 L-type LRs investigated, 30 (73%) clearly formed a ROL barrier. In contrast, strong blue coloration was observed in the basal parts of S-type LRs, and the blue staining was weaker in the middle and apical parts than in the basal parts (Fig. 1b and 1d). None of the 465 S-type LRs formed ROL barriers.

ROL profiles along L-type LRs measured by a root-sleeving oxygen electrode

The ROL profiles of the longer L-type LRs (55–60 mm long) of rice were investigated by measuring the ROL using a root-sleeving oxygen electrode (Fig. 2a and 2b). The ROL from the L-type LRs of plants grown in aerated nutrient solution were relatively high (210–220 nmol O$_2$ m$^{-2}$ sec$^{-1}$) throughout all parts of the roots (i.e., from 10–50 mm from the root tip) (Fig. 2a). In contrast, ROL from the L-type LRs of plants grown in stagnant deoxygenated solution was the highest (280 nmol O$_2$ m$^{-2}$ sec$^{-1}$) at 10 mm from the root tip, gradually decreasing to the basal part of the root, and was 71 nmol O$_2$ m$^{-2}$ sec$^{-1}$ at 50 mm from the root tip (Fig. 2b). We also investigated the ROL profile of the shorter L-type LRs (35–40 mm in length) (Fig. 2c and 2d). Under aerated conditions, the ROL values were relatively high at all root parts examined (10, 20, and 30 mm from the root tips) (Fig. 2c), as in longer L-type LRs (Fig. 2a). Under aerated conditions, the ROL values were relatively high at all root parts examined (10, 20, and 30 mm from the root tips) (Fig. 2c), as in longer L-type LRs (Fig. 2a). Under stagnant deoxygenated conditions, the ROL values were 243, 158, and 126 nmol O$_2$ m$^{-2}$ sec$^{-1}$ at 10, 20, and 30 mm from the root tips, respectively (Fig. 2d). However, it was difficult to measure the ROL profile of S-type LRs because they were too short (10–20 mm long) and thin to measure ROL using the method chosen for this study.

Aerenchyma formation in LRs

In the L-type LRs (55–60 mm long) of plants grown in aerated conditions, aerenchyma formation was initiated at 20 mm from the root tip (7%), and the aerenchyma areas were increased to the basal parts along the root, reaching 12% at 50 mm (Fig. 3a and 3c). Stagnant treatment enhanced aerenchyma formation in L-type LRs. Aerenchyma was observed at 20 mm from the root tip (15%), and the aerenchyma areas markedly increased in the more basal parts and reached 33% at 50 mm from the root tip (Fig. 3a and 3c). Thus, the areas of aerenchyma formed at 20, 30, 40, and 50 mm from the tips of L-type LRs were 2.0-, 3.5-, 2.2-, and 2.8-fold greater, respectively, under stagnant deoxygenated conditions than under aerated conditions (Fig. 3c). Nevertheless, no aerenchyma was observed in the S-type LRs of plants grown under either aerated or stagnant deoxygenated conditions (Fig. 3b).

Suberin staining of cross sections of LRs

In the L-type LRs (55–60 mm long) of rice plants grown under aerated conditions, no suberin
deposition was observed at 10, 20, 30, 40, or 50 mm from the root tip (Fig. 4a). In contrast, under stagnant deoxygenated conditions, suberin was deposited in the exodermis/hypodermis and sclerenchyma, as well as in the endodermis of L-type LRs (55–60 mm long) at 20–50 mm from the root tips (Fig. 4a). In the S-type LRs of rice plants grown under either aerated or stagnant deoxygenated conditions, no suberin deposition was observed in any root cross sections (Fig. 4b).

**Discussion**

In this study, we evaluated whether a ROL barrier was formed in rice LRs, distinguishing L- and S-type LRs. We verified that a ROL barrier was formed in L-type LRs, but not in S-type LRs under stagnant deoxygenated conditions (Figs. 1 and 2). Moreover, L-type LRs formed aerenchyma, but S-type LRs did not (Fig. 3). These results suggest that L- and S-type LRs play distinct roles in the growth of rice plants under deoxygenated soil conditions. The longitudinal diffusion of O₂ along the aerenchyma within the roots towards the root tips of L-type LRs or their branched roots (i.e., secondary LRs) may be promoted by ROL-barrier formation. Thus, the L-type LR itself and its branched roots may elongate by directing O₂ for cell division in the root apical meristem under deoxygenated soil conditions. However, some oxygen leakage (i.e., 126 and 71 nmol O₂ m⁻² sec⁻¹ at 40 and 50 mm, respectively, from the root tips; Fig. 2b) at the basal parts of L-type LRs may contribute to oxygenation in the rhizosphere to decrease the amount of harmful substances around the root surface. Additionally, L-type LRs are longer than S-type LRs and may be able to oxygenate a broad area in the rhizosphere. In contrast, ROL was observed mainly in the basal parts of S-type LRs under stagnant deoxygenated conditions (Fig. 1b and 1d), suggesting that the lack of aerenchyma formation (Fig. 3b) causes interference in the efficient movement of O₂ to the root tips of S-type LRs. Thus, ROL, which contributes to oxygenation in the rhizosphere, is observed mainly in the basal parts of S-type LRs (Fig. 1b and 1d). Our findings may modify the previous understanding that, in rice, a ROL barrier is formed only in adventitious roots; thus, LRs work as sources of substantial ROL in the rhizosphere (Armstrong 1970).

In *Z. nicaraguensis* and the maize introgression line IL #468 grown in stagnant deoxygenated solution, the ROL values were 250–350 nmol O₂ m⁻² sec⁻¹ at 5 mm from the root tips and steeply declined to less than 100 nmol O₂ m⁻² sec⁻¹ at 10 and 20 mm from the root tips in 30 mm long LRs (Pedersen et al. 2021). In contrast, in 35–40 mm long L-type LRs of rice grown in stagnant deoxygenated solution, the ROL values were higher (243 and 158 nmol O₂ m⁻² sec⁻¹ at 10 and 20 mm from the root tips, respectively; Fig. 2d) than the ROL values at the same positions of *Z. nicaraguensis* LRs (30 mm long). These results suggest that ROL barriers are more tightly formed in *Z. nicaraguensis* LRs than in rice L-type LRs. It was proposed that the LRs in *Z. nicaraguensis* can grow to a maximum length of 74 mm with a ROL barrier and only 33 mm without a ROL barrier under stagnant deoxygenated conditions (Pedersen et al. 2021). Thus, the 30 mm long LRs of *Z. nicaraguensis*, with their tightly formed ROL barriers, have the potential to elongate to ~74 mm. Taken together, it is possible that the maximum length of LRs with tightly formed ROL barriers may be longer than those with less tightly formed ROL barriers, when plants grow under stagnant deoxygenated conditions. Indeed, this possibility may be consistent with the maximum length (~80 mm) of LRs observed in *Z. nicaraguensis* (Pedersen et al. 2021) and the evidence that the maximum length and the average length of L-type LRs are ~60 mm and ~50 mm, respectively, under stagnant deoxygenated conditions (data not shown).

However, the maximum and average length of S-type LRs, which do not form ROL barriers or aerenchyma, are ~23 mm and ~10 mm, respectively (data not shown).

Previous studies (Colmer et al. 2006, Shiono et al. 2011) reported that ROL barriers were tightly formed in longer (>110 mm long) adventitious roots of rice grown in waterlogged soils or under stagnant deoxygenated conditions; however, these barriers were less tightly formed in shorter (~60 mm) adventitious roots. Similarly, in L-type LRs, the prevention of ROL at the basal parts was greater in longer roots (50 mm from the root tips of 55–60 mm long roots; Fig. 2b) than in shorter roots (30 mm from the root tips of 35–40 mm long roots; Fig. 2d) under stagnant deoxygenated conditions. These results suggest that root length is involved in the extent and strength of the ROL barrier in both L-type LRs and adventitious roots in rice.

In rice L-type LRs, aerenchyma was formed under aerated conditions as well as stagnant deoxygenated conditions (Figs. 3a and 3c). The areas of aerenchyma in L-type LRs were 2.0–3.5-fold greater under stagnant deoxygenated conditions than those under aerated conditions (Fig. 3c), indicating that aerenchyma is constitutively formed under aerated conditions, and its formation is further induced under stagnant deoxygenated conditions in rice L-type LRs, similarly to adventitious roots. In *Z. nicaraguensis* and the maize introgression line IL #468 grown in stagnant deoxygenated solution, the ROL values were higher (243 and 158 nmol O₂ m⁻² sec⁻¹ at 10 and 20 mm from the root tips, respectively; Fig. 2d) than the ROL values at the same positions of *Z. nicaraguensis* LRs (30 mm long). These results suggest that ROL barriers are more tightly formed in *Z. nicaraguensis* LRs than in rice L-type LRs. It was proposed that the LRs in *Z. nicaraguensis* can grow to a maximum length of 74 mm with a ROL barrier and only 33 mm without a ROL barrier under stagnant deoxygenated conditions (Pedersen et al. 2021). Thus, the 30 mm long LRs of *Z. nicaraguensis*, with their tightly formed ROL barriers, have the potential to elongate to ~74 mm. Taken together, it is possible that the maximum length of LRs with tightly formed ROL barriers may be longer than those with less tightly formed ROL barriers, when plants grow under stagnant deoxygenated conditions. Indeed, this possibility may be consistent with the maximum length (~80 mm) of LRs observed in *Z. nicaraguensis* (Pedersen et al. 2021) and the evidence that the maximum length and the average length of L-type LRs are ~60 mm and ~50 mm, respectively, under stagnant deoxygenated conditions (data not shown).

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nicaraguensis LRs, the aerenchyma areas at 10 and 20 mm from the root tips were respectively 1.3- and 1.4-fold greater under stagnant deoxygenated conditions than under aerated conditions (Pedersen et al. 2021). Therefore, this evidence suggests that wetland-adaptable plant species, such as rice and Z. nicaraguensis, have the abilities of both constitutive and inducible aerenchyma formation in LRs, as well as in adventitious roots.

In L-type LRs, cell wall suberization (i.e., suberin lamellae) of the exodermis/hypodermis, sclerenchyma, and endodermis was observed 20–50 mm from the root tip under stagnant deoxygenated conditions, but not under aerated conditions (Fig. 4a). As shown in Figure 2b, the ROL value (200 nmol O₂ m⁻² s⁻¹) at 20 mm from the root tips was significantly lower than that (280 nmol O₂ m⁻² s⁻¹) at 10 mm (Fig. 2b), indicating that ROL has been restricted at 20 mm from the root tips. This is because the ROL barrier starts forming at 20 mm. This observation is consistent with the pattern of suberin staining. Similarly, suberin lamellae were formed in the exodermis of the LRs of Z. nicaraguensis, grown under stagnant deoxygenated conditions, that formed ROL barriers (Pedersen et al. 2021). In contrast, no suberization was observed in the cells of S-type LRs under either stagnant deoxygenated or aerated conditions (Fig. 4b). These results support the hypothesis that suberin lamellae are one of the components of the ROL barrier. However, maize adventitious and LRs, which are much more permeable to oxygen, can also form suberin lamellae in the exodermis under stagnant deoxygenated conditions (Abiko et al. 2012, Watanabe et al. 2017, Pedersen et al. 2021). Therefore, to confirm this hypothesis, we need to determine if the suberin monomer composition or structure, or both, are involved in the permeability of suberin lamellae to O₂, using LRs as well as adventitious roots.

In conclusion, we have demonstrated that the L-type LRs of rice can form a ROL barrier, but S-type LRs do not form a barrier when grown under stagnant deoxygenated conditions. The functional implication of the ROL-barrier formation in L-type LRs may be that some O₂ is diffused and transported to the root tips through the aerenchyma for further elongation of the LRs, and some O₂ is radially leaked for oxidation of the rhizosphere in waterlogged soil. Moreover, substantial ROL from S-type LRs may play a role in oxygenation in the rhizosphere. Taken together, the coexistence of longer L-type LRs and shorter S-type LRs may contribute to oxygenation in a wider area of the rhizosphere in waterlogged, deoxygenated soil. To understand the individual roles of L- or S-type LRs in the adaptation of rice plants to waterlogged soil, it would be of interest to investigate how oxygenation in the rhizosphere and plant growth under stagnant deoxygenated conditions are affected by using L- or S-type LR-deficient rice mutants. Unfortunately, these mutants are not yet available and their screening will first be necessary.

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