

Invited Review

A barrier to radial oxygen loss helps the root system cope with waterlogging-induced hypoxia

Masato Ejiri, Takeshi Fukao, Tomoki Miyashita and Katsuhiko Shiono*

Graduate School of Bioscience and Biotechnology, Fukui Prefectural University, 4-1-1 Matsuoka-Kenjojima, Eiheiji, Fukui 910-1195, Japan

Internal aeration is crucial for root growth under waterlogged conditions. Many wetland plants have a structural barrier that impedes oxygen leakage from the basal part of roots called a radial oxygen loss (ROL) barrier. ROL barriers reduce the loss of oxygen transported via the aerenchyma to the root tips, enabling long-distance oxygen transport for cell respiration at the root tip. Because the root tip does not have an ROL barrier, some of the transferred oxygen is released into the waterlogged soil, where it oxidizes and detoxifies toxic substances (e.g., sulfate and Fe^{2+}) around the root tip. ROL barriers are located at the outer part of roots (OPRs). Their main component is thought to be suberin. Suberin deposits may block the entry of potentially toxic compounds in highly reduced soils. The amount of ROL from the roots depends on the strength of the ROL barrier, the length of the roots, and environmental conditions, which causes spatiotemporal changes in the root system's oxidization pattern. We summarize recent achievements in understanding how ROL barrier formation is regulated and discuss opportunities for breeding waterlogging-tolerant crops.

Key Words: aerenchyma, apoplastic barrier, flooding, planar O_2 optode, rhizosphere oxidation, root system, suberin.

1. Introduction

Plants can suffer from hypoxia or even anoxia when soils become waterlogged (i.e., only the root system is immersed in water). Well-drained soil is porous and normally filled with gas; excess water fills the pores, preventing the entry of atmospheric oxygen, as the diffusivity of oxygen in water is approximately 10,000 times slower than it is in air (Jackson *et al.* 1985). Other problems associated with waterlogging are the accumulation of phytotoxic compounds in the soil (Ernst 1990, Kreuzwieser *et al.* 2004, Lamers *et al.* 1998, Ponnampereuma 1984) and a decline in the availability of some nutrients (e.g., NO_3^- and Fe^{3+}) (Laanbroek 1990). Waterlogging damages many crops, including soybean, wheat, maize, oats, and barley (Bertholdsson 2013, Setter and Waters 2003). Moreover, the number of flooding events is increasing worldwide due to climate change (Hirabayashi *et al.* 2013), causing severe damage to crop yields (Fukao *et al.* 2019, Pedersen *et al.* 2017).

Plants can adapt to waterlogging-induced hypoxia by shifting metabolic pathways (Bailey-Serres and Voesenek

2008, Fukao *et al.* 2019) and reducing energy consumption (Bailey-Serres and Voesenek 2010, Fukao *et al.* 2019). When waterlogging is prolonged, some plants can survive by internal oxygen transport via aerenchyma, which is an interior gas space connected between the shoot and the root tip (Colmer 2003a, Colmer and Voesenek 2009, Pedersen *et al.* 2021b, Seago *et al.* 2005, Shiono *et al.* 2008, Yamauchi *et al.* 2018). A radial oxygen loss (ROL) barrier, together with aerenchyma, contribute to long-distance oxygen transport and waterlogging tolerance. This review examines how ROL barriers affect root growth, how their formation is regulated, and how they might be used to breed waterlogging-tolerant crops.

2. Adaptive significance of an ROL barrier for roots in waterlogged soil

2-1. Long-distance oxygen transport for respiration at the root tip

Under waterlogged conditions, oxygen molecules diffusing longitudinally through aerenchyma toward the root apex may be either consumed by respiration of root cells or diffused radially to the rhizosphere (Armstrong 1979, Colmer 2003a, Nishiuchi *et al.* 2012). Oxygen moving towards the root apex via the aerenchyma can leak into the surrounding soil (Armstrong 1979, Colmer and Voesenek 2009) in a process called radial oxygen loss (ROL). The

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*Corresponding author (e-mail: shionok@fpu.ac.jp)

amount of ROL is determined by the oxygen concentration gradient, the physical resistance to radial oxygen diffusion between aerenchyma and soil, and the consumption of oxygen by cells along the radial diffusion path (**Fig. 1A**) (Armstrong 1979, Armstrong and Beckett 1987, Colmer 2003a, Nishiuchi *et al.* 2012). ROL aerates the rhizosphere (Armstrong 1979), where it nourishes the oxidative microbial community (Martin *et al.* 2019) and detoxifies toxic reduced substances in the waterlogged soil (Blossfeld *et al.* 2011, Colmer 2003a, Martin *et al.* 2019, Neubauer *et al.* 2007, Nishiuchi *et al.* 2012). However, ROL reduces the oxygen supply to the root apex, preventing the roots from growing deeper into anaerobic soil (Armstrong 1979, Pedersen *et al.* 2021b, Sorrell *et al.* 2000).

Some wetland species form an ROL barrier in the outer cell layers exterior to the aerenchyma (**Fig. 1B**) (Nishiuchi *et al.* 2012, Yamauchi *et al.* 2018). Radial oxygen profiles across root tissues obtained with an oxygen microelectrode show that the ROL barrier is located in the outer cell layers, including sclerenchyma, exodermis/hypodermis, and epidermis (Armstrong *et al.* 2000, De Simone *et al.* 2003, Soukup *et al.* 2007). An ROL barrier along the basal part of roots enhances longitudinal oxygen diffusion to the root apex. This enables cells at the root tips that are surrounded by anaerobic soil to continue respiring [**Fig. 1B** (i)] (Armstrong 1979, Colmer 2003a, Nishiuchi *et al.* 2012, Pedersen *et al.* 2021b). Multiple mathematical models sup-

port the idea that an ROL barrier leads to increased root length (Armstrong 1979, Pedersen *et al.* 2021b, Sorrell *et al.* 2000). For instance, the maximum root length in roots with an ROL barrier was about twice that of roots without an ROL barrier when the cortex-to-stele area ratio was set at 10 (Pedersen *et al.* 2021b).

2-2. Detoxification of reduced substances around the root tip

In waterlogged soil, some soil phytotoxins are produced by anaerobic bacteria (**Fig. 1B**). Because the root tip does not have an ROL barrier, the ROL around the root tip can neutralize (oxidize) toxic reduced substances such as sulfate and Fe^{2+} [**Fig. 1B** (ii)] (Armstrong 1979, Colmer 2003a, Yamauchi *et al.* 2018). Recent studies using a planar O_2 optode, an instrument that measures 2D *in situ* oxygen levels in vertical planes in the soil and rhizosphere, have shown that light conditions dynamically change oxygen levels in the rhizosphere (Larsen *et al.* 2015, Lenzewski *et al.* 2018, Martin *et al.* 2019) and that Fe^{2+} oxidation was spatiotemporally associated with ROL (Maisch *et al.* 2019). The use of diffusive gradients in thin films (DGTs) is a technique for measuring 2D metal flux and concentrations at the surface of sediments (Davison and Zhang 1994, Davison *et al.* 1997). A study using a planar O_2 optode combined with sulfide DGTs in the seagrasses *Halophila ovalis* and *Zostera muelleri* grown in marine sediments showed that ROL from the root tips reduces sulfate concentrations in the rhizosphere (Martin *et al.* 2019). This detoxification of reduced toxic substances around the root tip enables the roots to elongate into anaerobic soils (Armstrong 1979, Pedersen *et al.* 2021b).

2-3. Spatiotemporal dynamics of rhizosphere oxidization Effect of root length on ROL barrier formation

ROL barrier formation depends on the degree of soil waterlogging and the root developmental stage. In long roots of rice (*ca.* 110 mm), ROL barrier formation begins within nine hours of stagnant treatment and is completed within 24 h, while in short roots (*ca.* 60 mm), it takes 72–120 h to form a barrier (Shiono *et al.* 2011). Tissue age appears to affect ROL barrier formation because basal tissues of long roots are older than those of short roots, and suberin accumulation can be influenced by the developmental stage (Kotula *et al.* 2009, Schreiber *et al.* 1999). The length distribution of adventitious roots with an ROL barrier in rice (*cv.* Nipponbare) grown under stagnant conditions is shown in **Supplemental Fig. 1**. Roots that leaked oxygen only in the root tips were considered to have an ROL barrier. The figure shows the percentages of roots of a given length that formed an ROL barrier. In agreement with these results, Colmer *et al.* (2006) observed that an ROL barrier was formed along both long (>110 mm-long) and short (*ca.* 60 mm) adventitious roots of rice grown in stagnant conditions for 3–4 weeks. However, only less than half of very short (<40 mm-long) adventitious roots had an ROL

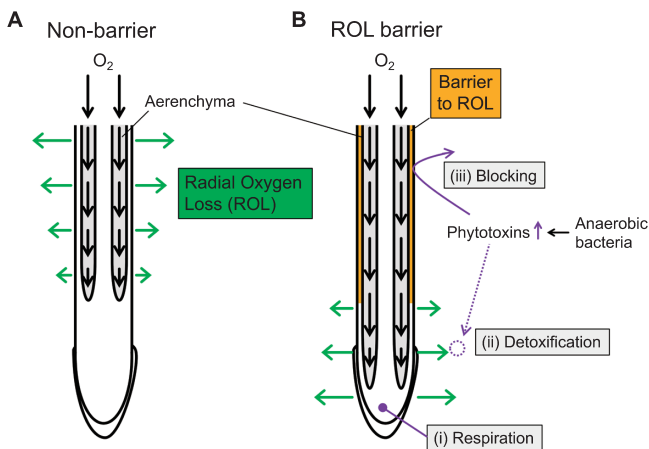


Fig. 1. Adaptive significance of an ROL barrier for roots in waterlogged soil. (A) Oxygen molecules diffusing longitudinally through aerenchyma toward the root apex may either be consumed by respiration of root cells or diffuse radially to the rhizosphere (called radial oxygen loss, ROL). Although ROL aerates the rhizosphere, it also reduces the supply of oxygen to the root apex. (B) Many wetland species form an ROL barrier in the basal parts of roots. ROL barrier promotes longitudinal oxygen diffusion by preventing losses to anaerobic soils. (i) In anaerobic soils, enhanced movement of oxygen toward the apex enables active respiration of the cells at the root tips. Because the root tip does not have an ROL barrier, (ii) the ROL around the root tip detoxifies toxic reduced substances in the waterlogged soil. (iii) The barrier also blocks the entry of potentially toxic compounds in highly reduced soils.

barrier (**Supplemental Fig. 1**). In the very short roots, an ROL barrier might not be essential for supplying sufficient oxygen for root tip respiration because the distance for oxygen diffusion from root-shoot junction to tips of the very short roots is much shorter than it is for long roots. Curiously, some of the very short roots formed an ROL barrier (**Supplemental Fig. 1**). After five days of incubation at 26°C without plants, the agar solution at a depth of 5 mm was anoxic (0 mg/L) (Shiono, unpublished). We speculate that emergence of roots into the anoxic and reduced solution induces barrier formation even in very young adventitious roots.

In rice and common reed (*Phragmites australis*), short lateral roots along adventitious roots leak oxygen due to the lack of an ROL barrier (Armstrong 1970, Armstrong *et al.* 1996, Colmer 2003a). Thus, the soil around the lateral roots and the very short adventitious roots was thought to be oxidized. Recently, oxygen levels imaged with a planar O₂ optode indicated that the soil around the plant basal-region where many short lateral roots and very short roots are located had high oxygen levels even though waterlogged soil without plants was anoxic (Larsen *et al.* 2015, Maisch *et al.* 2019). **Fig. 2** illustrates how an ROL barrier mediates oxygen distribution in the root-system in soil-grown rice. Rice plants have many very short (<40 mm-long) roots around the root-shoot junction, where new roots emerge (**Fig. 2A**). Although most short roots (between 40 and 80 mm) have an ROL barrier, very short roots (<40 mm) tend to lack an ROL barrier (**Fig. 2B, Supplemental Fig. 1**), thereby leading to oxygen leakage and detoxification of reduced toxic substances to promote new root growth. On the other hand, most long roots form a strong ROL barrier along the subapical-to-basal regions, resulting in oxygen leakage only around the root tip (**Fig. 2A, 2C**). This allows long roots to serve as pioneers that detoxify reduced substances around the root tips, contributing to their active growth under waterlogging.

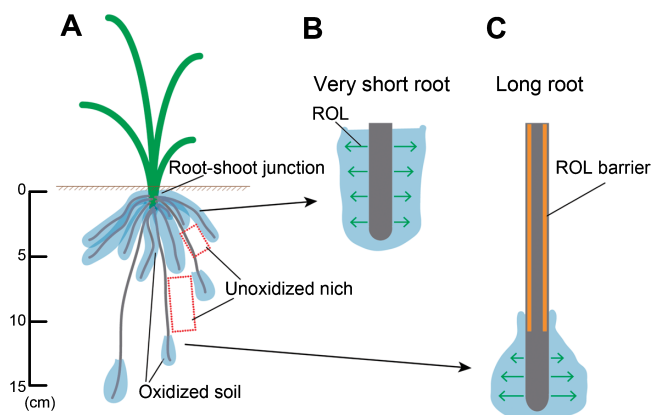


Fig. 2. Predicted locations of oxidized rhizosphere and ROL barrier formation along short and long roots. (A) Predicted locations of oxidized soil (blue) in the rhizosphere. (B, C) Radial oxygen loss (green arrows) in very short (<50 mm) roots (B) and long roots (>100 mm) (C).

ROL barrier formation in long lateral roots

In rice and *P. australis*, short lateral roots do not form an ROL barrier (Armstrong 1970, Armstrong *et al.* 1996, Colmer 2003a). However, it was not known whether long lateral roots generate the barrier or not. Pedersen *et al.* (2021a) recently reported that *Zea nicaraguensis*, a wild relative of maize that grows in flood plains, forms an ROL barrier along the long lateral roots under stagnant conditions. The grass species has a sparse root system: Abiko *et al.* (2012) reported that 25-d-old *Z. nicaraguensis* grown under waterlogged soil for 21 d had only 15 adventitious roots. We speculate that the rhizosphere of *Z. nicaraguensis* has large unoxidized niches (rectangles in **Fig. 2A**) due to its sparse root system, and that long lateral roots elongate into these niches. Like the ROL barrier of adventitious roots (**Fig. 1**), the ROL barrier of long lateral roots may help the roots to grow into the reduced soils in the unoxidized niches. Monitoring the oxygen status in the niches during lateral roots elongation with a planar O₂ optode would clarify whether long lateral roots help to adapt to waterlogged conditions.

Effect of photosynthetic activity on ROL

The photosynthetic activity of the shoot dynamically changes the oxygen level in the rhizosphere (Larsen *et al.* 2015, Lenzeński *et al.* 2018, Martin *et al.* 2019). Under light conditions, oxygen release is pronounced, resulting in a larger oxygenated zone in waterlogged soils. During the subsequent dark period, the oxygen concentration in the oxygenated region declined continuously, reaching the anoxic background level (Larsen *et al.* 2015, Lenzeński *et al.* 2018, Martin *et al.* 2019). Under field conditions, oxygen levels in the rhizosphere must be affected by light conditions and other environmental factors. For the evaluation or screening of crop waterlogging tolerance, the effect of the light condition also need to be considered in the field experiments.

3. Distribution of plant species that have ROL barriers

For the past two decades, many studies have evaluated ROL barrier formation in mono- and dicotyledonous species grown in wetland, non-wetland, and brackish water regions (**Table 1**). In general, ROL barriers do not form in waterlogging-sensitive non-wetland species including major crops, e.g., maize (*Z. mays* ssp. *mays*) (Abiko *et al.* 2012), wheat (*Triticum aestivum*) (McDonald *et al.* 2001b), barley (*Hordeum vulgare*) (McDonald *et al.* 2001a), *Brassica napus* (Voesešek *et al.* 1999), *Pisum sativum* (Healy and Armstrong 1972), and *Sorghum bicolor* (McDonald *et al.* 2002). Most waterlogging-tolerant species that can form an ROL barrier are distributed in wetlands (**Table 1**). Typical examples are rice and wild *Echinochloa* weeds that grow in paddy fields (Colmer *et al.* 1998, Ejiri and Shiono 2019), *P. australis* growing on riversides (Armstrong and Armstrong 2001), and *Z. nicaraguensis* and *Oryza glumaepatula*

Table 1. Plants species that have a tight barrier to ROL

Species	Habitat	Induction	References
Dicotyledonous			
<i>Tabernaemontana juruana</i>	W	Unknown	De Simone <i>et al.</i> (2002, 2003)
<i>Caltha palustris</i>	W	Inducible	Visser <i>et al.</i> (2000)
<i>Bruguiera gymnorhiza</i>	B	Unknown	Cheng <i>et al.</i> (2012)
Monocotyledonous			
<i>Echinochloa colona</i>	N	Constitutive	Ejiri and Shiono (2019)
<i>E. crus-galli</i> var. <i>praticola</i>	N	Constitutive	Ejiri and Shiono (2019)
<i>Colocasia esculenta</i>	N/W	Unknown	Abiko and Miyasaka (2020)
<i>E. crus-galli</i> var. <i>crus-galli</i>	N/W	Constitutive	Ejiri and Shiono (2019)
<i>Cyperus eragrostis</i>	W	Constitutive	Manzur <i>et al.</i> (2015)
<i>Critesion marinum</i>	W	Inducible	McDonald <i>et al.</i> (2001a)
<i>E. crus-galli</i> var. <i>formosensis</i>	W	Constitutive	Ejiri and Shiono (2019)
<i>E. crus-galli</i> var. <i>mitis</i>	W	Constitutive	McDonald <i>et al.</i> (2001a, 2002)
<i>E. oryzicola</i>	W	Inducible	Ejiri and Shiono (2019)
<i>Glyceria maxima</i>	W	Unknown	Soukup <i>et al.</i> (2007)
<i>Hordeum marinum</i> ssp. <i>marinum</i> (Acc. H21)	W	Inducible	Garthwaite <i>et al.</i> (2003), Malik <i>et al.</i> (2011)
<i>Juncus effusus</i>	W	Constitutive	Visser <i>et al.</i> (2000)
<i>Oryza glumaepatula</i>	W	Constitutive	Ejiri <i>et al.</i> (2020)
<i>O. rufipogon</i>	W	Inducible	Ejiri <i>et al.</i> (2020)
<i>O. sativa</i>	W	Inducible	Colmer <i>et al.</i> (1998)
<i>Phragmites australis</i>	W	Unknown	Armstrong and Armstrong (2001)
<i>Urochloa humidicola</i>	W	Constitutive	Jiménez <i>et al.</i> (2019)
<i>Zea nicaraguensis</i>	W	Inducible	Abiko <i>et al.</i> (2012)
<i>Halophila ovalis</i>	B	Unknown	Connell <i>et al.</i> (1999)
<i>Zostera muelleri</i>	B	Unknown	Martin <i>et al.</i> (2019)

W: Wetland, N: Non-wetland, B: Brackish water region, N/W: non-wetland or wetland.

Induction of an ROL barrier: Constitutive means an ROL barrier is formed under aerated (or well-drained) and stagnant (or waterlogged) conditions. Inducible means an ROL barrier is formed under stagnant (or waterlogged) conditions, but that is not formed under aerated (or well-drained) conditions. Unknown means an ROL barrier formed under stagnant (or waterlogged conditions), but that it was not evaluated under aerated (or well-drained) conditions. Thus, these species can form an ROL barrier, but it remains unclear whether it is constitutive.

growing in flood plains (Abiko *et al.* 2012, Ejiri *et al.* 2020). However, ROL barriers were not found in some wetland species, including *Paspalidium geminatum* (Manzur *et al.* 2015), *Ranunculus sceleratus* (Visser *et al.* 2000), *Rumex crispus* (Manzur *et al.* 2015), *R. palustris* (Visser *et al.* 2000), and *Salix martiana* (De Simone *et al.* 2002). This may be because these species live in areas where soil substrates are not highly reduced (e.g., low in organic matter; flowing water) and thus may not need an ROL barrier (Colmer 2003a, Smits *et al.* 1990). Alternatively, these species may possess genetic factor(s) related to tolerance to toxic soil constituents under flooded reducing soil conditions.

ROL barriers are classified as inducible and constitutive (Table 1). In some wetland species, including rice (Colmer *et al.* 1998), *H. marinum* (Garthwaite *et al.* 2003), *Z. nicaraguensis* (Abiko *et al.* 2012), and *Caltha palustris* (Visser *et al.* 2000), an ROL barrier is induced in roots by growth in waterlogged soil or stagnant conditions, while roots remain leaky to oxygen under well-drained soil or aerated conditions. ROL barriers form constitutively (i.e., in the absence of waterlogging) in several wetland species, including *Cyperus eragrostis* (Manzur *et al.* 2015), *Juncus*

effusus (Visser *et al.* 2000), wild *Echinochloa* species (Ejiri and Shiono 2019), *O. glumaepatula* (Ejiri *et al.* 2020), and *Urochloa humidicola* (Jiménez *et al.* 2019) (Table 1). The ability to form a constitutive or inducible barrier is considered an adaptation to waterlogging (Colmer 2003a). Recently, the patterns of ROL barrier formation were evaluated in annual wild *Echinochloa* species (Ejiri and Shiono 2019). All three *Echinochloa crus-galli* varieties including var. *crus-galli*, *formosensis*, and *praticola* formed a constitutive ROL barrier, whereas *E. oryzicola*, like rice (Colmer 2003b), had an inducible ROL barrier (Ejiri and Shiono 2019). Phylogenetic analyses suggest that *E. crus-galli* (which is hexaploid) is derived from *E. oryzicola* (which is tetraploid) (Aoki and Yamaguchi 2008, 2009). *E. oryzicola* is limited to waterlogged paddies, while *E. crus-galli* adapts to wet and dry areas depending on the variety (Rao *et al.* 2007, Tanesaka *et al.* 2010, Yamasue 2001). It is expected that constitutive suberization in the outer root cell layers that form an ROL barrier also increases drought and salinity tolerance (Enstone *et al.* 2003, Kreszies *et al.* 2018, Ranathunge *et al.* 2011b). The ability of *E. crus-galli* to grow in a wide range of habitats may be due to its constitutive barrier that increases its tolerance for multiple stresses.

4. ROL barrier components that prevent radial oxygen diffusion

4-1. Suberin at the exodermis/hypodermis

Which compounds physically prevent oxygen loss through an ROL barrier? Lignin and suberin act as diffusion barriers in the extracellular spaces (apoplast) in the outer part of roots (OPRs) and endodermis. Lignin is a complex of polyphenolic polymers that provides mechanical strength and plant defense due to its resistance to degradation (Barros *et al.* 2015, Campbell and Sederoff 1996). Suberin is a hydrophobic macromolecule built from long-chain fatty acids, glycerol, and aromatic polymers (Graça 2015). Suberin lamellae are composed of suberin deposited on exodermal cell walls (Schreiber and Franke 2011).

When wetland plants, e.g., *Phragmites australis* (Soukup *et al.* 2007) and rice (Kotula *et al.* 2009), are grown under waterlogged or stagnant conditions for a long period (i.e., 2–3 weeks), both suberized and lignified cell walls are frequently observed in the OPRs at the basal part of roots. However, lignification in the OPRs is often unassociated with ROL barrier formation, depending on ecotypes, different developmental stages, or different genetic backgrounds (De Simone *et al.* 2003, Ejiri and Shiono 2019, Ejiri *et al.* 2020, Shiono *et al.* 2011, Watanabe *et al.* 2017). Four lines of evidence show that suberin in the OPRs is a major component of ROL barriers: 1) *Z. nicaraguensis* with ROL barrier develops suberin lamellae in the exodermis and lignin in the epidermis (Abiko *et al.* 2012, Watanabe *et al.* 2017). However, an introgression line of *Z. nicaraguensis* in a maize background (IL#468) with ROL barrier under stagnant conditions develops a well-suberized exodermis/hypodermis along the basal parts of adventitious roots, but the epidermis is not lignified as it is in maize (inbred line Mi29) (Watanabe *et al.* 2017). 2) Roots of five wild *Echinochloa* species that constitutively form an ROL barrier under aerated conditions also have a well-suberized exodermis/hypodermis (Ejiri and Shiono 2019). However, two of the five species (a wetland ecotype *E. crus-galli* var. *crus-galli* and a non-wetland ecotype *E. colona*) do not have lignified sclerenchyma (Ejiri and Shiono 2019). 3) A transcriptome analysis using laser-microdissected tissues of the OPRs in rice showed that many genes involved in suberin biosynthesis (but not in lignin biosynthesis) are upregulated during ROL barrier formation (Shiono *et al.* 2014b). 4) A metabolomic analysis in rice indicated that rice roots that form an ROL barrier accumulate malic acid and very-long-chain fatty acids, which are needed to synthesize suberin (Kulichikhin *et al.* 2014).

The apoplast is a continuum of the extracellular space outside the plasma membrane (e.g., cell walls, xylem, and phloem), while the symplast is a continuum of intracellular space (e.g., cytosol and plasmodesmata). Roots take up water and ions via apoplastic and symplastic pathways. In general, suberin lamellae at the exodermis/hypodermis pre-

vent water (Aloni *et al.* 1998, Kreszies *et al.* 2020), ions (Ranathunge *et al.* 2011a), and mycorrhizal fungi from moving through the apoplast (Enstone *et al.* 2003). In several grasses that form an ROL barrier with well suberized OPRs [e.g., rice (Ejiri *et al.* 2020), *P. australis* (Soukup *et al.* 2007), *Z. nicaraguensis* (Watanabe *et al.* 2017), wild *Echinochloa* species (Ejiri and Shiono 2019), and *O. glumaepatula* (W2165) (Ejiri *et al.* 2020)], tracers (e.g., periodic acids) were blocked from moving through the apoplast to the interior part of roots. In fact, rice roots that form an ROL barrier also have reduced permeability to NaCl (but remain permeable to water) (Ranathunge *et al.* 2011a). The difference in permeabilities may be due to different transport mechanisms and pathways (Ranathunge *et al.* 2011a). Generally, oxygen moves through the plasma membrane by diffusion, whereas water predominantly moves through the apoplast by bulk flow (Ranathunge *et al.* 2011a). Substances that are potentially toxic to plants (e.g., reduced metal ions) often accumulate in waterlogged soil (Colmer 2003a, Ernst 1990, Kreuzwieser *et al.* 2004, Lamers *et al.* 1998, Ponnampereuma 1984). Another benefit of ROL barriers in waterlogged soils is that their main component (suberin) can reduce the entry of such phytotoxins [Fig. 1B (iii)] (Kreszies *et al.* 2018, Ranathunge *et al.* 2011b, Watanabe *et al.* 2013, Yamauchi *et al.* 2018).

4-2. Oxygen permeable windows

In roots of several wetland species that form an ROL barrier, such as rice (Armstrong 1971), *P. australis* (Armstrong *et al.* 2000), *H. maritimum* (H819) (Garthwaite *et al.* 2008), and wild *Echinochloa* species (Ejiri and Shiono 2019), the sites of the emergence of lateral roots contain passage cells that leak oxygen. Areas of passage cells where oxygen leaks and lateral roots emerge have been called “windows” (Armstrong *et al.* 2000). Armstrong *et al.* (2000), using a microelectrode, showed that oxygen levels in *P. australis* were higher at the surface of window sites than at non-window sites. Similarly, Ejiri and Shiono (2019), using methylene-blue staining, showed that oxygen was leaking in a spotty manner (probably from window sites) along the basal part of roots in *Echinochloa* accessions. Interestingly, in rice (Justin and Armstrong 1987), *P. australis* (Armstrong *et al.* 2000) and wild *Echinochloa* species (Ejiri and Shiono 2019, 2020), the exodermis at window sites lacks suberin lamellae, and the cortical cells are left intact without programmed cell death (i.e., without aerenchyma) (Fig. 3). However, the lateral root primordium does not reach the cortex and the exodermis. At window sites of *E. crus-galli*, lignin deposits at the sclerenchyma and the exodermis were also clearly reduced (Ejiri and Shiono 2020).

Passage cells possess Casparian strips that are composed of lignin and small deposits of suberin (Schreiber and Franke 2011) but not suberin lamellae such as those that cover the plasma membrane of the exodermal cells (Peterson and Enstone 1996). The development of exodermal passage

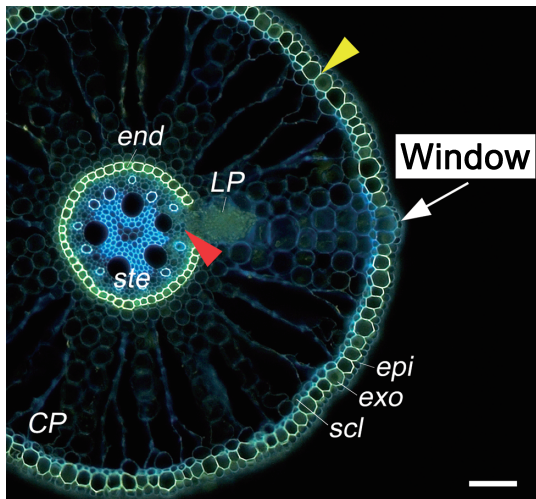


Fig. 3. An oxygen-permeable window site in the adventitious roots of *Echinochloa colona*. Cross-section of an adventitious root showing the location of a window (white arrow) at a site in the exodermis where a lateral root is expected to emerge. The window is composed of passage cells that lack suberin lamellae. Suberin lamellae are indicated as yellow-green fluorescence with Fluorol Yellow 088 (yellow arrowhead). Red arrowhead indicates the pericycle from which lateral root primordia are predicted to emerge. Blue fluorescence indicates autofluorescence. Fluorol Yellow 088 staining was conducted as described in Ejiri and Shiono (2019). The plants were grown in an aerated nutrient solution for ten days and then transferred into a deoxygenated stagnant nutrient solution for 14 days. Abbreviations: CP, cortical parenchyma; end, endodermis; epi, epidermis; exo, exodermis; LP, lateral root primordia; scl, sclerenchyma; ste, stele. Scale bars: 100 μm .

cells is well described in maize (Enstone and Peterson 1997, 2005), although the oxygen permeability of the passage cells has not been evaluated. In the younger part of maize root, the exodermis matured in a patchy manner with some unsuberized cells, some developing suberin lamellae, and some mature suberin lamellae (Enstone and Peterson 2005). Exodermal cells surrounding emerged lateral roots remained unsuberized longer than other cells in the layer, thus becoming exodermal passage cells (Enstone and Peterson 2005). Application of toxic sulfide to rice roots induced unusually high suberin and/or lignin accumulations in the OPRs, preventing lateral roots from penetrating the sclerenchyma and growing through the cortex of adventitious roots (Armstrong and Armstrong 2005). This suggests that well-suberized and/or well-lignified cells in the OPRs block the emergence of lateral roots.

4-3. Another potential component of an ROL barrier

Regions that histochemically stain for suberin do not always function as ROL barriers. In maize, well-developed suberin lamellae were histochemically detected at the exodermis under stagnant conditions, but the roots were leaky to oxygen (Watanabe *et al.* 2017). Suberin lamellae in the OPRs were not histochemically visualized in the roots of

Halophila ovalis, in which an ROL barrier was observed (Martin *et al.* 2019). In rice, ROL barrier formation was induced by 48 h of stagnant conditions but suberin lamellae were not histochemically detected in the exodermis (Shiono *et al.* 2011). This failure to detect suberin may be due to a difference in suberin monomer composition and/or structure (Watanabe *et al.* 2017).

Rice appears to have another, unidentified component that impedes oxygen diffusion. It is an electron-dense material that is packed in the intercellular space between the exodermis and the epidermis. It is observed in rice roots after 48 h of stagnant treatment using transmission electron microscopy (Shiono *et al.* 2011). These roots do not have histochemically stained suberin lamellae. Similar electron-dense materials are found in the intercellular spaces in the outer part of soybean nodules (James *et al.* 1991, Parsons and Day 1990). The electron-dense material contains glycoproteins and forms an oxygen diffusion barrier (James *et al.* 1991, Parsons and Day 1990). This is necessary because the interior of the nodules must be anaerobic because nitrogenase activity that catalyzes the conversion of N_2 to ammonia is inactivated by oxygen (Denison *et al.* 1992, Denison 1998). Further studies are needed to identify the electron-dense components that inhibit oxygen transport in the OPRs.

5. Mechanisms underlying ROL barrier formation

5-1. Environmental triggers for ROL barrier formation

ROL barriers are induced in some wetland species including rice by stagnant or waterlogged conditions (Colmer *et al.* 1998). How does waterlogging induce ROL barrier formation? Prolonged waterlogging causes the depletion of oxygen and the accumulation of other products, such as ethylene, CO_2 , and phytotoxins (e.g., Fe^{2+} and organic acids) in the soil, as well as pH changes in the soil (i.e., a decrease in alkaline soil and an increase in acidic soil) (Ponnamperuma 1984). Low oxygen and high ethylene initiate aerenchyma formation, promoting longitudinal diffusion of oxygen (Shiono *et al.* 2008, Voesenek and Sasidharan 2013, Yamauchi *et al.* 2018). Interestingly, in rice, ROL barrier formation is not triggered by low oxygen, elevated ethylene and CO_2 (Colmer *et al.* 2006), or low pH (4.7 or 5.7) (Colmer *et al.* 2019). On the other hand, ROL barrier formation can be stimulated by some soil phytotoxins including Fe^{2+} (Mongon *et al.* 2014), sulfide (Armstrong and Armstrong 2005), and organic acids (Armstrong and Armstrong 2001, Kotula *et al.* 2014) that are produced by anaerobic bacteria in waterlogged soils. However, adding sulfide (0.174 mM) or organic acids (e.g., acetic acids 0.4–1.5 mM) to a hydroponic solution was found to inhibit root growth due to their toxicity and result in increased suberization and/or lignification of cell walls in the OPRs (Armstrong and Armstrong 2001, 2005, Kotula *et al.* 2014). Recently, Colmer *et al.* (2019) demonstrated

that non-toxic levels (<0.05 mM) of organic acids such as acetic, propionic, butyric, and hexanoic acids initiate ROL barrier formation in rice roots, without affecting root tissue respiration. Mongon *et al.* (2014) also showed that non-toxic levels of Fe²⁺ (0.36–0.54 mM) induce an ROL barrier under aerated conditions, without inhibiting root growth. Thus, ROL barrier formation in rice is considered to be triggered by accumulations of soil phytotoxins in waterlogged soil (Pedersen *et al.* 2021b).

The sensing and signal mechanisms underlying phytotoxin-induced ROL barrier formation in rice remain unknown. However, ethylene, a crucial hormone in acclimation to waterlogging, does not appear to be involved as it did not induce ROL barrier formation (Colmer *et al.* 2006).

5-2. Molecular mechanisms of ROL barrier formation

In rice, an ROL barrier can be induced by waterlogging (Colmer *et al.* 1998), making rice a model plant for understanding the molecular regulation of ROL barrier formation. For example, analyses of gene expressions in rice forming an ROL barrier under stagnant nutrient solution (Kulichikhin *et al.* 2014, Shiono *et al.* 2014b), non-aerated nutrient solution with high Si levels (1.78 mM) (Fleck *et al.* 2011), or non-aerated nutrient solution with organic acids (Colmer *et al.* 2019) were demonstrated. A transcriptome analysis using laser-microdissected tissues of the OPRs identified 98 genes that were strongly upregulated during ROL barrier formation in rice (Shiono *et al.* 2014b). These included suberin biosynthesis genes such as *CYTOCHROME P450* (*OsCYP86B3*, LOC_Os10g34480) and *ABC TRANSPORTER* (*OsABCG5*, LOC_Os03g17350), and transcription factor genes containing *WRKY*, *AP2*, and *MYB* domains (Shiono *et al.* 2014b). Many (65–78%) of the promoters of the upregulated transcription factors had putative *cis*-elements such as *WRKY*, *AP2*, *NAC*, and *MYB* (Shiono *et al.* 2014b). Application of low concentrations of organic acids also upregulated two suberin biosynthesis genes (*OsABCG5* and *OsCYP86B3*) (Colmer *et al.* 2019).

Waßmann (2014) analyzed suberin formation in the OPRs using a rice mutant lacking a suberin biosynthesis gene, *OsCYP86B3*. In *cyp86b3* mutant roots, C₂₄ to C₃₀ ω-OH fatty acids, which are major aliphatic suberin monomers, were hardly detectable. However, the level of other aliphatic suberin compounds (α-OH acids) and aromatic suberin compounds (coumaric acid and ferulic acid) were similar in the mutant and wild type. Additionally, in the mutant, suberin lamellae at the exodermis/hypodermis were detected by histochemical staining. ROL barrier formation in *cyp86b3* was evaluated qualitatively with methylene blue staining and quantitatively with a cylindrical oxygen electrode. As in the wild type, in *cyp86b3* grown under stagnant conditions, oxygen leaked from the root apex but not from the basal parts, indicating the presence of a tight ROL barrier. Unexpectedly, the lack of C₂₄–C₃₀ ω-OH fatty acids did not affect the oxygen permeability of rice roots.

Shiono *et al.* (2014a) examined another rice mutant of *OsABCG5*, called *reduced culm number1* (*rcn1*). In this mutant, Fluorol Yellow 088 staining did not reveal any suberin lamellae in the exodermis/hypodermis, even under stagnant conditions. The amounts of the major aliphatic suberin monomers originating from C₂₈ to C₃₀ fatty acids and ω-OH fatty acids were significantly lower in *rcn1* than in the wild type. Apoplastic tracers (periodic acid and berberine) rapidly passed through the OPRs and into the roots of *rcn1*, indicating that the apoplastic barrier at the exodermis/hypodermis was impaired (Shiono *et al.* 2014a). Another study found that *rcn1* hyper-accumulated sodium under waterlogged conditions (Matsuda *et al.* 2014). Under saline waterlogged soil conditions, the sodium contents of *rcn1* were about 2 times greater in the shoots and about 1.4 times greater in the roots than those in the wild type (Matsuda *et al.* 2014). Under these conditions, the growth of *rcn1* was significantly inhibited, suggesting that the inhibited growth was due to salinity stress (Matsuda *et al.* 2014). However, ROL barrier formation in the *rcn1* mutant has not been evaluated. Further studies on the ROL barrier formation of suberin mutants are required to reveal its molecular mechanisms.

6. Future perspectives for improving waterlogging tolerance in crops

Most waterlogging-sensitive crops cannot form an ROL barrier and/or a significant amount of aerenchyma. Some attempts have been made to improve waterlogging tolerance in crops by introducing genes or quantitative trait loci (QTLs) associated with ROL barrier formation from waterlogging-tolerant wild relatives. *H. maritimum*, a waterlogging-tolerant wild relative of wheat, inducibly forms an ROL barrier under stagnant conditions (Garthwaite *et al.* 2003). To obtain wheat varieties with inducible ROL barriers in the roots, wheat cultivars [e.g., Chinese Spring (CS)] were hybridized with *H. maritimum* accessions (e.g., H21), producing amphiploid (Malik *et al.* 2011) and disomic chromosome addition lines for H21 in CS wheat (Konnerup *et al.* 2017). Under stagnant conditions, four of the amphiploid lines, including the H21-CS amphiploid, had tight ROL barriers (Malik *et al.* 2011). On the other hand, Konnerup *et al.* (2017) did not detect ROL barriers in any of six disomic chromosome addition lines for chromosomes 1, 2, 4, 5, 6, or 7 of H21 in CS wheat. The gene involved in ROL barrier formation may be located on chromosome 3 of H21, but unfortunately a disomic chromosome addition line of chromosome 3 of H21 was not isolated and could not be tested (Konnerup *et al.* 2017). The wild relative of maize, *Z. nicaraguensis*, generates an inducible ROL barrier under stagnant conditions, while maize inbred line “Mi29” does not (Abiko *et al.* 2012). Analyses of *Z. nicaraguensis* introgression lines in the genetic background of domesticated maize identified a major locus associated with ROL barrier formation

in a segment of the short arm of chromosome 3 of *Z. nicaraguensis* (Watanabe *et al.* 2017). Recently, we found that some accessions of a wild rice species, *O. glumaepatula*, have a constitutive ROL barrier under aerated conditions (Ejiri *et al.* 2020). *O. glumaepatula* has an AA-genome, making it possible to cross it with cultivated rice that does not form a constitutive ROL barrier. We are presently mapping ROL-related QTLs in introgression lines of *O. glumaepatula* to identify the genes that regulate constitutive ROL barrier formation.

Plants form an ROL barrier as a strategy for adapting to waterlogged soils, but an ROL barrier alone is not enough to survive. For adequate root aeration under waterlogging, well-developed aerenchyma formation is also essential. Additionally, soil-surface roots that develop in shallow soil with relatively higher oxygen levels even under waterlogging would escape the toxic reduced-substances in deeper soil. Several studies have attempted to identify QTLs that regulate aerenchyma formation (Mano and Omori 2008, 2009, Mano *et al.* 2016, Zhang *et al.* 2016, 2017) and soil-surface roots (Mano *et al.* 2005, Uga *et al.* 2012). A gene that regulates the development of soil-surface roots in rice was recently identified as *qSOR1* (*quantitative trait locus for SOIL SURFACE ROOTING1*) and was found to involve gravitropic responses that act through negative regulation via auxin signaling (Kitomi *et al.* 2020). A combination of QTLs involved in various agronomic traits is useful for applied breeding, which is called QTL pyramiding (Ashikari and Matsuoka 2006, Nagai *et al.* 2012). A combination of QTLs regulates root aeration (i.e., an ROL barrier and aerenchyma formation) and a shallow root system by QTL pyramiding must be one of the promising approaches to breed waterlogging-tolerant crops.

Author Contribution Statement

M.E. drafted the manuscript with contributions of T.M., T.F., and K.S. K.S. wrote the article with contributions of M.E., T.M., and T.F. All authors edited the manuscript and approved the final manuscript.

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