Short report

Waterlogging tolerance and capacity for oxygen transport in *Brachypodium distachyon* (Bd21)

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Abstract: Many crops are sensitive to waterlogging. A small, fast-growing grass, *Brachypodium distachyon* (Bd21), whose genome has been sequenced, is a new model for studying cereal crops such as wheat and barley, and for developing novel biomass grasses. However, its waterlogging tolerance and oxygen transport properties are not known. Here, we show that in stagnant deoxygenated nutrient solution, which mimics waterlogged soil, *B. distachyon* grows poorly and does not increase the number of newly formed roots. In both aerated and stagnant conditions, aerenchyma was hardly observed in roots, and root porosities were low. Suberin and lignin, which are thought to be constituents of the barrier to radial oxygen loss, did not develop in the outer part of roots in either aerated or stagnant conditions. Our results suggest that the abilities of oxygen transport in *B. distachyon* are insufficient to grow and survive in stagnant deoxygenated conditions.

Keywords: adventitious root, aerenchyma, barrier to radial oxygen loss (ROL), Casparian strip, lignin, suberin

Introduction

Many crops, including wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), are sensitive to waterlogging, and grow more slowly under waterlogged conditions than under drain (aerated) conditions because their roots suffer from hypoxia, or even anoxia, when soils become waterlogged. When the amount of oxygen is insufficient in roots, plants shift to anaerobic metabolism (e.g. activation of fermentation pathways, reduction of energy consumptions) (Kennedy et al. 1992, Sachs et al. 1996, Dolferus et al. 2003). Many crop species cannot survive such a shift for more than two or three weeks without sufficient internal aeration.

Plants adapted to waterlogging have three major adaptation strategies for survival under waterlogged conditions. These involve forming (1) newly adventitious roots, (2) aerenchyma and (3) a barrier to radial oxygen loss (ROL) (Shiono et al. 2008, Colmer and Voesenek 2009). Aerenchyma is large interconnected gas spaces that connect the shoot to near the tips of roots. Oxygen moves via diffusion in the root aerenchyma (Jackson and Armstrong 1999, Drew et al. 2000, Evans 2003, Seago et al. 2005). Newly formed adventitious roots emerge during waterlogging and may replace the function of the previous root system if it is damaged (Jackson and Drew 1984). These newly formed roots typically contain aerenchyma and can support future shoot growth (Laan et al. 1989, Colmer and Voesenek 2009). In a process called ROL, 30-40% of the oxygen transported via the root aerenchyma can leak from root (Armstrong 1979). The function of the barrier to ROL is to diminish ROL along the basal part of roots, which can enhance the longitudinal diffusion of oxygen via the aerenchyma to the root tip and allow root elongation into anoxic substrates (Armstrong 1979, Colmer 2003b). The barrier might also impede entry of phytotoxins from chemically-reduced waterlogged soils (Armstrong 1979). In Amazonian tree species (De Simone et al. 2003), *Glyceria maxima* and *Phragmites australis* (Soukup et al. 2007), the barrier to ROL has been linked to suberin deposits in the exodermis/hypodermis. In *Oryza sativa* (rice) and *Hordeum marinum*, suberin and/or lignin in the outer part of roots are thought to contribute to the barrier (Garthwaite et al. 2008, Kotula et al. 2009a, Kotula et al. 2009b, Ranathunge et al. 2011), although barrier induction can occur before marked changes in rice root histochemistry

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can be detected (Shiono et al. 2011). These strategies for acclimating to waterlogged conditions are found in several wetland plants, including rice (Justin and Armstrong 1991, Colmer et al. 1998, Colmer et al. 2006), *Rumex palustris* (Visser et al. 1995, Visser et al. 2000) and *H. marinum* (Garthwaite et al. 2003, Garthwaite et al. 2008). However, other crops, such as wheat (McDonald et al. 2001b), barley (Garthwaite et al. 2003) and maize (*Zea mays*) (Drew et al. 1979, Abiko et al. 2012b), can form aerenchyma and newly formed roots, but cannot form an ROL barrier. These crops are more sensitive to waterlogging than wetland plants.

Brachypodium distachyon (Bd21) is a wild annual grass belonging to the Pooideae subfamily, which also includes common cereals such as wheat, barley, rye (*Secale cereale*) and oats (*Avena sativa*). It has all the features of a genetic model plant: a small genome, a short life cycle and easy to grow in a simple facility, such as the type used for *Arabidopsis thaliana*. The whole genome of the diploid line Bd21 was recently sequenced (The International Brachypodium Initiative 2010), and other resources (e.g., T-DNA tagging line and database of EST) are rapidly being developed and made available to the community (Opanowicz et al. 2008, Bevan et al. 2010). Additionally, biolistic- (Draper et al. 2001, Christiansen et al. 2005) and *Agrobacterium*-mediated transformation (Vogel and Hill 2008) systems have been developed for a wide range of *Brachypodium* genotypes. *B. distachyon* has recently emerged as an attractive experimental genetic model for the study of cereals and related grasses. However, the waterlogging tolerance and oxygen transport properties of *B. distachyon* are not known. In the present study, we evaluated the ability of *B. distachyon* to form newly adventitious roots and aerenchyma and localizations of suberin and lignin depositions in stagnant deoxygenated nutrient solution that mimics waterlogged soil conditions.

Materials and Methods

Growth conditions

Seeds of *Brachypodium distachyon* L., Bd21 the genome-sequenced line, were kindly provided by Dr. Keiichi Mochida (RIKEN, Japan). *B. distachyon* was grown in one-quarter strength nutrient solution used in earlier studies of rice (Colmer 2003a, Shiono et al. 2011). Plants were supported at the stem base in light-shielding pots so that roots were kept in darkness within a controlled-environment chamber (14-h light/10-h dark cycle; 20^{o}C/15^{o}C; relative humidity over 60%; photosynthetic photon flux density at 148 μmol m^{-2} s^{-1} during the light period). Seeds were surface sterilized in 2.5% (v/v) sodium hypochlorite for 1 min. These seeds were then rinsed thoroughly with deionized water, and incubated in deionized water for 1 d in darkness at 20^{o}C. One day after imbibition, seeds were placed on stainless mesh floating on aerated quarter-strength nutrient solution and exposed to light. Eight-day-old seedlings were transplanted into 5-L pots (width: 180 mm, depth: 120 mm, height: 250 mm; each pot held two seedlings) containing quarter-strength nutrient solution. Plants were grown in aerated nutrient solution for 29 d (ca. leaf 6 stage), because adventitious roots start to emerge from shoots by leaf 6-7 stage (Watt et al. 2009). Half of 29-d-old plants were continued in aerated nutrient solution or transferred into stagnant deoxygenated nutrient solution for 20 d. The stagnant nutrient solution contained 0.1% (w/v) dissolved agar and was deoxygenated prior to use by flushing with N_2 gas (Flow rate: about 2.2 L min^{-1} for 15 min per pot). During N_2-flushing, the topside of the 5-L pot was covered a wetted towel and aluminum foil to prevent atmospheric air influx. Before plants grew in the stagnant deoxygenated nutrient solution, its dissolved oxygen (DO) level was checked to be less than 1.0 mg L^{-1} by a DO meter with Galvanic DO sensor (SG6-ELK, Mettler Toledo, Greifensee, Switzerland). The dilute agar prevents convective movements in solution and better mimics the changes in gas composition found in waterlogged soils (e.g. decreased oxygen and increased ethylene) (Wiengweera et al. 1997). The solutions were renewed every 7 d. The pots were arranged in a completely randomized design.

Growth parameters

Plants were harvested after 20 d treatment in aerated or stagnant deoxygenated nutrient solution. Leaf age, the numbers of tillers and adventitious roots and the longest root length were recorded per plant. Shoots and roots were dried in an oven at 60°C for 3 d and weighed on an electric balance.

Root porosity and aerenchyma formation

Root porosity (% gas spaces per unit tissue volume) is a measure of gas volume in the roots. It is measured by determining root buoyancy before and after vacuum infiltration of gas spaces in the roots with water (Katayama 1961, Raskin 1983). Adventitious roots were sampled from plants grown in aerated or stagnant deoxygenated nutrient solution for 20 d, and they were cut into 50 mm segments. We measured root fresh weight in the air, clip weight in the water, weight of clip and roots in the water before infiltration, and weight of clip and roots in the water after infiltration. The submerged tissue was subjected to low pressures for three periods of 5 min to ensure vacuum infiltration. Root porosity was calculated as described previously (Thomson et al. 1990).

Formation of aerenchyma in aerated or stagnant deoxygenated conditions was measured in cross-sections 20 mm behind the apex of adventitious roots. Root portions were collected and stored in deionized water at 4°C. Fresh roots were embedded in 5% (w/v) agar and 100 µm sections were made using a vibrating microtome (Micro Slicer DTK-1000 ZERO1, DOSAKA EM, Kyoto, Japan). The cross-sections were viewed with Axio Imager.A2 microscope and photographed with an AxioCam MRc camera (both Carl Zeiss, Oberkochen, Germany). The proportion of each cross-section occupied by aerenchyma was determined using Image J software (Ver. 1.44c; NIH, Bethesda, USA).

Histochemical staining

Matured adventitious roots (59-121 mm length) were collected from plants grown in aerated or stagnant deoxygenated nutrient solution for 20 d. Root age affects anatomical development and root elongation rate is different under stress and non-stress conditions. To reduce these effects on the staining results, we collected sections from the middle part of roots and 20 mm behind the root apex. Then, fresh roots were embedded in 5% (w/v) agar and 100 µm sections were made using the vibrating microtome (Micro Slicer DTK-1000 ZERO1, DOSAKA EM). Root cross-sections were cleared by incubation in lactic acid saturated with chloral hydrate at 70°C for 1 h (Lux et al. 2005). Suberin lamellae were observed as described previously (Brundrett et al. 1991). Sections were stained with 0.01% (w/v) Fluorol Yellow 088 in polyethylene glycol 400 at room temperature for 1 h to visualize suberin lamellae as yellow-green fluorescence excited by UV light (UV filter set, 02, Carl Zeiss; microscopy: Axio Imager.A2, Carl Zeiss; CCD camera: AxioCam MRc, Carl Zeiss). Casparian strips were observed as described previously as follows Brundrett et al. (1988). Some sections were stained in 0.1% (w/v) berberine hemi-sulphate for 1 h. The sections were rinsed with water and soaked in 0.5% (w/v) aniline blue for 30 min. Casparian strips were visualized as bright white to golden fluorescence excited by UV light. The other sections were stained for 3 min with saturated phloroglucinol in 20% (w/w) hydrochloric acid at room temperature, to visualize lignin with cinnamyl aldehyde groups, which appear orange/red under white light (Jensen 1962).

Statistical analysis

Measurements were compared using a two-sample *t*-test at 5% probability level. Data were analyzed with SPSS 16.0J for Windows (SPSS Inc., Chicago, USA).

Results

Growth of *B. distachyon* was clearly reduced in stagnant deoxygenated conditions (Fig. 1, Table 1). Shoot and roots were smaller in stagnant deoxygenated conditions than in aerated conditions. Many of the leaves died in stagnant deoxygenated conditions (Fig.1). The dry weights of shoot and root were reduced 4-fold and 15-fold, respectively, in stagnant deoxygenated conditions compared with aerated conditions (Table 1). Lengths of longest adventitious roots were not significantly different between aerated and stagnant deoxygenated conditions (Table 1). However, the leaf age and tillers were reduced in stagnant deoxygenated conditions. Especially, the tiller number was 4-fold lower in stagnant

Table 1. Plant growth of *Brachypodium distachyon* (Bd21) in aerated and stagnant deoxygenated conditions.

Parameter (per plant)	Treatment		Significant difference
	Aerated	Stagnant	
Shoot DW (mg)	632 ± 119	170 ± 38	*
Root DW (mg)	272 ± 55	18 ± 5	*
Leaf age	8 ± 0	7 ± 0	*
Number of tillers	15 ± 1	4 ± 1	*
Longest adventitious roots (mm)	340 ± 28	250 ± 30	n.s.
Number of adventitious roots	30 ± 2	23 ± 3	n.s.

Values are mean ± SE. n = 4. Asterisks indicated significant differences between means of aerated and stagnant deoxygenated condition (two-sample *t*-test, *: $P < 0.05$). n.s.: not significant. Plants were grown in aerated nutrient solution for 29 d, and then continued in aerated nutrient solution or transplanted into stagnant deoxygenated nutrient solution for 20 d.

Fig. 1. Plant growth of *Brachypodium distachyon* (Bd21) in aerated and stagnant deoxygenated conditions. Plants were grown in aerated nutrient solution for 29 d, and then continued in aerated nutrient solution or transplanted into stagnant deoxygenated nutrient solution for 20 d. Scale bar, 50 mm.

deoxygenated conditions than in aerated conditions (Table 1). These results show that *B. distachyon* was sensitive to waterlogging.

One of the reasons for the sensitiveness of *B. distachyon* to waterlogging is that it was unable to form new adventitious roots: the numbers of adventitious roots in aerated (30 ± 2) and stagnant deoxygenated conditions (23 ± 3) were not different significantly ($P > 0.05$, Table 1). In addition, the root porosities were low both in aerated and stagnant deoxygenated conditions (1.7% and 5.1%, respectively) (Fig. 2A). In both aerated and stagnant deoxygenated conditions, hardly any aerenchyma was observed in roots (Fig. 2C), and the percentages of aerenchyma formation were very low (0.3% and 0.2%, respectively) (Fig. 2B). Root porosity and aerenchyma formation (% cross-section) did not change between aerated and stagnant deoxygenated conditions ($P > 0.05$; Fig. 2A, 2B).

In addition, in both aerated and stagnant deoxygenated conditions, lignin deposits, suberin lamellae and Casparian strips were not observed in the outer part of the middle part of adventitious roots (Fig. 3), but they were well developed in the endodermis of roots grown in deoxygenated conditions (Fig. 3G-L). In the roots grown in aerated conditions, only suberin lamellae was observed as a patch in the endodermis (Fig. 3B, 3E). Stagnant treatment enhanced suberin and lignin accumulation in the endodermis but not in the outer part of roots. The same trends were observed at the 20 mm behind the root apex (Supplemental Fig. S1). When the hypodermis has a Casparian strip, it is called an exodermis (Enstone et al. 2003). *B. distachyon* lacked an exodermis along adventitious roots. Neither suberin nor lignin was observed at the outer part of roots in both aerated and stagnant deoxygenated conditions.

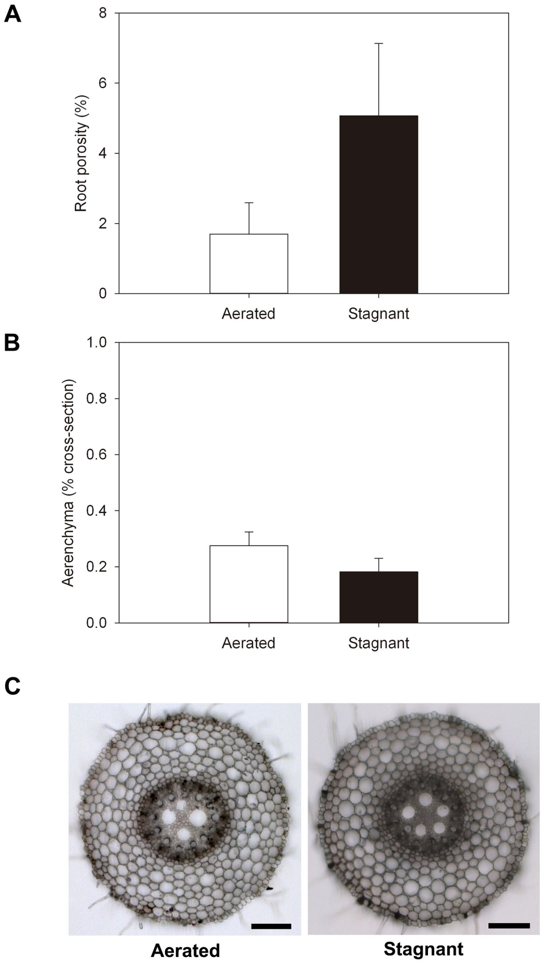

Fig. 2. Comparison of gas space in *Brachypodium distachyon* (Bd21) roots grown in aerated or stagnant deoxygenated nutrient solution. (A) Root porosity (%, total gas spaces in roots). Mean ± SE (Aerated conditions, n = 3; stagnant deoxygenated conditions, n = 3). (B) Aerenchyma formation (% cross-section) at 20 mm behind the root apex. Mean ± SE (Aerated conditions, n = 10; stagnant deoxygenated conditions, n = 7). (C) Representatives root cross-sections at the 20 mm behind the root apex. Scale bars, 100 μm. Plants were grown in aerated nutrient solution for 29 d, and then continued in aerated nutrient solution or transplanted into stagnant deoxygenated nutrient solution for 20 d.

Discussion

The preceding results demonstrate that *B. distachyon*

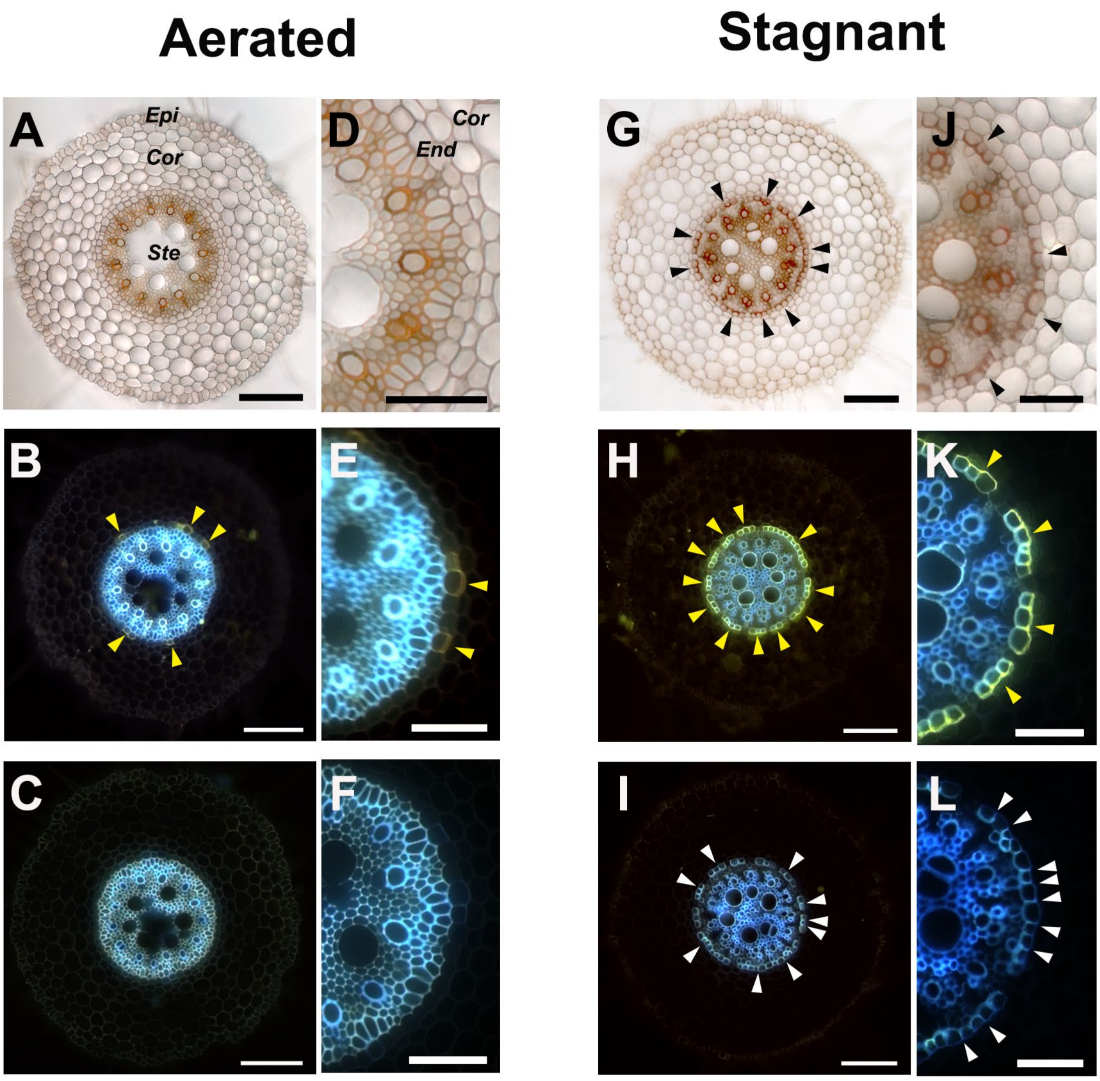

Fig. 3. Comparison of the development of lignin deposition, suberin lamellae and Casparian strips in the middle part of adventitious roots grown in aerated (A-F) or stagnant deoxygenated (G-L) conditions. Lignin with cinnamyl aldehyde groups stained orange/red with phloroglucinol-HCl (Black arrowheads in A, D, G and J). Suberin lamellae are indicated by yellow-green fluorescence with Fluorol Yellow 088 (Yellow arrowheads in B, E, H and K). Casparian strips are indicated by a bright white fluorescence with berberine-aniline blue (White arrowheads in C, F, I and L). In both staining, autofluorescence is detected as blue in the cell walls. Plants were grown in aerated nutrient solution for 29 d, and then continued in aerated nutrient solution or transplanted into stagnant deoxygenated nutrient solution for 20 d. *Cor*, cortex; *Epi*, epidermis; *End*, endodermis; *Ste*, stele. Scale bars, 100 µm (A-C, G-I); 50 µm (D-F, J-L).

significantly reduced their growth in stagnant deoxygenated conditions. Plants have three major strategies for acclimating to waterlogged conditions (Shiono et al. 2008, Colmer and Voesenek 2009): developing newly formed adventitious roots, forming aerenchyma and forming a barrier to ROL by accumulating suberin and/or lignin in the outer part of roots (De Simone et al. 2003, Garthwaite et al. 2008, Soukup et al. 2007, Kotula et al. 2009a, Kotula et al. 2009b, Ranathunge et al. 2011, Watanabe et al. 2013). In *B. distachyon*, newly formed adventitious roots were not increased. Under stagnant deoxygenated conditions, not only aerenchyma formation but also suberin and lignin (i.e. components of barrier to ROL) accumulation were not observed in the outer part of roots in adventitious roots. Because *B. distachyon* has no path for transporting oxygen and compositions of ROL barrier, it is thought that it cannot form an ROL barrier. Our results suggest that a susceptibility to waterlogging is a result of an inability to develop new roots and to transport oxygen in waterlogged conditions.

When wheat, barley and rye were grown in stagnant deoxygenated nutrient solution, the dry weights of their shoots and roots decreased by about 60% and 75%, respectively (McDonald et al. 2001a). When barley and wheat were grown in the waterlogged soil, their root dry weights decreased by about 90% and

85%, respectively (Arikado 1960). Another species of *Brachypodium*, *B. sylvaticum*, was also found to be sensitive to waterlogging (Smironff and Crawford 1983). *B. distachyon* had many dead leaves in stagnant deoxygenated conditions (Fig. 1). In stagnant deoxygenated conditions, the dry weight of shoot and roots decreased by 73.2% and 93.4%, respectively (Table 1). *B. distachyon* is highly sensitive to waterlogging stress similarly to those that are evolutionarily related to it.

Not only wetland plants but also many other plants can often form newly adventitious roots and aerenchyma in roots (Shiono et al. 2008, Colmer and Voesenek 2009). Waterlogging tolerant species tend to develop newly formed adventitious roots in waterlogged conditions compared with aerated and well-drain conditions (Smironff and Crawford 1983, Laan et al. 1989, McDonald et al. 2001a). Wheat and barley, which are waterlogging-intolerant species, did not increase newly formed adventitious roots in stagnant deoxygenated conditions (McDonald et al. 2001a). Many plants such as maize, wheat and barley do not normally form aerenchyma in well-drained conditions. However, these species develop aerenchyma following soil waterlogging (Drew et al. 1979, Drew et al. 1981, Thomson et al. 1990, Akhtar et al. 1998). Root porosities in wheat and barley are increased from about 6% to about 20% (McDonald et al. 2001a). In *B. distachyon*, root porosity was under 5% in both aerated and stagnant deoxygenated conditions (Fig. 2A). Stagnant treatment had no effect on the formation of aerenchyma (% cross-section) or root porosity (Fig. 2A, 2B). Waterlogging treatment also had no effect on the root porosity of *B. sylvaticum* (Smironff and Crawford 1983).

Little is known about the root anatomy of *B. distachyon* expect that an endodermis is present in adventitious roots grown in well-drained soil but not in the outer part of roots (Watt et al. 2009). In our results, suberin and lignin accumulated in the endodermis but not in the outer part of roots in stagnant deoxygenated conditions (Fig. 3, S1). It was difficult to observe formation of an ROL barrier using reduced/oxidized dyes (i.e. methylene blue staining) or root-sleeving oxygen electrode because roots were very thin and easy to be broken (data not shown). Further studies are needed to test the hypothesis that *B. distachyon* is unable to form barrier to ROL in roots.

The molecular mechanisms of aerenchyma and ROL barrier formation have been elucidated in rice, maize and teosinte by using microarrays (Fleck et al. 2011, Rajhi et al. 2011) or next-generation sequencing technology (Abiko et al. 2012a). Sequencing the genome of *B. distachyon* should help to reveal the differences between non-wetland plant and wetland plant. *B. distachyon* is commonly found in region from the Mediterranean sea to Southern Asia that have hot, dry summers and mild, wet winters. However, it is also found in Western United States, Peru, South Africa, Australia, and England. This raises the possibility that there exist natural genetic variation of *B. distachyon* that forms aerenchyma or exodermis in the roots. Such varieties would be good materials for functional genomic analysis to understand waterlogging tolerance in crops.

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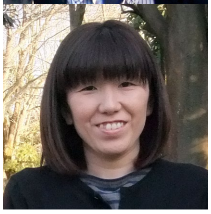

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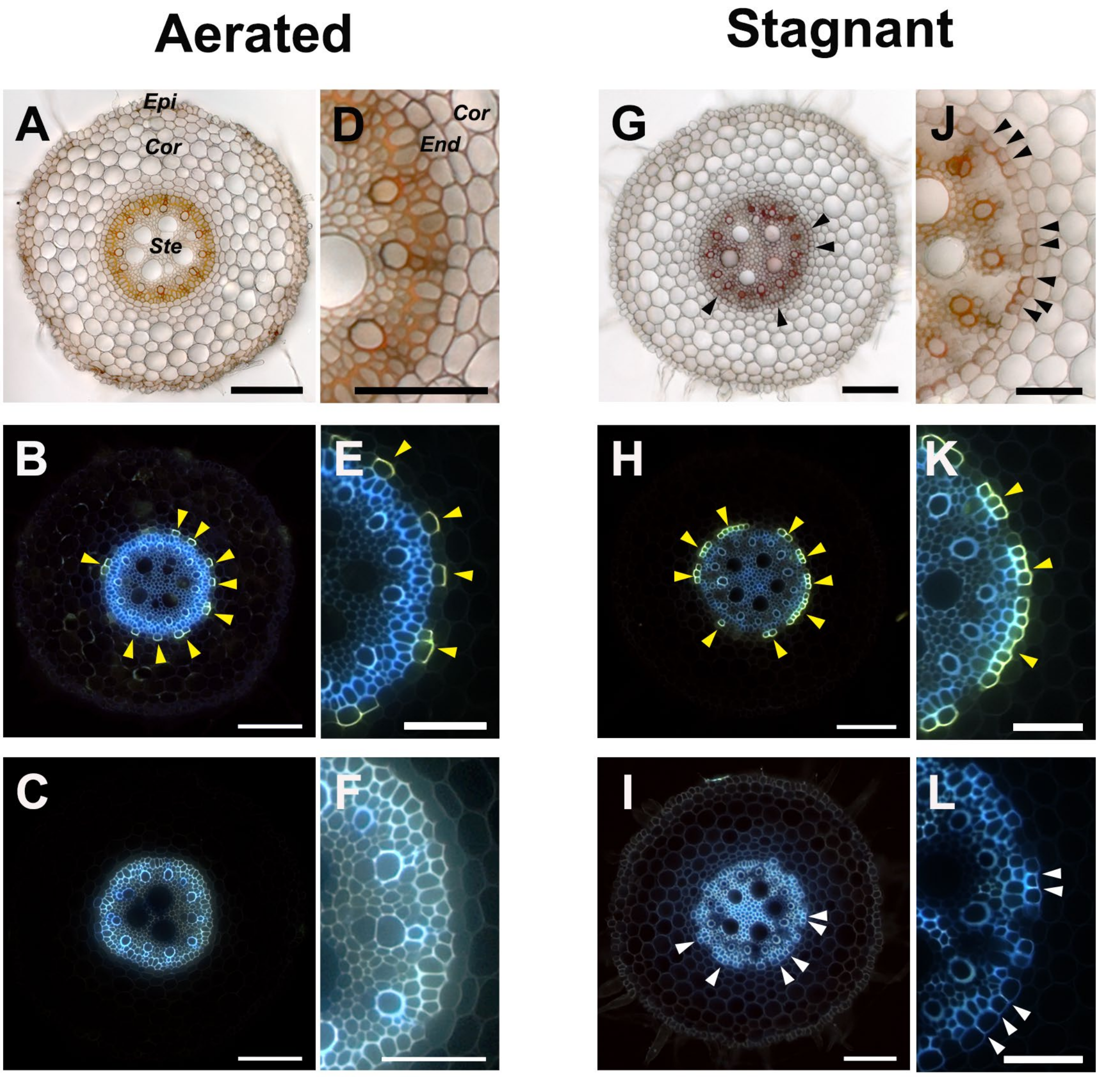

Supplemental Fig. S1. Comparison of the development of lignin deposition, suberin lamellae and Casparian strips at the 20 mm behind the root apex of adventitious roots grown in aerated (A-F) or stagnant deoxygenated (G-L) conditions. Lignin with cinnamyl aldehyde groups stained orange/red with phloroglucinol-HCl (Black arrowheads in A, D, G and J). Suberin lamellae are indicated by yellow-green fluorescence with Fluorol Yellow 088 (Yellow arrowheads in B, E, H and K). Casparian strips are indicated by a bright white fluorescence with berberine-aniline blue (White arrowheads in C, F, I and L). In both staining, autofluorescence is detected as blue in the cell walls. Plants were grown in aerated nutrient solution for 29 d, and then continued in aerated nutrient solution or transplanted into stagnant deoxygenated nutrient solution for 20 d. *Cor*, cortex; *Epi*, epidermis; *End*, endodermis; *Ste*, stele. Scale bars, 100 µm (A-C, G-I); 50 µm (D-F, J-L).