Transcriptional profiling of root hypodermal tissues during formation of a barrier to radial O$_2$ loss in rice (*Oryza sativa* L.)

Katsuhiro Shiono[1,2], Al Imran Malik[1,2,3,5], So Yamazaki[1], Naoko K. Nishizawa[1], Yoshiaki Nagamura[4], Nobuhiro Tsutsumi[1], Timothy. D. Colmer[3,5] and Mikio Nakazono[1]

[1] Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo, Tokyo, 113-8657, Japan
[2] JSPS Research Fellow, 8 Ichibancho, Chiyoda, Tokyo, 102-8472, Japan
[3] Future Farm Industries CRC, The University of Western Australia, 35 Stirling Highway, Crawley, 6009, Western Australia, Australia
[4] National Institute of Agrobiological Sciences, 2-1-2 Kannondai, Tsukuba, Ibaraki, 305-8602, Japan
[5] School of Plant Biology, Faculty of Natural and Agricultural Sciences, The University of Western Australia, 35 Stirling Highway, Crawley, 6009, Western Australia, Australia
Contact: Katsuhiro Shiono, Phone: +81-3-5841-5075, Fax: +81-3-5841-5183,
e-mail: ashionok@mail.ecc.u-tokyo.ac.jp

ABSTRACT

Internal aeration is crucial for plant growth in waterlogged soils. A barrier to radial O$_2$ loss (ROL) can enhance longitudinal diffusion of O$_2$ via the aerenchyma to the root tip, and thus promote root elongation into anoxic substrates. In rice, the barrier to ROL is inducible, forming in adventitious roots in stagnant or waterlogged conditions, but not in aerated conditions. However, the genes involved in regulating barrier formation have not been identified. The present study: (i) identified the timing of barrier formation in rice, using cylindrical root-sleeving O$_2$ electrodes, and (ii) identified candidate genes involved in barrier formation. We isolated root hypodermal tissues during formation of the barrier to ROL using laser microdissection, which is a technique for isolating specific cell-types from heterogeneous tissues. RNA extracted from these tissues was analyzed with a rice 44k oligo microarray. We 137 genes that were significantly up-regulated or down-regulated (P value of log ratio < 0.01) in the outer root cell layers during barrier formation. The up-regulated genes included some peroxidase and transporter genes that might be involved in forming putative suberin or lignin deposits, compounds that are considered to be involved in barrier formation.

KEYWORDS: Barrier to radial O$_2$ loss, Hypoxia, Laser microdissection, Rice 44k oligo microarray, Root aeration, Waterlogging

1. INTRODUCTION

Internal aeration is crucial for plant growth in waterlogged soils. Waterlogging-tolerant species, such as rice (*Oryza sativa* L.), contain large volumes of aerenchyma, which provides a low-resistance pathway for diffusion of O$_2$ within the roots. The roots of some wetland species including rice roots also contain a barrier against radial O$_2$ loss (ROL) from the basal zones (Armstrong, 1971; Colmer, 2003), so that ROL occurs predominately from short lateral roots and the apical few centimeters of the main axes of adventitious roots. A barrier to ROL can enhance longitudinal diffusion of O$_2$ via the aerenchyma to the root tip, and thus promote root elongation into anoxic substrates. In rice, the barrier to ROL is inducible (Colmer *et al.*, 1998). The barrier is created by suberin and lignin at the hypodermal tissues of adventitious roots under stagnant or waterlogged conditions, but not under aerated conditions (Kotula & Steudle, 2009). Although a barrier to ROL is regarded as key factors contributing to waterlogging tolerance in many plants, the genes involved in regulating the barrier formation are not known. In the present study, we

investigated the timing of, and the genes involved in, the formation of a barrier to ROL in rice. The timing was determined with cylindrical root-sleeving O_2 electrodes. Candidate genes at the root hypodermal tissues were identified with a rice 44k oligo microarray in conjunction with laser microdissection, a technique for isolating specific cell-types from heterogeneous tissues.

2. MATERIAL AND METHODS

2.1. Plant materials and culture

Lowland rice (*Oryza sativa* L. cv. Nipponbare) was grown in nutrient solution in pots within a controlled-environment room (25-30°C). Two experiments were conducted. In each experiment, seeds were soaked in pesticide for surface sterilization. Seeds were then washed thoroughly with deionized water, and then incubated in deionized water for 2 day in darkness at 30°C. Two days after imbibition, seeds were placed on stainless mesh floating on aerated quarter-strength nutrient solution and exposed to light. Six days after imbibition, each seedling was held with soft sponge floating on aerated full-strength nutrient solution. Nine days after imbibition, seedlings were transferred to pots containing full-strength nutrient solution. This nutrient solution was used in earlier studies of rice (Colmer, 2003). Solutions were renewed every 7 days. In each experiment, pots were arranged in a completely randomized design.

2.2. Measurement of radial O_2 loss (ROL) to monitor a barrier formation

To evaluate the dynamics in changes of ROL from the basal region of adventitious roots of plants transferred into the O_2-free medium, over 48 h, seedlings were grown until 25 to 28-d-old. Plants were then transferred to an O_2-free medium containing 0.1% (w/v) agar, 0.5 mM $CaSO_4$ and 5 mM KCl to measure rates of ROL from the basal region (i.e. at 15 mm below the root-shoot junction) of adventitious roots during 48 h, at 28°C. The equilibrium flux of O_2 from adventitious roots was measured at selected positions, with appropriate electrode polarization based on polarographs determined for each measurement (see Armstrong 1971). This method to measure ROL was used in earlier studies of rice (Colmer *et al.*, 1998). Adventitious roots were classified as shorter (65-85 mm long) or longer (105-115 mm long) when we commenced the treatment.

2.3. Microarray analysis in conjunction with laser microdissection (LM) technology

It is difficult to discriminate gene expression in root hypodermal tissues because not only hypodermal tissues but also endodermal tissues in roots also accumulate lignin and suberin which are constituents of the barrier to ROL (Kotula *et al*., 2009). To overcome this challenge, we used laser microdissection (LM) to isolate root hypodermal tissues in rice. Moreover, to identify the genes related to barrier formation, gene expression in root before and after barrier formation was compared (Table 1). For this experiment, 23 to 24-d-old seedlings were transferred to (i) aerated nutrient solution, (ii) N_2-flushed nutrient solution or (iii) stagnant deoxygenated agar nutrient solution for 9 h when the basal part of longer adventitious roots start to form the barrier (Table 1). The roots were placed in acetone fixative and were embedded in paraffin. Root hypodermal tissues were isolated from the remaining paraffin sections with a Molecular Devices LM system as shown in Figure 1. Hypodermal tissues-specific RNAs were isolated from each laser microdissected cell and applied to 44k rice oligo microarrays (Agilent technology). In one of the replicate experiments, the Cy3 and Cy5 labels were swapped between sample and control DNA (i.e. sample 1) to minimize the impact of inequalities in DNA incorporation and photobleachinig of the fluorescent dyes. In all analyses, gene expressions were compared between the control DNA (i.e. Sample1) and others (i.e. Sample 2, 3, 4 or 5). For each experiment, we selected genes

whose intensities were significantly higher or lower in each treatment than the control DNA (P value of log ratio < 0.01). Genes were classified into several categories based on their annotations in the rice annotation project database.

Table1. Root sample for microarray analysis in conjunction with laser microdissection technology.

Sample Name	Root type	Sampled region	Treatment type	Barrier formation
Sample 1	Longer adventitious root	Basal part	Stagnant deoxygenated agar nutrient solution	On forming
Sample 2	Longer adventitious root	Basal part	Aerated nutrient solution	Before initiation
Sample 3	Longer adventitious root	Basal part	N_2-flushed nutrient solution	Before initiation
Sample 4	Shorter adventitious root	Basal part	Stagnant deoxygenated agar nutrient solution	Before initiation
Sample 5	Longer adventitious root	Root tip	Stagnant deoxygenated agar nutrient solution	Before initiation

Root length of shorter and longer adventitious roots were 65-80 and 115-130 mm at the time of sampling. Basal part means 12-22 mm below root-shoot junction. Root tip means 17-22 mm behind the root apex. Only sample 1 started to form a barrier to ROL.
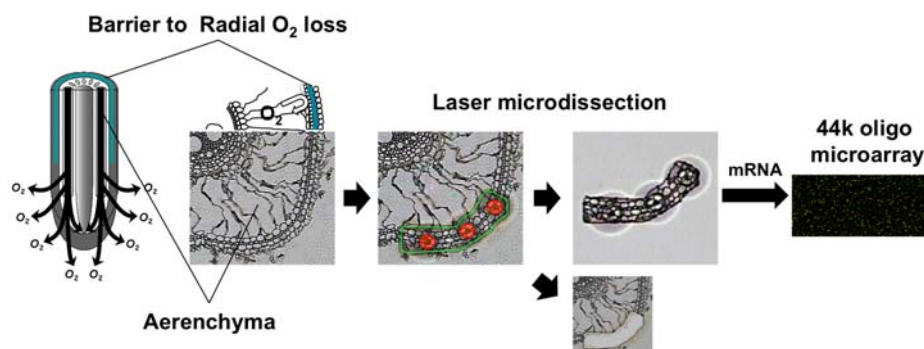

Figure 1. Procedures for transcriptional profiling of root hypodermal tissues using LM and microarray techniques

3. RESULTS AND DISCUSSION

3.1. Dynamics of radial O_2 loss (ROL) during the barrier induction

Rates of ROL from a basal position were measured during 48 h following transfer of roots from aerated solution to the O_2-free agar solution. For the shorter adventitious roots, O_2 fluxes from the basal regions remained high during the first 48 h in the stagnant agar solution; i.e. the shorter adventitious roots did not form a tight barrier to ROL within 48 h. By contrast, for the longer adventitious roots, O_2 fluxes from the basal regions declined soon after transfer into the O_2-free agar solution. At 24 h of stagnant treatment, O_2 fluxes from the basal region were under 5 ng cm^{-2} min^{-1}; the low O_2 fluxes were comparable to those observed for basal regions of adventitious roots with a tight barrier to ROL (Colmer *et al.*, 1998). Subsequently, the O_2 fluxes were reduced to 1.9 ng cm^{-2} min^{-1} after 48 h of stagnant treatment. This rate was 30-fold lower than that of the basal region of shorter adventitious roots at 48 h stagnant treatment ($P < 0.05$, two-sample t-test). In summary, a tight barrier to ROL along longer adventitious roots was induced within 24 h, whereas for shorter adventitious roots it was not formed within 48 h of stagnant treatment.

3.2. Elucidation of candidate genes in barrier formation

We identified 67 genes that were up-regulated and 70 genes that were down-regulated in the outer root cell layers during barrier formation, compared with roots that were not forming a barrier

(Figure 2). The up-regulated genes included (1) peroxidase and (2) ATP-binding cassette transporter genes that might be involved in forming small molecular-weight phenolic compounds or lipid complexes, putative suberin or lignin deposits, compounds that are considered to be involved in barrier formation (Arrieta-Baez & Stark, 2006; Franke & Schreiber, 2007). Identification of these genes should help to develop hypotheses for the mechanism of barrier formation and oxygen transportation in rice roots.
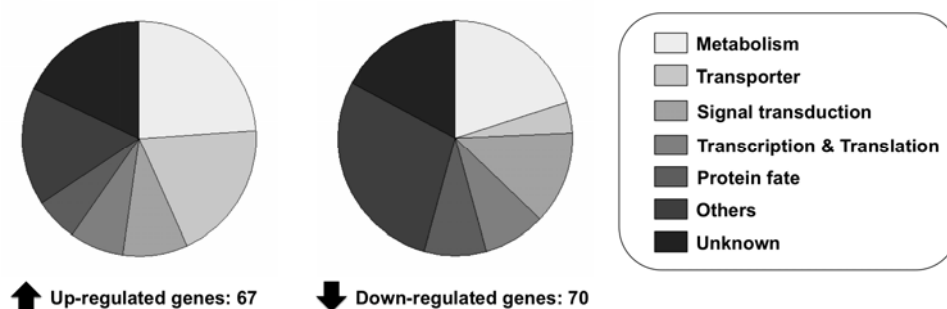

Figure 2. Up- and down-regulated genes during formation of a barrier to ROL

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