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TECHNICAL ARTICLE: PART OF A SPECIAL ISSUE ON ROOT BIOLOGY

Demonstration of osmotically dependent promotion of aerenchyma formation at different levels in the primary roots of rice using a 'sandwich' method and X-ray computed tomography

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• *Background and Aims* The effect of environmental factors on the regulation of aerenchyma formation in rice roots has been discussed for a long time, because aerenchyma is constitutively formed under aerated conditions. To elucidate this problem, a unique method has been developed that enables sensitive detection of differences in the development of aerenchyma under two different environmental conditions. The method is tested to determine whether aerenchyma development in rice roots is affected by osmotic stress.
• *Methods* To examine aerenchyma formation both with and without mannitol treatment in the same root, germinating rice (*Oryza sativa*) caryopses were sandwiched between two agar slabs, one of which contained 270 mM of mannitol. The roots were grown touching both slabs and were thereby exposed unilaterally to osmotic stress. As a non-invasive approach, refraction contrast X-ray computed tomography (CT) using a third-generation synchrotron facility, SPring-8 (Super photon ring 8 GeV, Japan Synchrotron Radiation Research Institute), was used to visualize the three-dimensional (3-D) intact structure of aerenchyma and its formation *in situ* in rice roots. The effects of unilateral mannitol treatment on the development of aerenchyma were quantitatively examined using conventional light microscopy.
• *Key Results* Structural continuity of aerenchyma was clearly visualized in 3-D in the primary root of rice and *in situ* using X-ray CT. Light microscopy and X-ray CT showed that the development of aerenchyma was promoted on the mannitol-treated side of the root. Detailed light microscopic analysis of cross-sections cut along the root axis from the tip to the basal region demonstrated that aerenchyma developed significantly closer to the root tip on the mannitol-treated side of the root.
• *Conclusions* Continuity of the aerenchyma along the rice root axis was morphologically demonstrated using X-ray CT. By using this 'sandwich' method it was shown that mannitol promoted aerenchyma formation in the primary roots of rice.

Key words: *Oryza sativa*, root, aerenchyma, osmotic stress, synchrotron radiation X-ray computed tomography.

INTRODUCTION

Wetland plants develop aerenchyma in their roots as a pathway to diffuse oxygen from their shoots. Determining how these plants develop aerenchyma is essential to an understanding of the tolerance mechanism of these plants in response to waterlogging stress, and also for improving the capacity of crop plants to adapt to waterlogging stress.

Rice roots develop aerenchyma (Webb and Jackson, 1986) that is formed by cell death (i.e. lysigeny) (Kawai *et al.*, 1998; Seago *et al.*, 2005). Previous studies have reported that aerenchyma formation is enhanced under waterlogging stress (Armstrong, 1971; Pradhan *et al.*, 1973; Das and Jat, 1977) and recent studies have shown that stagnant conditions enhance aerenchyma formation in rice roots (Colmer *et al.*, 2006; Shiono *et al.*, 2010). It has also been discussed whether aerenchyma formation in rice roots is regulated by environmental conditions because aerenchyma is developed constitutively under aerated conditions (Armstrong, 1971; Jackson *et al.*, 1985; Webb and Jackson, 1986; Justin and Armstrong, 1991; Kawai *et al.*, 1998).

To elucidate this problem, it is necessary to accurately detect differences in aerenchyma development during root growth under different environmental conditions. However, it is not easy to determine whether the time-course of the cell differentiation process can be modulated in response to environmental stimuli unless a synchronized cell culture is used. By utilizing plant roots that have relatively simple tissue organization, we have developed a method to monitor changes in the rate of differentiation of a particular cell type in an organ (Karahara *et al.*, 2004, 2008; Chen *et al.*, 2011). A shortcoming of this method is that even for the purpose of analysing cell

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differentiation, cell production rate must be measured, which requires a laborious procedure. To overcome this difficulty, we have recently developed a unique method called the 'sandwich' technique (Karahara *et al.*, 2009*a*), in which roots are sandwiched between two different agar media and roots are thereby unilaterally exposed to different environmental conditions. The aim of this study was to use the 'sandwich' method to examine whether aerenchyma development is affected by different environmental conditions. A recent study has shown that drought stress promoted aerenchyma formation in maize roots (Zhu *et al.*, 2010). Drought stress is simulated experimentally by an exposure to osmoticum, which can be applied locally to roots and thereby causes osmotic stress. In the present study, the primary roots of rice were initially exposed to different osmotic environments on opposite sides of primary roots.

Furthermore, we focused on a recently developed technique, synchrotron X-ray computed tomography (CT), which has been shown to be useful for the nondestructive observation of intercellular spaces in plants (Cloetens *et al.*, 2006; Verboven *et al.*, 2008). This non-invasive technique enables us to visualize aerenchyma *in situ* and in three dimensions (3-D). In this study, we used this technique to visualize aerenchyma structure in 3-D in combination with a detailed light microscopic examination of cross-sections cut along the root axis for statistical analyses.

MATERIALS AND METHODS

Plant material and germination conditions

Rice (*Oryza sativa* L. ssp. *japonica* 'Nipponbare') caryopses were presoaked in distilled and deionized water (ddH_2O) for 4 d at 4 °C, soaked in 70 % (w/v) ethanol for 10 s in a 2·5 % (w/v) sodium hypochlorite solution for 5 min for sterilization and then rinsed with ddH_2O. They were then incubated in ddH_2O for 24 h at 30 °C for germination.

Mannitol treatment ('sandwich' method)

Germinating rice caryopses were sandwiched between two 2 % (w/v) agar slabs containing 1/10 strength Hoagland medium (Sigma-Aldrich Japan K.K., Tokyo, Japan). The surface of the agar slab facing the rice caryopses was covered with a sheet of filter paper. Spacers of 0·5 mm thickness, made of silicone rubber, were placed among the rice caryopses to ensure that the sheets of filter paper on each agar slab would not come into contact. For the unilateral treatment of roots with mannitol, mannitol was added to one of the two agar media to a final concentration of 270 mM. The two agar slabs sandwiching the roots were placed vertically in a dark box. Roots were allowed to grow at 25 °C in the dark for 3–5 d, attached to both agar slabs (Fig. 1).

To examine whether there was a difference in the growth of rice roots between those exposed to mannitol on one side or both sides, we compared the length of 4-d-old roots treated on one side (control/mannitol, referred to as the unilaterally treated roots) with those without treatment (control/control, referred to as the control roots), and with those treated on both sides (mannitol/mannitol, referred to as the bilaterally treated roots).

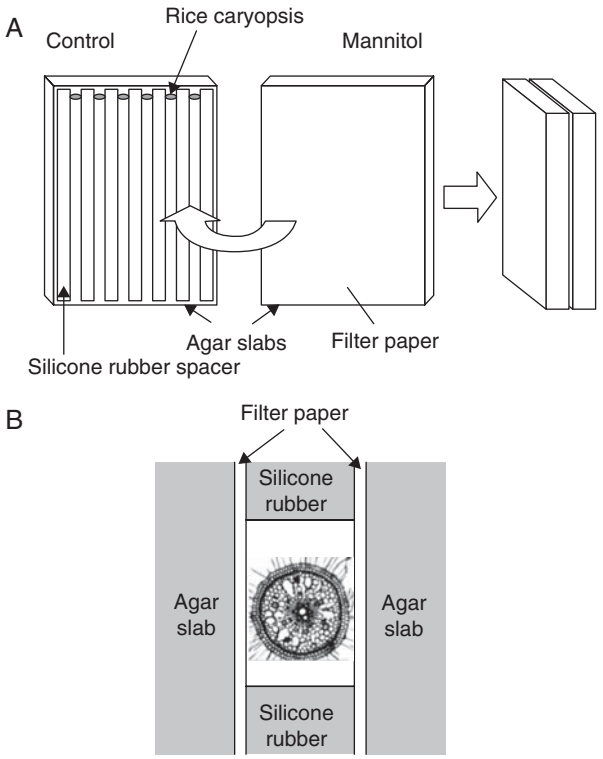

FIG. 1. Experimental set-up of the 'sandwich' method used in this study. Schematic illustrations showing how rice caryopses were sandwiched between two agar slabs (A), and a magnified cross-sectional view around a root in this system (B).

Aerenchyma, or root porosity, formation is shown to be enhanced under waterlogging stress in rice roots (Armstrong, 1971; Pradhan *et al.*, 1973; Das and Jat, 1977; Justin and Armstrong, 1987). Therefore, there is a possibility that water moved osmotically across the unilaterally treated roots from the control side to the mannitol-treated side, resulting in waterlogging of the latter side. To test this possibility, 0·1 % (w/v) Evans blue stain, that does not penetrate the plasma membrane (Gaff and Okong'O-Ogola, 1971), was added on the control side of the agar slab, and the roots were grown for 4 d. Infiltration of Evans blue, which shows red fluorescence under a fluorescence microscope (Matsuda *et al.*, 1995), within a root was examined by observing cross-sections under a fluorescence microscope.

Light microscopy

To distinguish between the control side and the mannitol-treated side on a cross-section, one side of the treated root (i.e. the control side or the mannitol-treated side) was marked along the root axis using black oil paint or by making a shallow incision using a razor blade before cutting cross-sections. The marked roots were then embedded in melted 5 % (w/v) agar aqueous solution just before the agar began to gel. Cross-sections (100 μm thick) were cut basipetally from the root tip to the base at 2·5- or 5-mm intervals through the entire primary roots using a linear slicer (PRO7; Dosaka EM, Kyoto, Japan). These cross-sections were observed under bright-field optics for aerenchyma. The

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Evans blue stain on the cross-sections was observed under a fluorescence microscope (BX-50 FLA; Olympus Corp., Tokyo, Japan) equipped with a filter assembly for excitation by UV light (U-MWU: excitation filter, BP330–385; absorption filter, BA420; dichroic mirror, DM-400; Olympus Corp.), as previously described (Karahara *et al.*, 2008). Bright-field or fluorescence micrographs were taken using a digital camera (Cool Snap cf; Nippon Roper KK, Tokyo, Japan) fitted to the microscope.

X-ray CT

Refraction contrast X-ray CT was performed at the experimental hutch (No. 1) of a bending magnet beamline BL20B2 of the SPring-8 synchrotron radiation facility (Super photon ring 8 GeV, Japan Synchrotron Radiation Research Institute, Hyogo, Japan). The hutch is located 42 m from the X-ray source. The X-ray from a bending magnet of the storage ring was monochromatized by a Si (111) double crystal monochromator, which was located 37 m from the source. The X-ray energy was adjusted to 10 keV. Four-day-old rice roots treated unilaterally with 270 mM mannitol solution were used for the observation. A root segment was placed on the inner surface of a plastic tube with water to avoid drying, and mounted on a rotation stage. X-rays passing through the sample mounted on the rotation stage were transformed into a visible image by the fluorescent screen. The images consecutively projected on the screen were recorded by a cooled CCD camera (C9100-02; Hamamatsu Photonics KK, Hamamatsu, Japan). Exposure time for each projection was 40 ms. For CT, a series of 900 projections was recorded over 180 °. The specimen-to-detector distance was 200 mm. The temperature in the facility was maintained at 25 °C. The seedlings were maintained under white light during image acquisition. The spatial resolution of the 3-D structure was estimated to be 4·86 μm pixel^{-1}. The convolution back projection method was used for tomographic reconstruction (Uesugi *et al.*, 2010). Tomographic slices were obtained and 3-D models were reconstructed using the IMOD software package (Kremer *et al.*, 1996), as previously described (Karahara *et al.*, 2009*b*).

Quantification of the development of aerenchyma

The effects of unilateral mannitol treatment on the development of aerenchyma were examined by light microscopy from a temporal and quantitative point of view by observing a series of cross-sections from the tip to the base of the root. The distance from the root tip to where aerenchyma were observed reflects the speed of its development (Karahara *et al.*, 2004, 2008). To assess the effects of unilateral mannitol treatment on aerenchyma development from a temporal aspect, the distance from the root tip to where aerenchyma was observed was measured.

Digital images of cross-sections obtained by light microscopy were divided into two sides (i.e. the control side and the mannitol-treated side). For the analysis of the effect of unilateral mannitol treatment on aerenchyma development based on the distance from the root tip, each side was divided into eight unit sectors. For each side, the percentage out of eight unit sectors where aerenchyma was observed was plotted against the distance from the root tip. The data were interpolated using binomial smoothing (seven passes) using Igor Pro v.5 software (WaveMetrics, Inc., Lake Oswego, OR, USA). The distances were compared between the control side (Control 3) and the mannitol-treated side when the percentage reached 40 % of the interpolated data. The distances were also compared between Controls 1 and 2.

Quantification of actual areas of aerenchyma and cortex (excluding endodermis, sclerenchyma and exodermis) on a cross-section image was performed using Openlab Darkroom software (Improvision, Coventry, UK). The total area of aerenchyma was calculated as the sum of the cross-sectional areas of

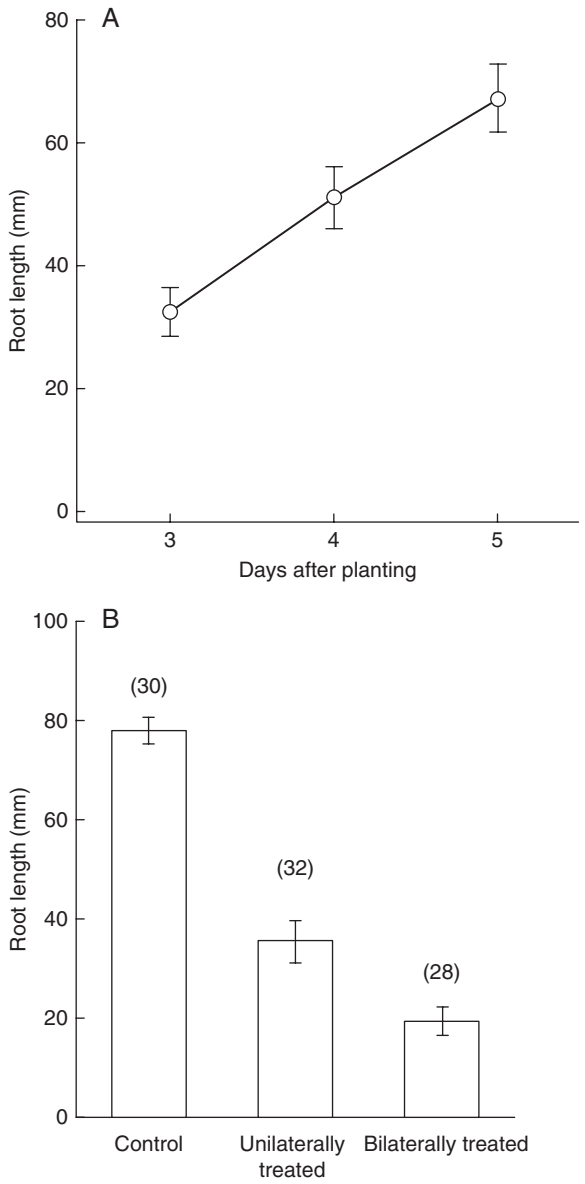

FIG. 2. The effects of unilateral treatment with 270 mM mannitol on growth of the primary roots of rice. (A) Time course changes in the length of roots treated unilaterally with 270 mM mannitol. Values are presented as the mean ± s.e.m., $n = 18$. (B) Comparison of the length of 4-d-old roots among the control roots, the unilaterally treated roots, and the bilaterally treated roots. Values are presented as the mean ± s.e.m. The numbers of samples are shown in parenthesis.

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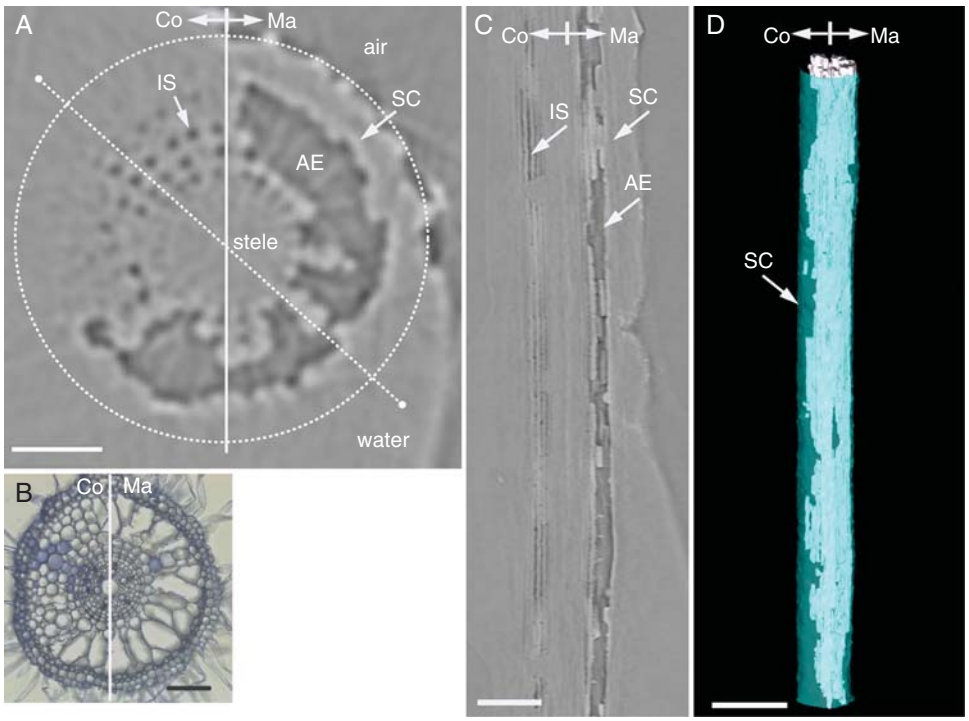

FIG. 3. Aerenchyma in a 4-d-old rice root treated unilaterally with 270 mM mannitol, visualized using X-ray CT. (A) A transverse tomographic slice obtained 30 mm from the root tip. The dashed line shows the position from where the longitudinal slice shown in (C) was obtained. The circle outlined by dashes shows the estimated position of the outer surface of the root. Abbreviations: Co, control side; Ma, mannitol-treated side; AE, aerenchyma; IS, intercellular space; SC, sclerenchyma. Scale bar = 100 μm. (B) Light micrograph of a cross-section of a rice root unilaterally treated with mannitol, which was obtained from a similar position in a different root to that shown in (A). Scale bar = 100 μm. (C) A longitudinal tomographic slice obtained from the same tomogram as used for (A). Scale bar = 250 μm. (D) Three-dimensional models showing the position of the outer surface of the aerenchyma (white) and the position of the inner surface of the sclerenchyma (blue), which were made using the same tomogram as used for (C). Scale bar = 500 μm.

aerenchymatous space observed on each side. The areas of aerenchyma obtained were plotted against the distance from the root tip and the data were interpolated using binomial smoothing as mentioned above. Total areas of aerenchyma were examined in the control roots as well as the unilaterally treated roots. The two sides of the control roots were designated as control side 1 (Control 1) and control side 2 (Control 2). The two sides of the roots unilaterally treated with mannitol were designated as control side 3 (Control 3) and the mannitol-treated side. Control side 2 corresponded to the mannitol-treated side in the experimental set-up using the 'sandwich' method in terms of its direction of placement in the dark box.

The percentage of the total area of aerenchyma in the cortex area in each side of the root was compared among the control sides and the mannitol-treated sides at the basal region of the roots. The total areas of aerenchyma were compared at positions near the base of the roots (i.e. at 45 and 65 mm from the root tip in unilaterally treated roots and control roots, respectively). Statistical tests were performed using JMP v.6 software (SAS Institute Inc., Cary, NC, USA).

RESULTS

Effects of unilateral treatment of roots with mannitol on root growth

As the length of 4-d-old primary roots grown with 270 mM mannitol was one-quarter of that of 4-d-old primary roots grown without mannitol, we selected a mannitol concentration of 270 mM for the experiments. The growth of roots unilaterally treated with 270 mM mannitol was monitored for 3–5 d. It was confirmed that root growth continued during this period (Fig. 2A). The effects of unilateral treatment with mannitol on one side on the growth of rice roots were compared with the treatment of both sides. The length of 4-d-old roots unilaterally treated on one side was shorter and longer than the length of the control roots and that of the roots treated on both sides, respectively (Fig. 2B).

Observation of aerenchyma formation using X-ray CT in a root treated unilaterally with mannitol on one side

A primary root segment excised from a 4-d-old root treated unilaterally with mannitol was observed using X-ray CT. Figure 3 shows slices of a tomogram obtained from a 6-mm-long root segment, excised in a region between 27 and 33 mm from the tip of a 63-mm-long primary root. In a transverse tomographic slice (Fig. 3A), the area occupied by air outside the root, which is transparent to X-rays, appeared darker compared with the area occupied by water or tissues containing water. Such darker areas within a root corresponded to aerenchyma also occupied by air, which was confirmed by light microscopy (Fig. 3B). The aerenchyma extended more on the mannitol-treated side in the cortex compared with the control side (Fig. 3A). Radial arrays of intercellular spaces

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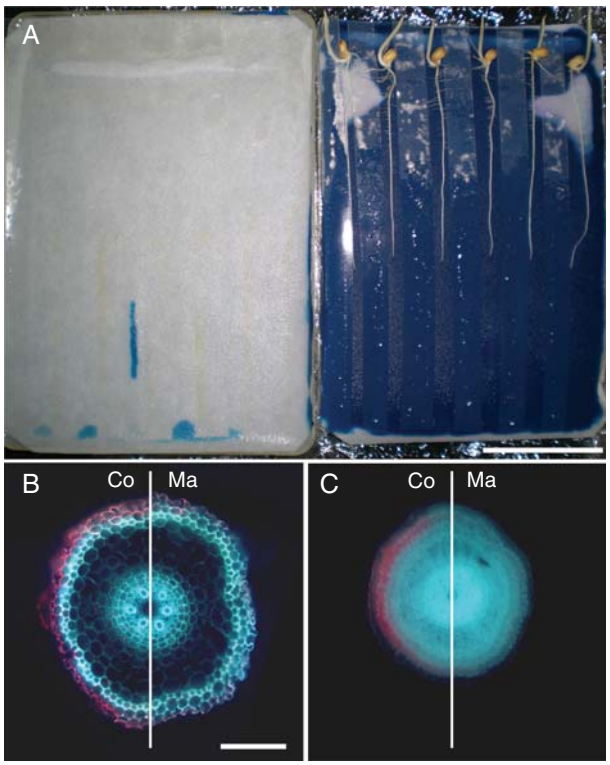

FIG. 4. Movement of Evans blue stain administered from the control side in the roots treated unilaterally with 270 mM mannitol. (A) A whole view of both agar slabs and rice seedlings. Evans blue was added to the right side of the agar slab (the control side). Scale bar = 40 mm. (B, C) Fluorescence micrographs of cross-sections cut from a root of length 54·5 mm at 7·5 mm (B) and 2·5 mm (C) from the root tip. Evans blue, showing red fluorescence, was administered from the control side (the left side). Co, Control side; Ma, mannitol-treated side. Scale bar = 100 μm.

were observed among these cells on the control side (Fig. 3A). No aerenchyma could be detected in the stele (Fig. 3A, C). The longitudinal tomographic slice showed that the side treated with mannitol promoted the formation of aerenchyma (Fig. 3C). Unfortunately, in the tomogram, it was difficult to distinguish the boundary where the root surface came into contact with water because there was no distinct contrast between them (Fig. 3A). Therefore, the positions of the inner surface of sclerenchyma were modelled instead of the root surface (Fig. 3D). The 3-D model also clearly demonstrated that the mannitol-treated side promoted the formation of aerenchyma (Fig. 3D). The continuity of aerenchyma along the root axis was clearly and morphologically demonstrated in the tomographic slice (Fig. 3C) and in the 3-D model (Fig. 3D).

Movement of Evans blue dye, which was administrated from the control side, in the roots laterally treated with mannitol

To test the possibility that water moved osmotically across the unilaterally treated roots from the control side to the mannitol-treated side, apoplastic tracer Evans blue was introduced by adding 0·1 % (w/v) to the agar medium on the control side. Even after 4 d, Evans blue hardly moved to the agar slab on the other side (Fig. 4A). Evans blue was localized

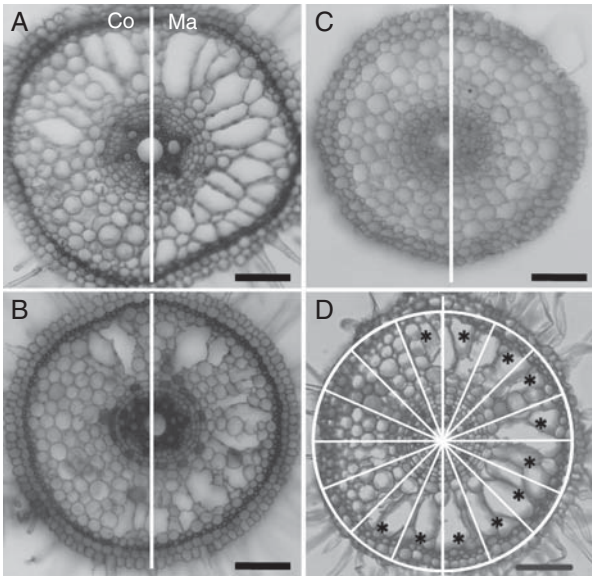

FIG. 5. Light micrographs of cross-sections cut from a 4-d-old rice root treated unilaterally with 270 mM mannitol on the right side of the agar slab. Cross-sections were cut from a treated root at 57·5 mm (A), 30 mm (B) and 5·5 mm (C) from the root tip. (D) A light micrograph showing how the formation of aerenchyma was quantified. Asterisks show the eleven unit sectors where aerenchyma was observed. In this case, aerenchyma was observed in 37·5 % of the sectors in the control side and in 100 % of the sectors in the mannitol-treated side. Abbreviations: Co, control side; Ma, mannitol-treated side. Scale bars = 100 μm.

only in the rhizodermis and outer part of the exodermis on the control side of the root cross-section (Fig. 4B, C).

Quantitative examination of the effects of the unilateral mannitol treatment on aerenchyma development by conventional light microscopy

The effects of unilateral mannitol treatment on the development of aerenchyma were examined by observing a series of cross-sections under a light microscope from the tip to the base of the root (Fig. 5A–C). First, the effects of unilateral mannitol treatment on aerenchyma development were assessed from a temporal aspect. The distance between the root tip and the region where 40 % of the sectors on each side included aerenchyma was determined and compared between the mannitol-treated side and the control side in the unilaterally treated roots. In the typical root shown in Fig. 6A, aerenchyma were formed in 40 % of the sectors at 17·8 mm from the root tip in the mannitol-treated side and at 24·7 mm on the control side. Statistical analysis of the differences in the distance from the root tip to where aerenchyma development reached 40 % between each side indicated that aerenchyma developed significantly earlier in the mannitol-treated side in the unilaterally treated roots (Fig. 6B). There was no significant difference in the distance between Controls 1 and 2 (Fig. 6B).

Second, the effects of unilateral mannitol treatment on aerenchyma development were assessed from a quantitative aspect (Fig. 7A). The percentage of actual aerenchyma area at the basal region significantly increased in the mannitol-treated side when compared to Control 3, in roots treated

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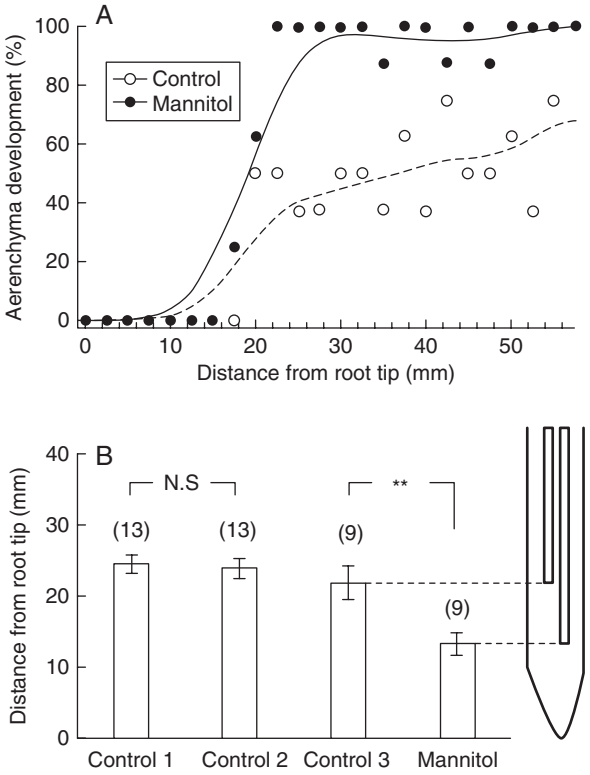

FIG. 6. Effects of unilateral mannitol treatment (270 mM) on aerenchyma development in rice roots, based on the distance from the root tip. (A) Development of aerenchyma in the mannitol-treated side and the control side (Control 3) along the axis of a typical 4-d-old primary root of rice. The percentage of the sectors in which aerenchyma was formed was plotted against the distance from the root tip. The data were interpolated using binomial smoothing (seven passes). (B) A statistical analysis of the effect of unilateral mannitol treatment on the distance from the root tip to where aerenchyma was observed in 40 % of the sectors in the mannitol-treated side as well as the control side (Control 3) of the 4-d-old rice primary roots. A comparison between Controls 1 and 2 is also shown. Values are presented as the mean ± s.e.m. The numbers of samples are shown in parenthesis. N.S., $P > 0{\cdot}05$; **, $P < 0{\cdot}01$ (*t*-test, paired, two-tailed).

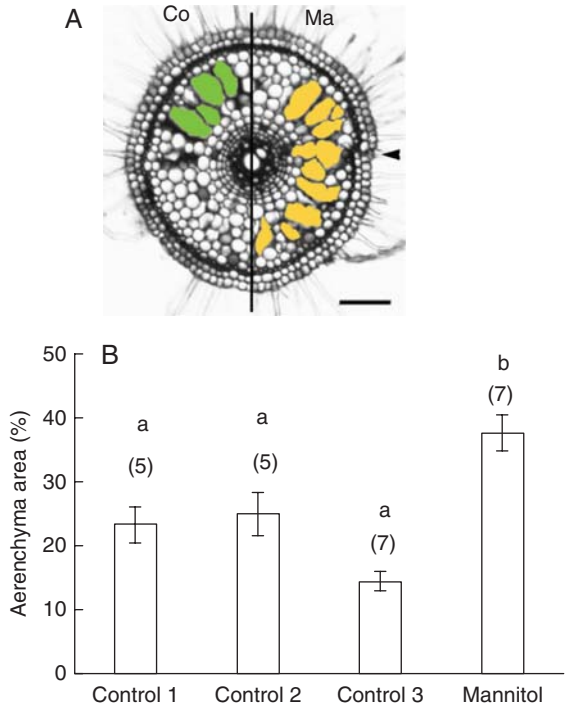

FIG. 7. Effects of unilateral mannitol treatment (270 mM) on the aerenchyma of rice roots. (A) Light micrograph of a cross-section of a 4-d-old rice root treated unilaterally with mannitol (the right side) showing how the actual area of aerenchyma was quantified. An area shown in green or yellow indicates an area of a piece of aerenchyma formed in the control side or the mannitol-treated side, respectively. An arrowhead indicates a shallow incision made using a razor blade on the mannitol-treated side as a marker. Abbreviations: Co, control side; Ma, mannitol-treated side. Scale bar = 100 μm. (B) Percentages of the cross-sectional area of an aerenchymatous cavity in the cortex area on each side, indicated as the aerenchyma area (%), compared among the control sides and the mannitol-treated side at the basal region of the roots. Different letters shown above the columns indicate significant differences assessed by the Tukey–Kramer HSD test ($P < 0{\cdot}05$). Values are presented as the mean ± s.e.m. The numbers of samples are shown in parenthesis.

unilaterally with mannitol (Fig. 7B). To examine whether there was any effect of the mannitol treatment on the control side in the unilaterally treated roots, the percentage of aerenchyma area was compared between the control roots and the unilaterally treated roots. The percentage of aerenchyma area in Control 3 tended to be smaller than in Controls 1 and 2, although no significant difference was observed (Fig. 7B).

DISCUSSION

The 'sandwich' method used in the present study has been shown to be successful in detecting changes in the development of aerenchyma in rice roots, from both temporal and quantitative points of view, in response to osmotic stress. By using this method, the present study has demonstrated that osmotic stress promoted the development of aerenchyma in rice roots, supporting a previous study reporting that drought conditions, that generally cause osmotic stress, promoted aerenchyma formation in maize roots (Zhu *et al.*, 2010). It is possible that the promotion of aerenchyma formation under osmotic stress might contribute to drought tolerance of the plants by reducing root metabolic costs, as previously discussed (Zhu *et al.*, 2010). Alternatively, promotion of lysigenous aerenchyma formation may simply be a generic response to all stressors of roots, perhaps originating with flooding stress because, in that case, it is clear that aerenchyma provides at least two benefits – reduced oxygen metabolism in the root and increased axial oxygen diffusion.

As shown in Fig. 4, Evans blue, administrated from the control side of a root treated unilaterally with mannitol, did not move across the root. It did not even penetrate the exodermis because this dye is an apoplastic tracer. However, this dye could move to the other side through the apoplast of the rhizodermis and to the outer part of the exodermis if there was an apoplastic flow of water across roots. This indicates that water absorbed in roots from the agar slab of the control side does not move osmotically to the other side of the roots. Therefore, the promotion of aerenchyma formation in the mannitol-treated side is not likely to be a secondary effect due to a presumed waterlogging-like condition caused by an excess flow of water from the control side. There is a possibility of active osmotic adjustment in the shoot system or the vascular tissue (such as that which generates root

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pressure) that could lower the water potential in the stele, which would result in root water fluxes that would tend to be radial and might prevent or diminish tangential flow through the epidermis.

The area of aerenchyma in Control 3 tended to be smaller than in Controls 1 and 2 (Fig. 7B), indicating that the unilateral treatment of roots with mannitol affected not only the treated side but also the control side. Aeration would be promoted in the mannitol-treated side due to the promotion of aerenchyma formation. This promoted aeration might affect the other side (Control 3) and possibly inhibit the aerenchyma formation on this side. Both cell division and elongation may be inhibited in the control side as well as in the mannitol-treated side because the growth of the unilaterally treated roots was inhibited when compared with the control roots (Fig. 2B). We cannot state this clearly until cell division and elongation are analysed on both sides of the unilaterally treated roots. In contrast, the development of aerenchyma was regulated locally in response to the environmental conditions, as has been previously suggested by Karahara *et al.* (2009*a*) and Lux *et al.* (2011).

In conclusion, microscopical analysis of aerenchyma development, in combination with our unique 'sandwich' method, has clearly demonstrated that this development is promoted under osmotic stress in rice primary roots. The continuity of aerenchyma in the longitudinal direction was morphologically demonstrated along the root axis through a non-invasive approach using X-ray CT (Fig. 3). It was also confirmed by reconstructing its 3-D model from the tomogram. This technique will allow us to perform a quantitative morphological analysis and time-course, and also *in-situ* observations of aerenchyma formation, which are in progress.

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LITERATURE CITED

Armstrong W. 1971. Radial oxygen losses from intact rice roots as affected by distance from the apex, respiration and waterlogging. *Physiologia Plantarum* **25**: 192–197.

Chen T, Cai X, Wu X, Karahara I, Schreiber L, Lin J. 2011. Casparian strip development and its potential function in salt tolerance. *Plant Signaling and Behavior* **6**: 1499–1502.

Cloetens P, Mache R, Schlenker M, Lerbs-Mache S. 2006. Quantitative phase tomography of Arabidopsis seeds reveals intercellular void network. *Proceedings of the National Academy of Sciences of the USA* **103**: 14626–14630.

Colmer TD, Cox MCH, Voesenek L. 2006. Root aeration in rice (*Oryza sativa*): evaluation of oxygen, carbon dioxide, and ethylene as possible regulators of root acclimatizations. *New Phytologist* **170**: 767–778.

Das DK, Jat RL. 1977. Influence of three soil-water regimes on root porosity and growth of four rice varieties. *Agronomy Journal* **69**: 197–200.

Gaff DF, Okong'O-Ogola O. 1971. The use of non-permeating pigments for testing the survival of cells. *Journal of Experimental Botany*, **22**: 756–758.

Jackson MB, Fenning TM, Jenkins W. 1985. Aerenchyma (gas-space) formation in adventitious roots of rice (*Oryza sativa* L.) is not controlled by ethylene or small partial pressures of oxygen. *Journal of Experimental Botany* **36**: 1566–1572.

Justin S, Armstrong W. 1987. The anatomical characteristics of roots and plant response to soil flooding. *New Phytologist* **106**: 465–495.

Justin S, Armstrong W. 1991. Evidence for the involvement of ethene in aerenchyma formation in adventitious roots of rice (*Oryza sativa* L.). *New Phytologist* **118**: 49–62.

Karahara I, Ikeda A, Kondo T, Uetake Y. 2004. Development of the Casparian strip in primary roots of maize under salt stress. *Planta* **219**: 41–47.

Karahara I, Matsuda K, Honma Y. 2008. Effects of ethylene on the production, elongation, and differentiation of endodermal cells in maize primary root: an integrative analysis of the developmental process of a particular cell type. *Plant Root* **2**: 29–37.

Karahara I, Ito Y, Akai Y, Abe J. 2009*a*. Effects of lateral mannitol treatment on the development of tissues in rice seminal roots. In: Himmelbauer M, Loiskandl W. eds. *Proceedings of the 7th International Society of Root Research symposium on Roots: research and applications (RootRAP).* Vienna, Austria.

Karahara I, Suda J, Tahara H, *et al*. 2009*b*. The preprophase band is a localized center of clathrin-mediated endocytosis in late prophase cells of the onion cotyledon epidermis. *The Plant Journal* **57**: 819–831.

Kawai M, Samarajeewa PK, Barrero RA, Nishiguchi M, Uchimiya H. 1998. Cellular dissection of the degradation pattern of cortical cell death during aerenchyma formation of rice roots. *Planta* **204**: 277–287.

Kremer JR, Mastronarde DN, McIntosh JR. 1996. Computer visualization of three-dimensional image data using IMOD. *Journal of Structural Biology* **116**: 71–76.

Lux A, Martinka M, Vaculik M, White PJ. 2011. Root responses to cadmium in the rhizosphere: a review. *Journal of Experimental Botany* **62**: 21–37.

Matsuda R, Nishikawa A, Tanaka H. 1995. Visualization of dystrophic muscle fibers in mdx mouse by vital staining with Evans blue: evidence of apoptosis in dystrophin-deficient muscle. *Journal of Biochemistry* **118**: 959–963.

Pradhan SK, Varade SB, Kar S. 1973. Influence of soil water conditions on growth and root porosity of rice. *Plant and Soil* **38**: 501–507.

Seago JL, Marsh LC, Stevens KJ, Soukup A, Votrubova O, Enstone DE. 2005. A re-examination of the root cortex in wetland flowering plants with respect to aerenchyma. *Annals of Botany* **96**: 565–579.

Shiono K, Ogawa S, Yamazaki S, *et al*. 2010. Contrasting dynamics of radial O_2-loss barrier induction and aerenchyma formation in rice roots of two lengths. *Annals of Botany* **107**: 89–99.

Uesugi K, Hoshino M, Takeuchi A, Suzuki Y, Yagi N, Nakano T. 2010. Development of fast (sub-minute) micro-tomography. *AIP Conference Proceedings* **1266**: 47–50.

Verboven P, Kerckhofs G, Mebatsion HK, *et al*. 2008. Three-dimensional gas exchange pathways in pome fruit characterized by synchrotron X-ray computed tomography. *Plant Physiology* **147**: 518.

Webb J, Jackson MB. 1986. A transmission and cryo-scanning electron microscopy study of the formation of aerenchyma (cortical gas-filled space) in adventitious roots of rice (*Oryza sativa*). *Journal of Experimental Botany* **37**: 832.

Zhu J, Brown KM, Lynch JP. 2010. Root cortical aerenchyma improves the drought tolerance of maize (*Zea mays* L.). *Plant, Cell & Environment* **33**: 740–749.

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