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Short Communications

Visualization of water transport into soybean nodules by Tof-SIMS cryo system

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ABSTRACT

This paper examined the route of water supply into soybean nodules through the new visualization technique of time of flight secondary ion mass spectrometry (Tof-SIMS) cryo system, and obtained circumstantial evidence for the water inflow route. The maximum resolution of the Tof-SIMS imaging used by this study was 1.8 μm (defined as the three pixel step length), which allowed us to detect water movement at the cellular level. Deuterium-labeled water was supplied to soybean plants for 4 h and the presence of deuterium in soybean nodules was analyzed by the Tof-SIMS cryo system. Deuterium ions were found only in the endodermis tissue surrounding the central cylinder in soybean nodules. Neither xylem vessels nor phloem complex itself did not indicate any deuterium accumulation. Deuterium-ion counts in the endodermis tissue were not changed by girdling treatment, which restricted water movement through the phloem complex. The results strongly indicated that nodule tissues did not receive water directly from the phloem complex, but received water through root cortex apoplastic pathway from the root axis.

Introduction

Water absorbed by plant roots moves to the shoots and most of it transpires to atmosphere through stomata on the shoot surface. During the transpiration process, the absorbed water is distributed to each organ via water transport pathways, such as xylem, phloem, and apoplastic or symplastic routes in parenchyma tissues. Water transport in root nodules, formed by the symbiosis of plants and rhizobia, has been studied by several researchers, beginning with the work of Sprent (1972) who used tritium (^{3}H) labeled water. She suggested that a significant amount of water passed from the root system into the nodules rather than going directly to the shoot. Sprent et al. (1987) also indicated that measurable quantities of ^{45}Ca were transported into infected and cortical tissues of soybean nodules 2 h after feeding from the lower part of the root system. Since then, there have been a number of reports that have described the water pathway for soybean root nodules. However, to date, direct evidence has not been available due to the experimental difficulties involved. In root nodules, xylem vessels coming from distal region of the root apex ends up inside and disconnects to the proximal direction toward the shoot system. This disconnection inside the nodule means that the phloem or the root cortex apoplastic route may be the main source of water for the nodule rather than xylem (Walsh et al., 1989a). Raven et al. (1989) and Streeter and Salminen (1992) speculated that parenchyma transport may also help water supply to the nodules together with phloem transport. As of now reviews (Walsh, 1995; Serraj et al., 1999) indicated that the phloem may be the main channel for water transport to nodules. However, experimental evidence is necessary to support this hypothesis.

There are various experimental techniques that can be used to observe water movement in plants. Stem flow measurement (Higuchi and Sakuratani, 2006; Helfter et al., 2007), the dye tracer method (Varney et al., 1993; Sano et al., 2005; Keller et al., 2006), hydrostatic pressure measurement (Gould et al., 2004, 2005), neutron beam analysis (Nakanishi et al., 2003), and nuclear magnetic resonance imaging (MRI) (Peuke et al., 2006; Windt et al., 2007; Van As, 2007; Scheenen et al., 2007) are widely used techniques that make it possible to analyze not only the water flow, but also

Abbreviations: Tof-SIMS, time of flight secondary ion mass spectrometry.

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plant water uptake. However, water movement from phloem cells to growing parenchyma cells, which has been hypothesized as the major route into the nodule, is not easy to detect using these techniques. For example, the dye tracer method is limited to the root cortex apoplastic water uptake or water movement along xylem or phloem because the dye can't move from the vascular tissues to the parenchyma tissues. Imaging of water movement between phloem and growing cells is possible by the use of the time of flight secondary ion mass spectrometer (Tof-SIMS) cryo-system (Iijima et al., 2011). This system can determine the surface chemical structure as the positional image and/or mass spectrum information. When a pulsed primary ion beam is bombarded onto the surface of a solid specimen, the secondary ions from the top two to three atomic layers (10–20 Å) are emitted from the surface and are detected as a positional image.

In this study, we employed the Tof-SIMS cryo-system to trace the accumulation of deuterium ions in nodules of soybean plants that had been supplied deuterium-labeled water in order to detect water movement from the roots to the root nodules. The specific purpose of this study is to acquire any evidence of the route of water supply to the soybean nodule by a visualization technique of cellular level of water movement using the cryo-Tof SIMS system.

Materials and methods

Plant growth, deuterium uptake and sample preparation

Pregerminated seeds (under dark at 25 °C for 60 h) of soybean [*Glycine max* (L.) Merr.] cultivar "Fukuyutaka" were sown on 25 August 2008 in plastic pots (inner diameter 5 cm, height 15 cm) filled with Akadama soil inside the glass house at Nagoya University, Japan (latitude 35°154′N, longitude 136°971′E). The plants were irrigated regularly with modified nitrogen-free Arnon's medium (Matsumoto et al., 1975) (0.16 mM KH_2PO_4, 2.07 mM KCl, 2.07 mM $MgSO_4{\cdot}7H_2O$, 1.45 mM $CaCl_2{\cdot}2H_2O$, 9.0 μM $MnCl_2{\cdot}4H_2O$, 3.7 μM H_3BO_3, 0.9 μM $ZnSO_4{\cdot}7H_2O$, 0.3 μM $CuSO_4{\cdot}5H_2O$, 0.03 μM $(NH_4)6Mo_7O_{24}{\cdot}4H_2O$, 0.054 mM Fe-EDTA, pH 5.9, EC 75.8 ms m^{-1}) supplied from the bottom of the plastic pots. At 26 to 30 days after sowing, the plants were carefully transferred to an aerated nutrient solution so as not to disturb the root growth. At 2 d after transfer, girdling was conducted by peeling off the outer cortex layer (e.g. Walsh et al., 1987) along a 3 cm length of the stem just above the cotyledon to stop phloem sap flow. On the same day, the plants were transferred into 99.9% deuterium-labeled water (D_2O), which was used as the tracer for plant water uptake (Iijima et al., 2007; Zegada-Lizarazu et al., 2007). Only the lower portion of the root system, approximately 10 cm below the shoot, was dipped into the D_2O solution to avoid any contamination of the root nodules growing near the root base. Deuterium-labeling lasted for 4 h from 9:00 to 13:00 on sunny days, when photosynthesis and transpiration were high inside the glass house. Preliminary trials indicated that less than 4 h of labeling was not effective to observe the accumulation of deuterium ions in root nodules. The nodule samples free from D_2O solution were quickly excised with a scalpel blade and were rapidly shock-frozen by plunging them into liquid nitrogen (−196 °C). The frozen nodule samples were kept in a deep freezer at −85 °C until the sectioning. The clean and even surfaces needed for time of flight secondary ion mass spectrometry (Tof-SIMS) analysis were obtained by sectioning the samples with a cryo-microtome (OT/FAS; Bright Co. Ltd., UK) at −20 °C. The frozen samples were quickly moved into the cryo-chamber for sectioning. The 30 μm thickness were mounted on a silicon plate by the aid of carbon tape and kept again in the deep freezer until Tof-SIMS measurement.

Cryo-time of flight secondary ion mass spectrometer measurement

The analysis was conducted using a Tof-SIMS spectrometer (TRIFT III; ULVAC-PHI Co. Ltd., Japan) equipped with a cryo-stage (Physical Electronics Co. Ltd.). The Tof-SIMS analysis procedures were same as in our previous study (Iijima et al., 2011). Briefly, samples were fixed in a cryo-holder immersed in liquid nitrogen and then inserted into a sample chamber maintained at below −130 °C. The chamber vacuum was kept below 1×10^{-5} Pa after transfer and operated with a pulsed mass-filtered 22-keV Au_1^+ primary ion beam at a raster size 200 × 200 μm (for surface analysis) or 150 × 150 μm (for enlarged image analysis). The actual resolution of the present work was three pixel step length = 1.77 μm to distinguish two separate dots at maximum to observe the lateral water transport of nodule sections. The deuterium ion counts in cells of root nodule were analyzed by the Win Cadence Tof-SIMS software (ULVAC-PHI).

Bright-field microscopy

Hand sections of nodule samples were made using a razor and observed with a stereomicroscope (SMZ-U, Nikon). Then semi-ultrathin sections of transverse direction of the nodules were observed with a light microscope (BX51; Olympus) to identify the tissue structure in the Tof-SIMS images. The samples were excised from about the same positions in each nodule. The segments were fixed in 0.05 M sodium phosphate buffer (pH 7.2) containing 1% glutaraldehyde and 2% paraformaldehyde at 20 °C for 5 h. They were washed with 0.1 M sodium phosphate buffer and post-fixed in 0.1 M sodium phosphate buffer containing 1% osmium tetroxide at 4 °C for 8 h. The samples were then embedded in Spurr's resin and polymerized at 70 °C for 24 h. Semi-ultrathin sections (1 μm in thickness) were cut with a glass knife on an ultramicrotome (EM UC6; Reichert). The sections were stained with toluidine blue O.

Statistical analysis

A total of 71 samples from 20 replicated plants (7 plants for the girdling treatments) by 5 batches of experiments were used for the surface analysis. One-way analyses of variance (ANOVA) was used for the comparisons of the secondary ion counts of the Tof-SIMS images.

Results

The transverse freehand section (B) of the soybean root nodule samples (A) is shown in Fig. 1. The outer layer of the root nodule on the opposite side of the soybean root, where vascular tissues were present (C) was selected for water transport analysis. The enlarged semi thin section (D) indicates the vascular tissues of the xylem and the phloem complex, other parenchyma tissues, such as endodermis, and the sclerenchyma boundary layer between the outer and inner cortex tissues. Similar region of D samples were analyzed for deuterium accumulation in Fig. 2. Vascular (A) and parenchyma tissues (B) around the central cylinder within the inner cortex of the root nodule were visible in the total ion images generated by cryo-Tof-SIMS. In both control (C) and girdling (D) treatments, deuterium ions were only seen at the cellular level in a limited region of the field of view. Judging from the position and the shape of these cells in the superimposed images (E and F), we concluded that the deuterium accumulation region was the cells in the endodermis tissues. The images of all the other tissues and/or cells indicated that only natural abundance levels of deuterium ions (around 0.0156%) were present.

Quantification of the deuterium ions in an image would give us information on how water was distributed within the root