I. Project Research

Project 9

PR9 Project Research on Boron Dynamics in Plants using Neutron Capture Reaction: Development of Boron Analytical Method and Elucidation of its Physiological Function

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BACKGROUND AND OBJECTIVE

Boron is an essential micronutrient for all plants. In general, boron in the soil dissolves in rainwater to form boric acid, which migrates into the groundwater. Therefore, in Japan or Southeast Asia, where there is a large amount of precipitation, the concentration of boron in the soil tends to decrease. As a result, various crops suffer from a growth disorder, called "boron deficiency," and it significantly reduces the value and productivity as the agricultural products. While the use of fertilizers, which contain boron, can restore them from its deficiency, boron overload causes other disorders for plants, such as sterility. Especially in semi-arid areas such as central Asia, in which the amount of precipitation is small, in fact the agricultural damage caused by the boron overload stress is more serious rather than by a simple lack of water. Despite this situation, drastic measures have not made, since there is not enough information on the physiological functions of boron to develop the effective measures compared to other essential micronutrients. In order to understand the physiological functions of micronutrients, research has often been carried out to investigate their kinetics in plants by tracer experiments using their radioisotopes. In fact, Tsukamoto *et al*. revealed that iron (Fe) distribution was different depending on the process of plant growth by a tracer experiment using ^{52}Fe (*Plant Cell Physiol.* 2009; 50(1): 48–57). On the other hand, recently it was put to practical use for a novel method by injecting boric-acid water into wood building material. This method is an epoch-making one that incorporates antiseptic properties to the wood by applying the insecticidal activity and cross-linking function in the cell wall of boron. However, if this development goes on without paying due attention to the leaching of boron from this wood, it may cause great adverse impacts on the environment.

If a method to analyze multi-dimensional information of boron in plants is developed, the productivity of agricultural products will be improved by advancing understanding of the physiological functions of boron. Moreover, it is very promising in the contribution to the public interest in many ways, such as reinforcing historical wooden buildings by the injection of a proper dose of boron. Therefore, we decided to establish a method to perform precise detection and quantification of boron in plant tissue using a neutron capturing reaction.

ALLOTTED RESEARCH SUBJECTS

This project research is composed by three individual subjects as follows;

PR-1: Analysis of boron transport within roots using neutron capture radiography (M. Kobayashi and T. Kinouchi)

PR-2: Analysis of Localization of Boron in Root Tissues by Neutron Capture Radiography (S. Kitajima, M. Kobayashi and T. Kinouchi)

PR-3: Improvement of *In Situ* Visualization of Boron Distributed in Plants by Neutron Capture Reaction (T. Kinouchi)

MAIN RESULTS

PR-1: Kobayashi *et al.* performed a time-course analysis of the localization of boron newly taken up into tap roots of radish seedlings. The result suggests that the delivery of boron to the innermost pith and medullary ray takes more time compared to the other parts, as the endodermis blocks an influx of boron from the external medium, and hence the acquisition relies on the diffusion of boric acid from xylem.

PR-2: Kitajima analyzed vertical distribution of boron in Arabidopsis tap roots by neutron capture radiography. High amount of boron was observed in the root cap and apical meristem.

PR-3: Kinouchi tried to improve *in situ* visualization technique of boron distribution in plants, which was grown in a boron-10 enriched medium, by neutron capture radiography. As a result, it was visualized that a large amount of boron was detected at the center tissues and cambium of the radish tap root.

PR9-1 Analysis of Boron Transport within Roots using Neutron Capture Radiography

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INTRODUCTION: Boron (B) is one of the essential micronutrients for vascular plants. At cellular level, B occurs mainly in apoplast to form a borate diester with the rhamnogalacturonan II regions of pectin. In addition to this "bound form" of B, plants contain free boric acid as well, under the condition of sufficient B supply. However, it still remains unclear how B in these forms distribute within plant tissues. To address the issue, we have been trying to develop a technique for *in situ* visualization of B in plants using neutron capture radiography [1-3]. Results of our previous analyses suggest that B distributes unevenly within roots. To understand the physiological relevance of the uneven distribution, it would be useful to analyze the movement of B within the roots. Thus, in this study we performed a time-course analysis of the localization of B newly taken up into tap roots of radish seedlings.

EXPERIMENTS: Seeds of radish (*Raphanus sativus* L. *sativus*) were germinated on vermiculite and cultivated under a 16-h light/8-h dark cycle in a growth chamber set at 23°C and 60% relative humidity. A week later, the seedlings were transferred to the hydroponic media containing 1 mM $Ca(NO_3)_2$, 0.5 mM KH_2PO_4, 0.5 mM K_2SO_4, 1 mM $MgSO_4$, 1.5 mM NH_4NO_3, 75 μM EDTA-Fe, 46 μM $H_3{}^{11}BO_3$, 9 μM $MnSO_4$, 0.8 μM $ZnSO_4$, 0.3 μM $CuSO_4$, and 0.8 μM Na_2MoO_4 under the same cultivation condition. After 6 days of cultivation, seedlings were transferred to the hydroponic medium of the same composition except that $H_3{}^{11}BO_3$ was replaced with $H_3{}^{10}BO_3$, then cultivated further for additional 24–96 h.
Detached tap roots were fixed with glutaraldehyde and embedded in OCT compound (Tissue-Tek), frozen in liquid nitrogen, and sectioned at 10-μm thickness with a sliding microtome. The section was mounted on CR-39 nuclear tracker detector (20 mm×30 mm) and irradiated with epithermal neutron by applying to the pneumatic tube in the graphite thermal column (Tc-Pn) of Kyoto University Research Reactor (KUR). The CR-39 plate after irradiation was etched in 6 M NaOH solution for 90 min, and the resulting etch-pits were observed under an optical microscope.

RESULTS: The boron neutron capture technique detects ^{10}B but not ^{11}B. In this study, radish seedlings were grown in an ^{11}B-enriched medium and then transferred to the medium containing ^{10}B, hence the time-course change of the signal should represent the movement of B taken up after the treatment. Figure 1 shows a representative set of radiographs of the roots sampled at 48–96 h after the treatment. Etch pits could be observed clearly in the section of the roots sampled at 48 h after treatment (Fig. 1B), indicating that significant amount of ^{10}B had already been taken up by that time. Nonetheless, further intensification of the signal was discernible over time, especially in the innermost pith and medullary ray inside the endodermis (Fig. 1D, F). The result suggests that the delivery of B to the regions takes more time compared to the other parts, as the endodermis blocks an influx of B from the external medium, and hence the acquisition relies on the diffusion of boric acid from xylem. The regions seem to coincide with the area where brown heart of tap roots, which has been suspected to be related with B deficiency, occurs in Japanese radish. Further analyses with shorter periods of ^{10}B feeding may give better resolution of the movement of B within tissues.

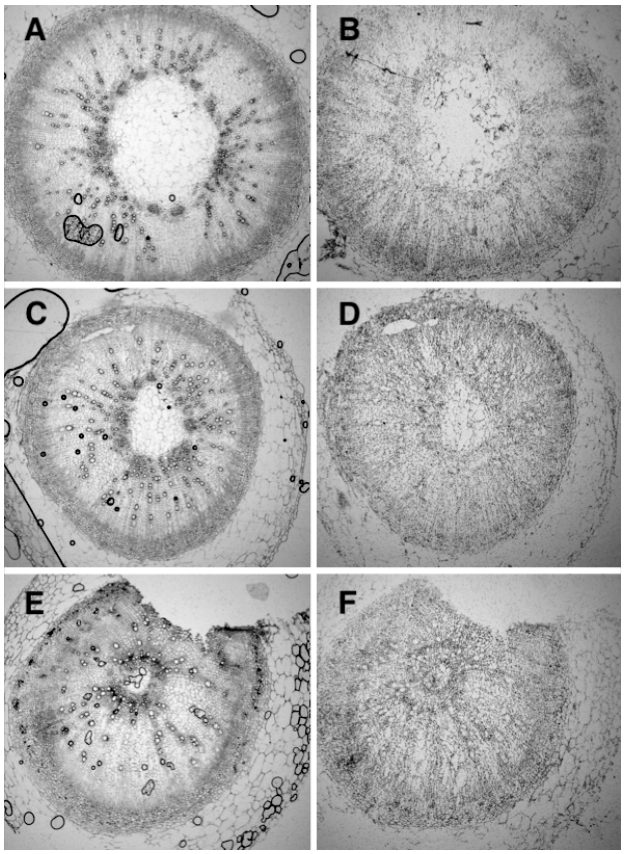

Fig. 1. Detection of B in radish roots using neutron capture radiography. (A), (C), and (E): Optical microscopic images of cross-sections of radish tap roots taken before irradiation; (B), (D), and (F): etch-pit images of (A), (C), and (E) after irradiation, respectively. (A) and (B): 48 h, (C) and (D): 72 h, (E) and (F): 96 h after transfer to ^{10}B-medium, respectively.

REFERENCES:

[1] M. Kobayashi and T. Kinouchi, KURRI Progress Report, **2014** 12929-12929 (2014).
[2] T. Kinouchi and M. Kobayashi, KURNS Progress Report 2018, 122 (2019).
[3] T. Kinouchi, KURNS Progress Report 2018, 121 (2019).

PR9-2 Analysis of Localization of Boron in Root Tissues by Neutron Capture Radiography

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INTRODUCTION: In plant primary cell walls, boron provides a cross-link as a borate ester between apiose residues of rhamnogalacturonan II (RG-II) in pectin [1]. Thus Borate-RG-II complex is an essential factor in the formation of the pectic network, which contributes to cell adhesion or physical characteristics of plants. In fact, boron deficiency causes various physiological disorders in plants, such as necrosis of tap roots or reduced fertility. On the other hand, the distribution of boron within plant tissues still remains unclear. In our previous analyses, it was suggested that a large amount of boron would be distributed in the central part and cambium of radish tap roots. In order to study the vertical distribution of boron in plants, we tried to analyze Arabidopsis tap roots by neutron capture radiography.

EXPERIMENTS: Plant material> Seeds of Arabidopsis (*Arabidopsis thaliana*) were germinated in the hydroponic media containing 1 mM $Ca(NO_3)_2$, 0.5 mM KH_2PO_4, 0.5 mM K_2SO_4, 1 mM $MgSO_4$, 1.5 mM NH_4NO_3, 75 μM EDTA-Fe, 46 μM H_3BO_3, 9 μM $MnSO_4$, 0.8 μM $ZnSO_4$, 0.3 μM $CuSO_4$, and 0.8 μM Na_2MoO_4, and cultivated at 23°C under a 16-h light/8-h dark cycle in a 60%-humidified growth chamber.
In situ visualization of boron by neutron capture radiography> After 6 days of cultivation, seedlings were fixed with 3% glutaraldehyde solution containing 250 mM sucrose. Chilled Arabidopsis in liquid nitrogen was sectioned at 10-μm thickness by a cryostat. The section was mounted onto CR-39 nuclear tracker detector and irradiated with epithermal neutron for 15 min by application to the pneumatic tube in the graphite thermal column (Tc-Pn) of Kyoto University Research Reactor (KUR). The irradiated CR-39 was etched in 6 M NaOH solution, and the resulting etch-pits were observed under an optical microscope.

RESULTS: Etch pit could be observed clearly in the vertical section of the Arabidopsis roots (Fig. 1C). As well as radish tap roots [1], boron in Arabidopsis roots was concentrated at the center and outer peripheral tissues as indicated by arrows. Interestingly, at the tip of the root, high amounts of boron were observed (Fig. 1E, G). Fig. 1D and F shows the root cap and apical meristem, which consists of undifferentiated cells (meristematic cells). Since both tissues cause cell division actively and require physical strength by synthesizing new cell walls, a large amount of boron would be applied. Recently, we improved the resolution of this visualization technique by culturing plants in a special solution replacing natural boron with boron-10. Further analyses based on the detailed boron-distribution might help understand the molecular mechanism regarding controlling root elongation growth.

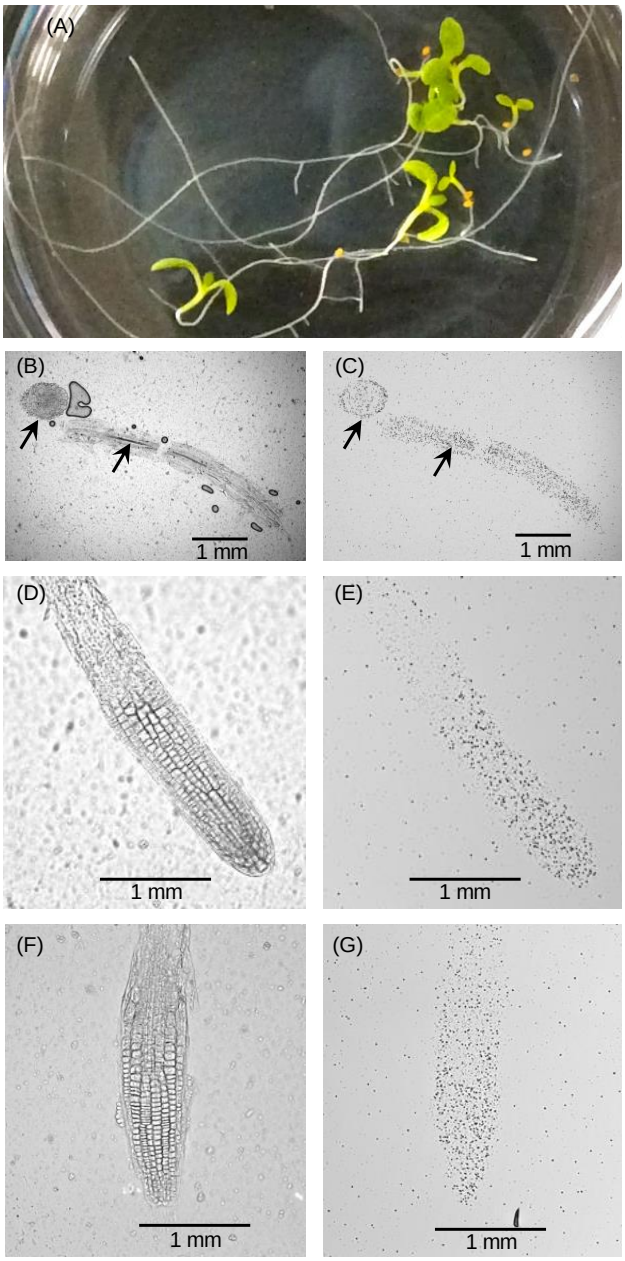

Fig. 1 Detection of boron in Arabidopsis tap roots using neutron capture radiography. (A): Stereomicroscopic image of Arabidopsis on the sixth day after seeding. Tap roots applied with neutron capture radiography. (B), (D) and (F): Optical microscopic images of cross-sections of Arabidopsis roots taken before irradiation; (C), (E), and (G): etch-pit images of (B), (D), and (F) after irradiation, respectively.

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[1] M. Kobayashi, T. Matoh, J. Azuma, Plant Physiol., **110**:1017–1020 (1996).

PR-9-3 Improvemnt of *In Situ* Visualization of Boron Distributed in Plants by Neutron Caputure Reaction

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INTRODUCTION: Boron is an essential nutritional element for all plants, however, a deficiency or an excess of boron causes various growth disorders of them. For all crops due to boron-toxicity which occurs frequently all over the world, a drastic solution has not been developed, because boron analysis methods with high resolution has not yet been well-developed. We still have a poor understanding about physiological functions of boron in plants. Thus, in order to collect multidimensional information on how much boron is localized in which tissue/cell at any stage of growth, we are developing an *in situ* visualization technique capable of detecting the localization of boron with high resolution, by applying neutron capture radiography with a solid-state nuclear track detector, CR-39[1,2]. On the other hand, boron captured by neutron is only boron-10, which is a stable isotope, and the ratio of these existing in nature is 20%. It means that, among boron absorbed from the roots of the plants, their ratio visualized by neutron capture radiography is also 20% at maximum. Since the low content of boron-10 made it difficult for visualization of the localization of boron in the tissue of a plant, we tried to increase the resolution by culturing the plant in a special solution replacing natural boron with boron-10 as a nutrient.

EXPERIMENTS: Plant materials and growth conditions> Seeds of radish (*Raphanus sativus* L.) were seeded on the moderately moisturized vermiculite and cultivated at 23°C under a 16-h light/8-h dark cycle in a 60%-humidified growth chamber. A week later, their seedlings were transferred to the hydroponic media containing major nutrients (1 mM $Ca(NO_3)_2$, 0.5 mM KH_2PO_4, 0.5 mM K_2SO_4, 1 mM $MgSO_4$, and 1.5 mM NH_4NO_3) and micronutrients (75 μM EDTA-Fe, 46 μM $H_3{}^{10}BO_3$, 9 μM $MnSO_4$, 0.8 μM $ZnSO_4$, 0.3 μM $CuSO_4$, and 0.8 μM Na_2MoO_4) under the same condition, and cultured for 10 days.

In situ visualization of boron in plants using neutron capture radiography> Mounted slice (10-μm thickness) of the radish tap root onto CR-39 (20 mm×30 mm) was irradiated with epithermal neutron for 15 min by applying to the pneumatic tube in the graphite thermal column (Tc-Pn) of Kyoto University Research Reactor (KUR). The irradiated CR-39 plate was etched in 6 M NaOH solution, and the resulting etch-pits were observed under an optical microscope [1].

RESULTS: Both Fig. 1(B) and 1(D) are radiographs of the radish tap root, which were cultured with natural boron and boron-10 including culture media respectively. As expected, a large of etch-pits, which were generated from boron-10 on CR-39 by boron-neutron capture reaction, were imaged as small black spots. Especially, Fig. 1(D) showed quite a number of etch-pit which were not equally distributed, but were concentrated at the center and outer peripheral tissues as indicated by arrows. To judge from the optical microscopic image of the original cross section (Fig. 1(C)), the outer peripheral tissues were supposed as cambium, and the vascular bundles arranged in ring shape were observed. The concentrated formation of etch-pits was considered as evidence for the distribution of a large numbers of boron, indicating cell dividing actively in such tissues.

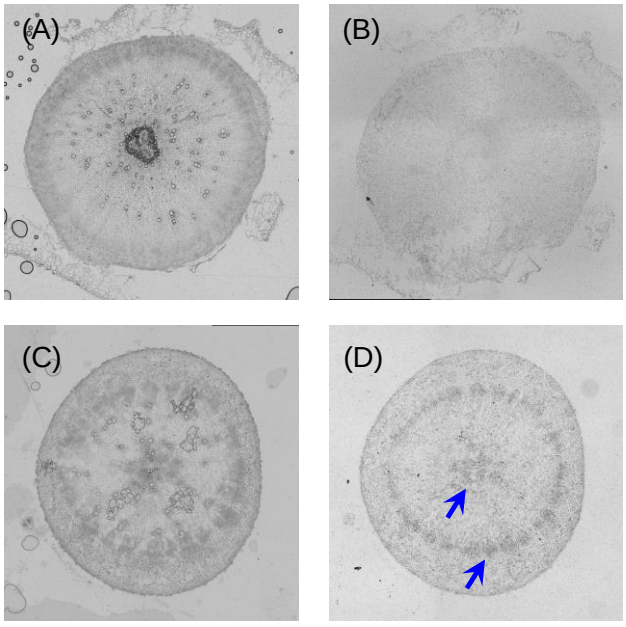

Fig.1 Distribution of boron in the radish tap root. (A) and (C): Optical microscopic images of cross sections of the radish tap root. Each root has a diameter of ~5 mm. (B) and (D): Etch-pit images of (A) and (C) after neutron radiation, respectively.

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[1] M. Kobayashi and T. Kinouchi, KURRI Progress Report 2014, 129 (2015).

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