I-1. PROJECT RESEARCHES

Project 12

PR12 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 agricultural products. While the use of fertilizers, which contain boron, can restore them from this deficiency, boron overload causes other disorders in plants, such as sterility. Particularly in semi-arid areas such as central Asia, in which the amount of precipitation is small, the agricultural damage caused by boron overload stress is more serious than a simple lack of water. Despite this situation, drastic measures have not been taken, 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: Localization of boron in plants using neutron capture radiography (M. Kobayashi and T. Kinouchi)

PR-2: Kinetics of boron artificially injected into wood and its environmental transfer analysis (S. Kitajima and T. Kinouchi)

PR-3: Development of *in situ* visualization of boron distributed in plants (T. Kinouchi)

MAIN RESULTS

PR-1: Kobayashi *et al.* examined various samples with the aim of confirming the usability of neutron capture radiography for the boron analysis, and looking for the suitable plant materials. Through the analyses, radish roots were found to be an excellent material for the boron localization analysis.

PR-2: Since suitable plant material was unobtainable due to unseasonable weather, this allotted research was not carried out.

PR-3: Kinouchi *et al.* tried to develop *in situ* visualization of boron distribution in the root by neutron capture radiography. As a result, it was observed that a large amount of boron was concentrated at the center and outer peripheral tissues of the radish tap root.

PR12-1 Localization of boron in plants using neutron capture radiography

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INTRODUCTION: Boron (B) is one of the essential micronutrients for vascular plants. Boron plays a role as the cross-linker of pectin in cell walls. The structure is considered important for the cell wall architecture, but the exact function of B-pectin cross-linking still remains elusive. Clarifying the localization of B within tissues may give us useful insights on the issue. Therefore, we have been trying to apply neutron capture radiography for the analysis, and reported that the method was applicable to the roots of radish seedlings [1]. Here, we examined the samples from some other plants as well, with the aim of confirming the usability of this method and looking for the suitable plant materials.

EXPERIMENTS: Suspension cultured cells of tobacco (*Nicotiana tabacum* L. cv. Bright Yellow-2) was maintained in the standard culture medium containing 1 mg B L^{-1} as boric acid and subcultured by transferring a 0.2-mL aliquot of 7-d-old culture into a 30-mL new medium. The CR-39 nuclear track detector was overlaid with a mixture of 0.1 mg mL^{-1} poly-L-lysine (PLL; Nacalai Tesque) and 0.1% (w/v) Tween 20 and air-dried at ambient temperature. The PLL-coated CR-39 was then overlaid with an aliquot of 5-d-old culture, incubated for 10 min at room temperature, and washed with new culture medium. The cells adhered onto the detector surface were fixed with 3% (w/v) glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7.0) for 2 h at room temperature. After washing in 0.1 M potassium phosphate buffer (pH 7.0), the cells were dehydrated in a graded ethanol series (20%, 40%, 60%, 80%, and 99.5%). Duckweed (*Lemna minor*) was cultured in a medium containing 0.5 mg B L^{-1} in the laboratory under sunlight through window. Fronds were fixed with glutaraldehyde and embedded in OCT compound (Tissue-Tek), frozen in liquid nitrogen, and subjected to section preparation using freezing microtome. Radish (*Raphanus sativus* L. *sativus*) seeds were grown and used for section preparation as described previously [1].

The bright field-images of the plant specimen on CR-39 nuclear track detectors were taken under microscope. The detectors were then irradiated with neutron for 15 min using Tc-Pn facility at Kyoto University Research Reactor Institute. The irradiated CR-39 plate was etched in NaOH solution, and the resulting pits were observed under microscope.

RESULTS: Figure 1 shows a representative result of analysis using tobacco BY-2 cells as the material, which is the merged image from the bright-field and pit images. The pits shown in pink were found mostly within the cell boundary, suggesting that the observed signals were derived from B in the cells. The result, together with our previous report on the analysis of radish roots, demonstrate the usability of this method to detect B in plants at a cellular level. However, the number of observed B signals was much lower than that expected from the distribution of RG-II as the B acceptor, which distributes in the entire cell wall [2][3]. Thus, the sensitivity of signal detection needs to be increased, to apply this method to subcellular localization of B in plant cells.

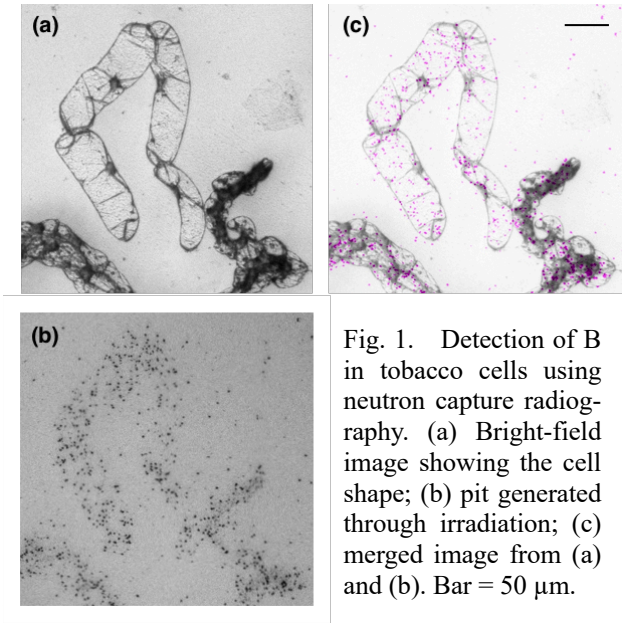

Fig. 1. Detection of B in tobacco cells using neutron capture radiography. (a) Bright-field image showing the cell shape; (b) pit generated through irradiation; (c) merged image from (a) and (b). Bar = 50 μm.

Duckweed is an aquatic monocotyledonous plant that accumulate exceptionally high amount of B. The high tissue concentration of B may lead to a stronger signal, hence we tried using duckweed for the analysis. However, it was difficult to obtain slices of duckweed fronds with high quality, probably because the fronds did not get in contact with OCT compound well. This might be due to water repellency of the fronds of the aquatic plants.

Through the analyses, radish roots were found to be excellent as the material for the B localization analysis. The tip region of tap roots in young seedlings has a diameter suitable for the microscopy used in the analysis. In addition, anthocyanin accumulated in the skin helps finding the specimen in the molded block of OCT during the section preparation. Using the material, we are now planning to analyze the concentration of B in different tissues within a root, based on pit density in each substructures.

REFERENCES:

[1] M. Kobayashi and T. Kinouchi, KURRI Progress Report, **2014** 12929-12929 (2014).
[2] Matoh T *et al*., Plant Cell Physiol. **39** 483-491 (1998).
[3] Zhou Y *et al*. Biosci. Biotech. Bioch. **81** 899-905 (2018).

PR12-2 Development of *In Situ* Visualization of Boron Distributed in Plants

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INTRODUCTION: Boron is known to be the essential element for the growth of 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 agricultural products.

Figure 1 shows the artificially caused boron deficiency in radish tap roots. As indicated by arrows, brown pigment, which is a kind of phenolics such as anthocyanin, was observed at the center and outer peripheral tissues of the tap root of radish, which was cultured in hydroponics in the absence of boron. In particular, since the phenolics accumulated in the outer periphery was formed a ring-shape, that tissue was considered to be cambium. Although the phenolics accumulation is known as typical symptoms of boron deficiency, it was unknown the reason why plants undergoing boron deficiency produce and accumulate phenolics in those specific tissues. Thus, in order to make clear the relation between boron deficiency and abnormal pigmentation within tissues, we tried to develop *in situ* visualization of boron distribution in the root by neutron capture radiography.

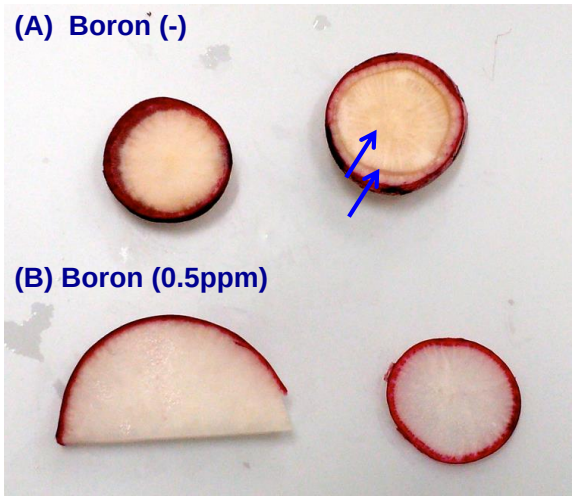

Fig.1 The artificially caused boron deficiency in radish tap roots. (A) Upper two cross sections of the tap root of radish, which were cultured in hydroponics in the absent of boron, exhibits symptoms of boron deficiency: deposition of brown pigment can be observed at the center and the periphery of the tap root as shown by arrows. (B) Lower two cross sections are also obtained from the tap root of radish, which were cultured in hydroponics with boron (0.5ppm).

EXPERIMENTS: Plant materials and growth conditions> Radish (*Raphanus sativus* L. *sativus*) was cultivated at 23°C in 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_3BO_3, 9 μM $MnSO_4$, 0.8 μM $ZnSO_4$, 0.3 μM $CuSO_4$, and 0.8 μM Na_2MoO_4) under a 16-h light/8-h dark cycle in a 60%-humidified growth chamber.
In situ visualization of boron in plants using neutron capture radiography> Mounted slice (10-μm thickness) of the radish tap root onto a solid-state nuclear track detector, 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: Pits generated by boron-neutron capture reaction were not distributed equally, but were concentrated at the center and outer peripheral tissues (fig. 2). Therefore, the total pit-image merged well with phenolics accumulating sites. Since cell division is very active at the central part and cambium, a large amount of boron would be distributed in such tissues. On the other hand, the relationship between phenolics accumulation and boron distribution still unknown. We would like to solve this question by analysis using cultured cells.

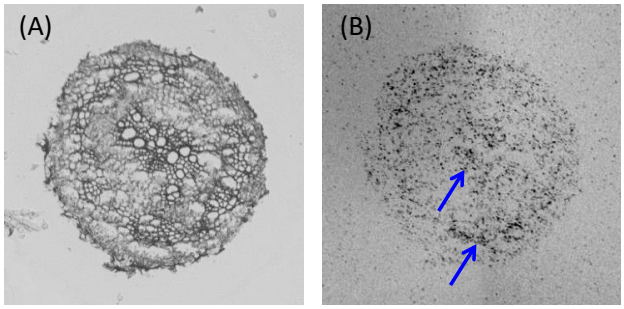

Fig.2 Distribution of boron in the radish tap root. (A) Optical microscopic image of the radish tap root. The root has a diameter of 2 mm. (B) Etch-pit image of the radish tap root after neutron radiation.

REFERENCES:

[1] M. Kobayashi and T. Kinouchi, KURRI Progress Report, **2014** 12929-12929 (2014).