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Development of Observation Method for Isoflavones Secreted by Soybean Roots in Rhizobox Using Sheets with Immobilized Bovine Serum Albumin

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A sensing method was developed to qualitatively evaluate the distribution of soybean isoflavones secreted by roots of soybeans transplanted into a simplified rhizobox by transferring them onto pieces of glass microfiber filter (sheets) with immobilized bovine serum albumin (BSA). To take advantage of fluorescence quenching by the interaction between BSA and soybean isoflavones, BSA was chemically bonded onto the sheets. The sheets with immobilized BSA were brought into contact with a nylon mesh (1 μm mesh aperture) of a simplified rhizobox, and secretions from soybean roots were transferred to the sheets. Fluorescence images before and after contact were captured with an electron-multiplying CCD camera to visualize and analyze fluorescence quenching. The results indicated that soybean isoflavone was spread within 2 to 3 mm from the roots.

1. Introduction

Plants are in constant interaction with a myriad of soil microorganisms in the soil region close to their roots, which is called the rhizosphere. The rhizosphere is home to a greater abundance of microorganisms than the soil away from the plant roots because of the continuous supply of nutrients from the roots.[1,2] Several microorganisms in the rhizosphere have been shown to improve plant growth by increasing tolerance to various stresses and providing mineral nutrients to plants. Therefore, understanding the molecular mechanisms of these interactions is particularly important to take advantage of soil microbial functions and to establish sustainable agriculture.[3,4] Metabolites that are not necessarily essential for growth are called secondary metabolites or plant specialized metabolites (PSMs). They have been shown to affect the abundance and function of soil microorganisms.[5,6]

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A rhizobox is often used to observe the growth and nutrient status of plant roots in their growing state.[7–9] This observation device consists of an acrylic box and nylon mesh sheets for compartmentalization, allowing the movement of metabolites. Furthermore, the observation of metabolites for the same individuals can be continued at the appropriate time without uprooting the plant. However, only a few studies to visualize secretions from roots using the rhizobox have been attempted, and the visualizations of oxygen and carbon dioxide using sensor probes called optodes and foils coated with fluorescent reagents are only a few examples.[10–12] Other examples of the visualization of substances secreted by plant roots include the visualization of phosphorus by enzymatic zymography,[13] the visualization of rhizosphere nutrients by Fourier transform infrared spectroscopy imaging,[14] the visualization of glucose oxidase and glucose with horseradish peroxidase,[15] and the two-dimensional determination of inorganic nutrients in rhizobia by laser ablation inductively coupled plasma mass spectrometry.[16]

In our previous papers, we developed pieces of glass microfiber filter (sheets) with immobilized bovine serum albumin (BSA) to visualize isoflavones secreted by soybean roots and kaempferols secreted by pea roots.[17,18] BSA is chemically immobilized on sheets by utilizing the fluorescence quenching phenomenon of BSA when it interacts with phenolic compounds. By bringing soybean and pea roots dug out from the soil into contact and transferring the root exudates directly onto the sheets with immobilized BSA, visualization was successfully achieved, showing that each metabolite can be qualitatively visualized with high spatial resolution. Although the distribution of daidzein was determined to be within a few millimeters of the root surface by simulation,[8] the variation in the level of daidzein secretion with the root area has not been clarified thus far. The elucidation of the amount and area of daidzein secretion from soybean roots is essential for further utilization of rhizosphere microorganisms in crop production, but techniques of visualizing isoflavone secretion and diffusion in the rhizosphere have not yet been established.[19] Therefore, in addition to the visualization method with high spatial resolution, a less destructive technique is required.

To tackle this issue, we developed a technique by combining our previous sheets with immobilized BSA and a simplified rhizobox with a nylon mesh sheet to observe isoflavone secretion from the roots. The advantage of using the rhizobox with a nylon mesh sheet is that the direction of root growth and the movement of soil particles can be controlled while observing the root growth, and only the movement of metabolites can be observed. In the rhizobox, secreted metabolites are transferred through the nylon mesh during the process of growing the plant. Exudates from soybean roots in a simplified rhizobox were transferred to the sheet with immobilized BSA through a nylon mesh. Then, isoflavone secretion was visualized by observing quenched fluorescence intensity using the sheets with immobilized BSA, and the extent of diffusion from the roots was investigated.

2. Materials and Methods

2.1 Materials

BSA (Cohn fraction V, pH 7.0) was purchased from Fujifilm Wako Pure Chemicals, Japan. A glass microfiber filter (GGF/A, 1.6 μm pore size, Whatman) was used as a substrate for the sheets with immobilized BSA. Water produced by the Milli-Q system (Millipore) was used as a solvent. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from Nacalai Tesque, Japan. 3-Aminopropyltriethoxysilane (APTES) manufactured by Sigma-Aldrich and ethanolamine hydrochloride from GE Healthcare Bioscience were used. Soybean seeds (Enrei) were obtained from Tsurushin Seed Co. (Matsumoto, Japan).

2.2 Preparation of sheets with immobilized BSA

Sheets with immobilized BSA were prepared by the method described in a previous paper,[17] with a slight change in the size of a glass microfiber filter. A glass microfiber filter was cut to the size of a quartz plate (Shimadzu, 36 × 36 mm^2) for fluorescence measurement. First, the filter was treated with oxygen plasma and then allowed to react with 5 ml of 20% APTES in ethanol solution in a fluoropolymer Petri dish. The Petri dish was shaken with a shaker for 60 min, and then the filter was washed with ultrapure water. 0.4 M EDC and 0.1 M NHS solutions were prepared, and 3 ml of 1×10^{-4} M BSA solution, 1 ml of EDC solution, and 1 ml of NHS solution were mixed to make a 5 ml mixture. The mixture was poured into a Petri dish and shaken for 60 min to activate the carboxyl groups of BSA and immobilize BSA on the filter by peptide bonding with the amino groups of APTES attached to the filter. The filter was then washed with ultrapure water. To block the carboxyl groups of activated BSA, 2 ml of ethanolamine hydrochloride was added dropwise onto the filter and allowed to react for 20 min. The filter was then washed with ultrapure water and dried on a quartz plate in an incubator with silica gel at 28 °C.

2.3 Fluorescence intensity distribution imaging

A xenon light source (LAX-C100, Asahi Spectra, Japan) capable of generating UV light was used for excitation. A UV Light-B (UVB) mirror module and a 280 nm bandpass filter (HQB280-UV, Asahi Spectra, Japan) were incorporated into the light source, which was connected to a rod lens (RLQ80-1, Asahi Spectra, Japan) via a quartz light guide. A 325 nm short-pass filter was attached to the rod lens to block visible light, and only 280 nm UV light was output in a plane. A cooled CCD camera (BU-66EM-UV, BITRAN, Japan) for the UV region was used in combination with a UV lens (FL-BC2528-VGUV, RICOH, Japan). A 340 nm bandpass filter (RRX340, Asahi Spectra, Japan) was attached to the lens to block the reflected excitation light, and only the BSA fluorescence at around 340 nm was captured. The light source rod lens, camera, and stage on which the BSA immobilization sheet was placed were set up in a

table-top darkroom, and dedicated accessory software (BU-60 series camera imaging) was used for imaging.

2.4 Visualization of soybean isoflavone secretion from soybean roots in simplified rhizobox

Vermiculite was placed in 500 mL polypropylene cups and soybean seeds were grown at room temperature (25 °C, 12-h light/dark cycle) for one week. Irrigation was performed two days before measurements. The soybean seedlings were then uprooted. Figure 1 shows a diagram of a soybean seedling in a simplified rhizobox and the positions of sheets with immobilized BSA. The lid of a square polystyrene box (194 × 104 × 26 mm^3, AS ONE, Japan) was removed and the case was filled with vermiculite. Soybean roots were placed on top of the vermiculite and wrapped with nylon mesh sheets (1 μm mesh openings, AS ONE, Japan). After obtaining fluorescent images of the sheets with immobilized BSA in the initial state, the sheets with immobilized BSA were placed in contact with the nylon mesh in the rooted area, the lid of the case was replaced, and the sheets with immobilized BSA were held down and left face down for 17 h. Then, fluorescence images of each sheet with immobilized BSA were taken.

2.5 Analysis of soybean isoflavone secretion from soybean roots

The method used is described in detail in a previous paper.[17] Therefore, only a brief description is given here. Image processing was performed on images in 8-bit TIFF format using

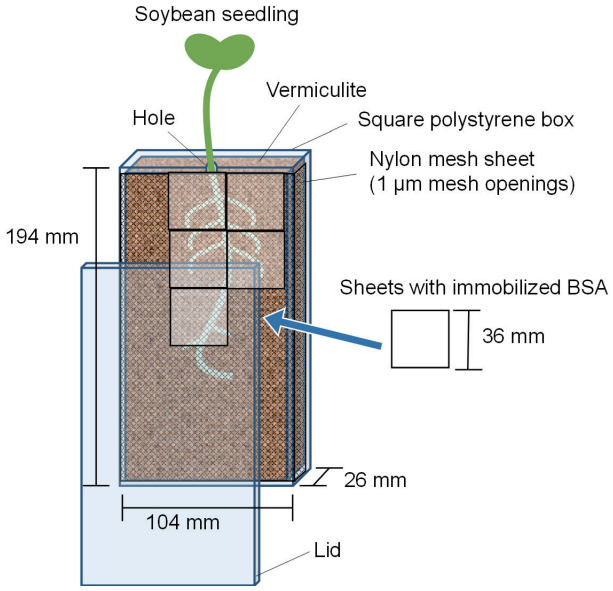


Fig. 1. (Color online) Diagram of soybean seedling in simplified rhizobox with nylon mesh sheet and positions of sheets with immobilized BSA.

the image processing software ImageJ (Fiji) by the following procedure: First, because the fluorescence intensity of the sheets with immobilized BSA tended to slightly decrease with time, the images before and after root contact were subjected to bleach correction. Subtracted and difference images were created from the pre- and post-root contact images (F0 and F1, respectively) after bleach correction. The subtracted images were applied a radius of 2.0 pixels of the median filter to remove noise and then binarized to produce mask images, in which the areas of fluorescence intensity change were represented in black and those of constant fluorescence intensity were represented in white. The region-of-interest (ROI) image was obtained by calculating the logical conjunction (AND) of the difference image and the mask. Then, the areas with fluorescence intensity changes were extracted from the ROI. Areas of strongly quenched intensity were converted to high luminance values. A 16-color look-up table was applied to the ROI image, and 16 pseudo-colors images were obtained.

3. Results and Discussion

Figure 2 shows photographs of a soybean seedling in a simplified rhizobox. Figure 2(a) is simplified rhizobox without a nylon mesh sheet and lid and a transplanted soybean. The left side of Fig. 2(b) shows a simplified rhizobox with a nylon mesh sheet and lid. Soybean root shape can be seen through the nylon mesh. The right side shows only vermiculite as a control. Fluorescent images of the sheets with immobilized BSA in the initial state (F0) were obtained and then sheets with immobilized BSA were placed on a nylon mesh sheet of a simplified rhizobox. Figure 2(c) shows the sheets with immobilized BSA on a quartz plate in contact with the nylon

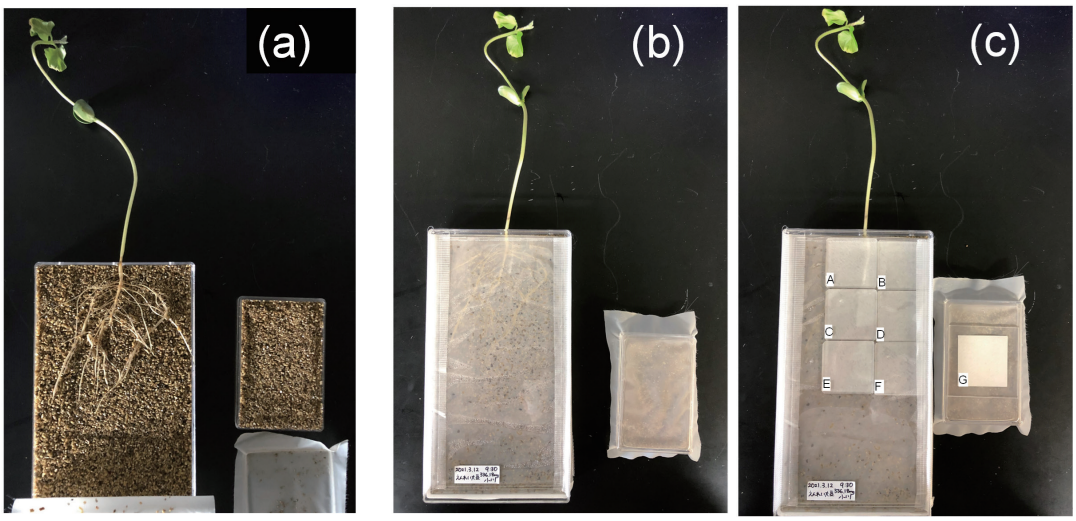


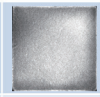
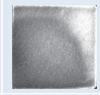
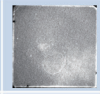
Fig. 2. (Color online) Photographs of soybean in simplified rhizobox. (a) Left: soybean in simplified rhizobox without nylon mesh sheet and lid; right: without soybean for control. (b) Left: soybean in simplified rhizobox with nylon mesh sheet and lid; right: without soybean for control. (c) Sheets with immobilized BSA were placed between the nylon mesh sheet and the lid in a simplified rhizobox .

mesh in the simplified rhizobox. Root exudates were transferred to the sheets with immobilized BSA for 17 h. After transferring the root exudates, fluorescence images (F1) were obtained using the fluorescence intensity distribution imaging system.

Table 1 shows the fluorescence images of the sheets with immobilized BSA and the results of the analysis. The luminance resolution range used for the analysis was adjusted using the software attached to the CCD camera after the images were taken. The range is the same as in the previous papers.[17,18] The fluorescence images of the sheets with immobilized BSA are F0 for the initial state before contact with the nylon mesh, F1 for the fluorescence image after contact with the nylon mesh, and F0' and F1' after bleach correction. The difference image between F1' and F0' is absolute (|F1'−F0'|), and the background is displayed in black (luminance value 0). The areas with high intensity become white as the luminance becomes high. The ROI image was obtained by taking the logical disjunction of the |F1'-F0'| and mask images and extracting the areas where quenching occurred around the root. As can be seen in the F1' images at positions A–E, fluorescence quenching that closely followed the shape of the root was observed in the area where there was contact with the nylon mesh.

Figure 3(a) shows an image of the simplified rhizobox in contact with the sheets with immobilized BSA superposed with the horizontally flipped 16 colors shown in Table 1. Positions A–E in Fig. 3(b) show quenching following the shape of the root. As reported in a previous paper, the fluorescence of sheets with immobilized BSA was quenched when BSA interacted

Table 1
(Color Online) Analysis process for fluorescence images of sheets with immobilized BSA before and after placement in simplified rhizobox.

	Original		Bleach correction		Mask image			Region of interest		
Position	F0 (0-1081)	F1 (0-1081)	F0'	F1'	F1'-F0'	Median	Mask	|F1'-F0'|	Results (|F1'-F0'| AND Mask)	Results 16colors
A										
B										
C										
D										
E										
F										
G										

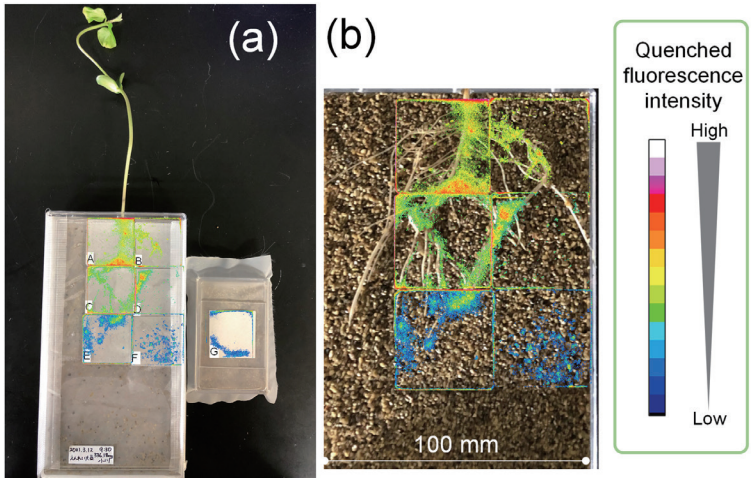


Fig. 3. (Color online) Analyzed fluorescence images on simplified rhizobox. (a) Figure 2(c) with horizontally flipped and superimposed pseudocolor-processed quenched fluorescence images. (b) Soybean root in rhizobox without nylon mesh with horizontally flipped and superimposed pseudocolor-processed quenched fluorescence images.

with soybean root exudates, and the degree of quenching intensity was correlated with the amounts of the six types of soybean isoflavone.[17] In that study, the sheets with immobilized BSA were in direct contact with the roots; however, in this study, the sheet was intermediated by a nylon mesh. Therefore, root exudates may have passed through the nylon mesh and transferred to the sheets with immobilized BSA. Although the fluorescence intensity of some parts of the roots was not quenched, most of the quenched intensity followed the shape of the main and lateral roots, and the quenching area was 2–3 mm from the roots and larger than the root thickness. In this study, the simplified rhizobox was kept face down to transfer secretions onto the sheets with immobilized BSA, which is different from the actual environment, but the results are generally consistent with those of the diffusion of metabolites and enzymes in the rhizosphere.[20]

On the other hand, weak fluorescence intensity quenching was observed in the rootless F and control G areas. In preliminary experiments, it was observed that when a 50 μm nylon mesh was used, vermiculite particles passed through and blocked the fluorescence. Therefore, it is possible that in this case, vermiculite particles smaller than 1 μm passed through and blocked the excitation light or fluorescence. However, the quenched intensity near the soybean root at positions A–D was medium to high and clearly quenched, indicating that the effect of the sub-micrometer vermiculite particles was not significant and that the distribution of soybean isoflavones could be visualized.

4. Conclusions

In this study, we proposed a visualization method for soybean isoflavone distribution in a simplified rhizobox using sheets with immobilized BSA. A nylon mesh (1 μm openings) was

used to transfer soybean root exudates from transplanted soybeans to the rhizobox and onto sheets with immobilized BSA. As a result, fluorescence quenching by soybean isoflavone could be observed within a few millimeters from the roots. In the future, this method can be applied to the visualization of the distribution of isoflavones diffusing from soybean roots in the rhizobox to the soil separated by the nylon mesh with respect to the distance from the root. To develop the quantitative measurement of isoflavone in the future, the selectivity of the isoflavone in the sheet should be further improved and excitation light irradiation should be considered uniformly.

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