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Onodera, Takeshi
Faculty of Information Science and Electrical Engineering, Kyushu University

Miyazaki, Haruna
Graduate School of Information Science and Electrical Engineering, Kyushu University

Li, Xinzhu
Graduate School of Information Science and Electrical Engineering, Kyushu University

Wang, Jin
Graduate School of Interdisciplinary Science and Engineering in Health Systems, Okayama University

他

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Development of Two-dimensional Qualitative Visualization Method for Isoflavones Secreted from Soybean Roots Using Sheets with Immobilized Bovine Serum Albumin

Takeshi Onodera[a]*, Haruna Miyazaki[b], Xinzhu Li[b], Jin Wang[c], Masaru Nakayasu[d], Rui Yatabe[a], Yusuke Tahara[e], Ai Hosoki[f], Nozomu Sakurai[f], Akifumi Sugiyama[d]

[a]Faculty of Information Science and Electrical Engineering, Kyushu University, 744 Motooka, Fukuoka 819-0395, Japan

[b]Graduate School of Information Science and Electrical Engineering, Kyushu University, 744 Motooka, Fukuoka 819-0395, Japan

[c]Graduate School of Interdisciplinary Science and Engineering in Health Systems, Okayama University, 3-1-1 Tsushimanaka, Kitaku, Okayama 700-8530, Japan

[d]Research Institute for Sustainable Humanosphere (RISH), Kyoto University, Uji, Kyoto 611-0011, Japan

[e]Faculty of Textile Science and Technology, Shinshu University, 3-15-1 Tokida, Ueda City, Nagano 386-8567, Japan

[f]Bioinformation and DDBJ Center, National Institute of Genetics, 1111 Yata, Mishima, Shizuoka 411-8540, Japan

*Corresponding author. 744-W2-457, Motooka, Fukuoka 819-0395, Japan

E-mail address: onodera@ed.kyushu-u.ac.jp (T. Onodera)

Abstract

A visualization method for the qualitative evaluation of soybean isoflavones secreted from soybean roots by transferring them onto a sheet with immobilized bovine serum albumin (BSA) was developed. BSA was chemically bonded onto a glass microfiber filter. The fluorescence quenching resulting from the interaction of BSA with soybean isoflavones such as daidzein and daidzin was utilized. Fluorescence images before and after soybean roots were placed in contact with the sheets with immobilized BSA were taken with an electron-multiplying charge-coupled device camera. The fluorescence quenching in the images was visualized and analyzed. Soybean isoflavones were extracted from the sheets for quantitative analysis, and the correlation coefficient between the quenched fluorescence intensity per sheet and the total amount of soybean isoflavones was 0.78 ($p<0.01$), indicating a high correlation. The quenched fluorescence intensity was lower in pumpkin roots, which do not secrete soybean isoflavone. It was found from analyzed images that soybean isoflavone is secreted in larger amounts from the basal region of the taproot and the tips of the lateral roots of soybean.

Keywords: bovine serum albumin, isoflavone, soybeans, fluorescence quenching, imaging, rhizosphere chemical

1. Introduction

Plants are in constant interaction with a myriad of soil microorganisms in an area of soil close to roots, which is called the rhizosphere. The rhizosphere harbors a greater abundance of microorganisms than the soil away from plant roots, owing to the continuous supply of nutrients from plant roots (Badri and Vivanco 2009; Hartmann et al. 2008) . Several microorganisms in the rhizosphere have been shown to improve plant growth by enhancing tolerance to various stresses and providing mineral nutrients to plants; thus, understanding the molecular mechanisms underlying these interactions is of particular importance to establish sustainable agriculture utilizing the functions of soil microorganisms (Compant et al. 2019; de la Fuente Cantó et al. 2020). Recently, root-derived metabolites, especially those belonging to plant specialized metabolites (PSMs), have been shown to affect the abundance and functions of soil microorganisms (Jacoby et al. 2020; Pascale et al. 2020) .

Globally, soybean is the fourth most produced crop after corn, rice, and wheat. Soybean is used in various foods. Forty percent of dried soybean is protein, and it is also rich in PSMs such as isoflavones and soyasaponins, which have various bioactive and pharmaceutical effects on humans, including antioxidative, anti-inflammatory, and anticancer effects (Chen et al. 2018; Górniak et al. 2019; Sparg et al. 2004) . Therefore, soybean is one of the most important crops that support a healthy life. The soybean plant establishes symbiotic interactions with soil bacteria called rhizobia, which form nodules on soybean roots and fix atmospheric nitrogen through nitrogenase to supply nitrogen to the plant. Daidzein, the most abundant isoflavone secreted through soybean roots, induces the expression of the nodulation (*nod*) gene, which initiates the formation of root nodules (Kosslak et al. 1987), and also determines the composition of the rhizospheric microbiota (Okutani et al. 2020). Although genes involved in root exudation of daidzein remain unknown, it has been shown that the amount of daidzein secreted varies depending on the nutritional

conditions and growth stages: the amount of daidzein secreted increases more than 10-fold under nitrogen-deficient conditions, which induces the initiation of soybean nodulation, and daidzein is mainly secreted during the vegetative stage (Sugiyama et al. 2016; Toyofuku et al. 2021). The distribution of daidzein was determined by simulation to be within a few millimeters from the root surface (Okutani et al. 2020), but the variation in the amount of daidzein secreted depending on the area of roots has not been clarified thus far. Clarification of the amount secreted and the area of daidzein secretion from soybean roots is essential to further utilize rhizospheric microorganisms in crop production; however, no technique to visualize the secretion and diffusion of daidzein in the rhizosphere has yet been established (Sugiyama 2021).

Examples of methods of visualizing substances secreted from plant roots are as follows: visualization of oxygen and carbon dioxide using a sensor probe called an optode and a foil coated with a fluorescent reagent (Lenzewski et al. 2018; Tschiersch et al. 2012; Tschiersch et al. 2011), visualization of oxygen in the rhizosphere by neutron radiography combined with fluorescence staining imaging (Rudolph et al. 2012), zymography using enzymes to visualize phosphorus (Spohn and Kuzyakov 2013), visualization of rhizospheric nutrients by FTIR imaging (Victor et al. 2017), visualization of glucose using glucose oxidase and horseradish peroxidase (Voothuluru et al. 2018), and two-dimensional quantification of inorganic nutrients in rhizoboxes using laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) (Wagner et al. 2020). However, no studies of the visualization of secondary metabolites such as soybean isoflavones have been reported.

Serum albumin can be excited at 285 nm and its fluorescence spectrum peaks at around 340 nm. Daidzein interacts with bovine serum albumin (BSA) and human serum albumin (HSA), and the fluorescence intensity of the tryptophan residue of albumin decreases when the residue binds to daidzein (Bourassa et al. 2010; Mahesha et al. 2006; Song et al. 2016). Serum albumin is a natural protein that functions in the transport and storage of nutritional substances and their metabolites, and medicines. The binding of various ligands to albumin is often regarded to involve nonspecific interactions. Although albumin does not have high selectivity such as antibodies or enzymes, it has “specificity” in its “nonspecific interactions” (Aoki et al. 1984).

In this study, as a preliminary step in observing soybean isoflavone secretion in a growth environment such as a rhizobox, we developed sheets with immobilized BSA for the visualization and qualitative analysis of secretion from soybean roots. The immobilized BSA interacts with soybean isoflavones such as daidzein and then the fluorescence of BSA is quenched. An electron-multiplying charge-coupled device (EM-CCD) camera was used to capture the fluorescence of BSA immobilized in sheets quenched owing to the transfer of chemicals from soybean roots to the sheets with immobilized BSA. Image analysis was carried out to visualize the distribution of the soybean isoflavones secreted. The amount of isoflavones recovered from the sheets with immobilized BSA was quantified, and then the correlation between the quenched fluorescence intensity and the total isoflavone amount per sheets was examined. We also attempted to qualitatively analyze the part of the roots from which isoflavone secretion was high.

2. Methods

2.1. Materials

BSA (Cohn fraction V, pH 7.0) was obtained from FUJIFILM Wako Pure Chemical, Japan. The substrate of the sheets with immobilized BSA was a glass microfiber filter (Glass Microfiber Filter, GF/A, 1.6 μm core size, Whatman). 3-Aminopropyl triethoxysilane (APTES) was purchased from Sigma Aldrich. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from Nacalai Tesque, Japan. Ethanolamine hydrochloride was obtained from GE Healthcare Biosciences. Daidzein, genistein, daidzin, dibutyl phthalate (Tokyo Chemical Industry, Japan), genistin (Nagara Science, Japan), malonyl daidzein, and malonyl genistin (FUJIFILM Wako Pure Chemicals, Japan) were used as isoflavones. All aqueous solutions were prepared using Milli-Q deionized water obtained from a Milli-Q- system (Merck Millipore, Burlington, MA, USA). Soybean seeds (Enrei) were purchased from Tsurushin Seed Co. (Matsumoto, Japan) and pumpkin seeds (Azumaebisu) from Tohoku Seed Co. (Japan).

2.2. Preparation of sheets with immobilized BSA

The glass microfiber filter was cut to a size of about 35 mm × 35 mm, which could be placed on a quartz plate (36 mm × 36 mm) for fluorescence measurement. First, the filter was treated with oxygen plasma for 5 min using an LF vacuum plasma cleaner (CUTE 1MP, Femto Science, Korea). The filter was then reacted with 5 mL of 20% APTES in ethanol solution in a Petri dish with shaking for 1 h. Then, the filter was rinsed with MilliQ water. Solutions of 0.4 M EDC (1 mL) and 0.1 M NHS (1 mL) were prepared and mixed with 3 mL of 1×10^{-4} M BSA solution to make a 5 mL mixture. The mixture was poured on the filter in a Petri dish and then the Petri dish was shaken for 1 h to immobilize BSA on the filter by the amide bonding of the carboxyl groups of BSA with the amino groups of APTES. The filter was then rinsed with MilliQ water, and then the filter was placed in ethanolamine-HCl for 30 min to block residues of the activated carboxyl group. Then, the filter was rinsed again with MilliQ water and dried on a quartz plate in an incubator at 28°C with silica gel until its moisture content reached around 20%, as determined with a moisture checker (SK-940A TYPE1, Sato Keiryoki MFG, Japan).

2.3. Fluorescence intensity distribution imaging

The principle of fluorescence quenching and the measurement of the interaction between BSA and soybean isoflavone using a fluorescence spectrometer are described in Chapter 5 of Supporting Information. Fig. 1 shows the system for imaging and measuring the fluorescence intensity of BSA immobilized in sheets. A xenon light source (LAX-C100, Asahi Spectra, Japan) capable of generating UV light was used for the excitation of BSA. The intensity of the excitation light was set to 15 at the light source setting. A UVB mirror module and a 280 nm bandpass filter (HQB280-UV, Asahi Spectra, Japan) were installed in the light source. A rod lens (RLQ80-1, Asahi Spectra, Japan) was attached to the end of a quartz light guide from the light source for irradiating planar light. The rod lens was equipped with a 280–310 nm shortpass filter (SWX325, Asahi Spectra, Japan) to remove visible light and output planar UV light. A cooled CCD camera for the UV region (BU-66EM-UV, BITRAN, Japan) equipped with a UV lens (FL-BC2528-VGUV, RICOH, Japan) was used. A 340 nm bandpass filter (RRX340, Asahi Spectra, Japan) was attached to the lens to remove the reflected excitation light and capture the fluorescence of BSA. The rod lens of the light source, the camera, and the stage for placing the sheets with immobilized BSA were installed in a tabletop darkroom. Fluorescence images were captured using the software supplied with the camera (BU-60 series camera).

The images were taken at an exposure of 1 s and a 16-bit gain of 5x with dark correction. The images were converted from the original format to the 8-bit TIFF format using the supplied software.

2.4. Visualization of soybean isoflavone secretion from soybean roots

Vermiculite was placed in 100 mL disposable cups, and soybean seeds were grown for one week at room temperature (25 ℃, 12 h light/dark cycle). Soybean seedlings were then uprooted and the vermiculite brushed off. First, a fluorescence image of the initial state of a sheet with immobilized BSA was taken immediately before placing the soybean roots, and then the roots were placed on the sheet. The lateral roots and the tip of the taproot were first pressed with a glass slide and followed by the main root for 60 min to transfer the secretion from the roots. After removing the roots, another fluorescence image was taken.

Images of the 8-bit TIFF format were processed using the image processing software ImageJ (Fiji). First, since the fluorescence intensity of BSA immobilized in sheets decreases with time, bleach correction was performed on the image before root contact F0 and after root contact F1. The bleach correction was carried out by exponential fitting, and in case of error, histogram matching was performed. F0 after bleach correction was defined as F0' and F1 after bleach correction as F1'. The difference image |F1'-F0'| and the subtraction image F1'-F0' were obtained from F0' and F1'.

F1'-F0' was the median filtered to obtain the F1'-F0' median image with noise removed. The F1'-F0'median was binarized to generate a mask image, mask, in which the areas with fluorescence intensity changes are shown in black and the areas without changes are shown in white. The image of the region of interest (ROI), result, was obtained using the AND operation of mask and |F1'-F0'|. The 16 colors in lookup table was applied to the result to obtain result_16colors with 16 pseudocolors. A control experiment was similarly conducted using pumpkin (Azumaebisu). The identification code of sheets with immobilized BSA contains the experimental date and gf (glass microfiber filter) position, for example, 0915 gf1.

2.5. Recovery of isoflavones from sheets with immobilized BSA and their quantitative analysis by LC-MS

After the images were taken, the sheets with immobilized BSA were wrapped in aluminum foil and placed in an aluminum zip bag with an oxygen absorber and 1 g of silica gel, and the bag was filled with nitrogen, sealed, and stored in a refrigerator until analysis. Compounds on the sheets were extracted as previously described by Toyofuku et al. (2021) with several modifications. Compounds adsorbed on the sheets were extracted twice with 1 mL of methanol in a 5-mL plastic tube by shaking on a Rotator RT-50 (TAITEC, Saitama, Japan) for 5 min. One milliliter of the combined extracts was dried, dissolved in 100 µL of methanol, and filtered through a 0.45-µm Minisart RC4 syringe filter (Sartorius, Gottingen, Germany). The filtered samples were subjected to liquid chromatography-mass spectrometry (LC-MS) analysis.

LC-MS analysis was conducted using an Acquity ultrahigh-performance liquid chromatography (UPLC) H-Class/Xevo TQD (Waters, Milford, MA, USA). Samples (2 µL) were injected onto an Acquity UPLC BEH C18 column (1.7 µm, 2.1 × 50 mm; Waters) with a UPLC BEH C18 VanGuard precolumn (1.7 µm, 2.1 × 5 mm) at 40 °C. The mobile phases were water containing 0.1% (v/v) formic acid (solvent A) and acetonitrile (solvent B). The elution program was as follows: 10–90% B from 0–10 min (linear gradient), 90% B from 10–12 min, 100% B from

12–16 min, and 10% B from 16–21 min. The flow rate was 0.2 mL min^{-1}. Mass spectra were obtained in the positive electrospray ionization mode under the following conditions: cone voltage, 30 V; collision energy, 30 eV; capillary voltage, 3.15 kV; source temperature, 150 °C; desolvation gas temperature, 400 °C; nebulizer and desolvation N_2 gas flow rates, 50 and 800 L h^{-1}, respectively. Isoflavones in samples were detected in multiple reaction monitoring modes (MRMs), as described in a previous study (Matsuda et al. 2020), with modifications of the MRM transitions as follows: *m/z* 254.7 > 136.43 for daidzein, *m/z* 270.7 > 152.5 for genistein, *m/z* 416.8 > 254.7 for daidzein, *m/z* 432.9 > 270.7 for genistin, *m/z* 502.8 > 254.7 for malonyldaidzin, and *m/z* 518.9 > 270.6 for malonylgenistin. Data were analyzed using MassLynx v. 4.1 software (Waters). The amounts of isoflavones were calculated from peak areas using calibration curves constructed using authentic compounds.

3. Results and discussion

3.1. Preparation of sheets with immobilized BSA

Fig. S1 shows the process of preparing a sheet with immobilized BSA. BSA was chemically bonded on a glass microfiber filter via APTES by amide bonding (Hermanson 2008; Wang et al. 2017). The sheet was irradiated with xenon light at 280 nm, which is the excitation wavelength of BSA, and the fluorescence at around 340 nm was observed using an EM-CCD camera. The original monochrome images were converted to the TIFF format and to 16-pseudocolor images using ImageJ without further image processing. The result is shown in Fig. 2(a). Areas with low fluorescence intensity are shown in cold colors, and areas with high fluorescence intensity are shown in warm colors. Soybean roots were placed on sheets with chemically bonded BSA as shown in Fig. 2(b). A glass slide was placed on top of the sheet and lightly pressed to ensure that the roots were sufficiently in contact with the sheet. As shown in Fig. 2(c), there are regions where the fluorescence intensity is low and the region shown in cold colors is root-shaped, suggesting that the fluorescence of the tryptophan residue of BSA was quenched owing to the interaction of the residue with root exudates. Similar results were obtained from both samples 1 and 2, as shown in Fig. 2.

The chemical immobilization of BSA on the glass microfiber filter is essential for the visualization of fluorescence quenching. As shown in Figs. S2(a)–(c), when BSA was physically adsorbed on the glass microfiber filter, the fluorescence intensity decreased in the contact areas of cotton threads soaked in daidzein solution and water. In the case of physical adsorption, BSA was not completely fixed to the glass microfiber filter; and it is possible that the BSA adsorbed to the cotton thread and then desorbed from the filter, or that the BSA diffused with the water molecules that migrated from the cotton thread. On the other hand, as shown in Figs. S2(d)–(f), no such phenomenon was observed in the glass microfiber filter with chemically bonded BSA.

After the fluorescence images of BSA immobilized in sheets were taken, to estimate daidzein concentration equivalent to the concentration of daidzein secreted, 1×10^{-2} M, 1×10^{-3} M, and 1×10^{-4} M daidzein solutions were dropped onto the right side of the sheets. The rate of diffusion of each solution was not constant, and the fluorescence of BSA immobilized in sheets was not quenched to the same degree depending on the ease of diffusion of the solutions. BSA quenches fluorescence upon its interaction with flavonoids and polyphenols (Bourassa et al. 2010; Papadopoulou et al. 2005). Therefore, BSA does not have high selectivity to daidzein, and it is assumed that it also

interacted with isoflavones and analogues other than daidzein. However, in terms of daidzein concentration, other compounds with concentrations equivalent to 10^{-3} to 10^{-2} M may also be secreted.

3.2. Analysis of fluorescence quenching by spotting of daidzein solution

Figs. 3(a)–(c) show the images of fluorescence quenching after dropping 15 µL each of daidzein solutions with concentrations of 1×10^{-2} M, 1×10^{-3} M, and 1×10^{-4} M onto two sheets with immobilized BSA at six locations and analyzed as described in 2.4. Fig. 3(a) shows that the fluorescence of the center of the spot is strongly quenched, probably owing to the high concentration of 1×10^{-2} M daidzein. In Fig. 3(b), compared with Fig. 3(a), the quenching was more uniform but slightly stronger at the center of the spots. In Fig. 3(c), there was a spot in which the concentration of daidzein was low and no clear quenching was obtained. In Fig. 3(d), the average quenched fluorescence intensity was calculated by multiplying the area (number of pixels) and the brightness of twelve spots of daidzein of the same concentration. Additionally, the results of spots with four different daidzein concentrations are shown in more detail in Fig. S3. The light source intensity is slightly different from Fig. 3 because the xenon lamp was changed. From these results, the quenched fluorescence intensity of increased in a concentration-dependent manner, it can be concluded from the results shown in Fig. 2 that visualization of soybean isoflavone in a two-dimensional distribution is possible.

3.3. Visualization of soybean root exudates using sheets with immobilized BSA

To visualize soybean isoflavone secreted from the roots in more detail, we obtained images before and after placing soybean roots in contact with the sheets with immobilized BSA and formed differential images from these fluorescence images. Fig. 4(a) shows the initial fluorescence images of the sheets with immobilized BSA, Fig. 4(b) shows photographs of soybean roots placed on the sheets, and Fig. 4(c) shows the fluorescence images after the roots came into contact with the sheets. The two dots in the periphery of Figs. 4(a) and (c) are the traces of moisture content measurements.

In Fig. 4(d), the ROI is extracted from the area of quenching by root exudates, and 16 pseudocolors were applied. The details of the image analysis procedure are described in Fig. S4. The ROI displayed fluorescence quenching caused by the interaction of BSA with root exudates, and areas without changes in fluorescence intensity are shown in black (brightness value 0). Thus, warm colors indicate strong quenching in the region. Images of gf2 and gf4 in Fig. 4(d) show that the areas in red and orange are near the root tips, indicating that the fluorescence quenching at root tips is stronger than that in the rest of the roots.

Fig. 5(a) shows a soybean seedling, Fig. 5(b) shows the soybean roots placed in contact with a sheet immobilized BSA, and Fig. 5(c) shows the images of Fig. 4(d) modified with a transparent background (brightness value, 0) superimposed on the image of soybean roots (Fig. 5(b)). Fluorescence was quenched at the contact region of the taproot and lateral roots, especially at the tips of lateral roots. Metabolites are easily secreted from the root tips of plants (Canarini et al. 2019), and in the case of soybean, it is probable that larger amounts of soybean isoflavone are secreted. Reproducible results were obtained from two other soybean samples, although there were individual differences in the growth rate (Fig. S5).

As a control experiment, pumpkin, which does not secrete isoflavones, was analyzed similarly to soybean, and

image analysis was performed. The details of the analysis process are shown in Fig. S6. Fig. 5(d) shows a pumpkin seedling 7 days after sowing. Fig. 5(e) shows the pumpkin roots placed in contact with a sheet with immobilized BSA, and Fig. 5(f) shows 16 pseudocolor images of ROI modified with a transparent background (brightness value, 0) superimposed on the image of pumpkin roots (Fig. 5(e)). As in the case of pumpkin, fluorescence is weakly quenched in the vicinity of contact between the roots and the sheet. The same tendency was observed in other samples, as shown in Fig. S7. As shown in Table S1, measurements of quenching caused by the interaction of isoflavone with BSA also showed that coumarin and lauric acid quenched the fluorescence, although the quenching constant was lower than that of daidzein, suggesting the effect of flavonoids, polyphenols, and fatty acids contained in plants in general (Papadopoulou et al. 2005; Spector and John 1968). Therefore, it is possible that weak quenching was caused by the interaction of pumpkin root exudates with BSA.

3.4. Correlation between the amount of soybean isoflavone extracted from sheets with immobilized BSA and fluorescence quenching intensity

Soybean isoflavones were recovered by solvent extraction from the sheets with immobilized BSA and analyzed as shown in Figs. 4 and S5, and daidzein, genistein, daidzin, genistin, malonyl-daidzin, and malonyl-genistin were quantified by LC-MS. Representative chromatograms are shown in Fig. S8 and the results are shown in Table S2. Among these analytes, daidzein and glycoside daidzin were recovered in the largest amounts, followed by genistin. As shown in Table S1, BSA quenches fluorescence upon its interaction with both daidzein and daidzin, and these substances are likely to be the main cause of quenching. According to previous studies, one-week-old soybean grown in hydroponic solution secreted significantly larger amounts of daidzein per plant than other isoflavones, whereas the amounts of the glucosides such as daidzin were at trace levels (Sugiyama et al. 2016). On the other hand, larger amounts of daidzin were recovered from the sheets than of daidzein in some cases. Daidzin is degraded in soil (Sugiyama et al. 2017); however, on the sheets, daidzin exuded from the roots was not degraded during the measurement time. Daidzin may have acted as a quencher.

The correlation between the total amount of the six soybean isoflavones bound to the sheets and the quenched fluorescence intensity of BSA immobilized in sheets (sum of brightness values of each sheet) was examined. The results are shown in Fig. 6, which shows a correlation coefficient r of 0.781, indicating a strong positive correlation, and the R^2 was 0.61. The results of the t-test (test of no correlation, $p<0.01$) showed a p-value of 0.004545; thus, this correlation was significant. Therefore, the intensity of quenched fluorescence of the ROI in images (and pseudocolor images) of soybean roots placed in contact with the sheets probably reflected the amount of soybean isoflavones secreted. In the case of thick taproots, the contact was not uniform at the basal region of the lateral roots, and the exudates were not transferred well, and quenching did not occur in some regions. Therefore, there is still room for improvement in the method of placing roots on the sheets for better contact. However, the flow of primary metabolites by root exudation is considered to be mostly at the root tips (Canarini et al. 2019), and the results in Figs. 5(c) and S5 suggest that isoflavones are stored and secreted in larger amounts at the root tips, even for secondary metabolites such as soybean isoflavones.

4. Conclusions

To stabilize soybean yield, it is necessary to understand the effect of the concentrations of exudates in the soybean rhizosphere, especially those of soybean isoflavones, on soybean growth. As a first step, in this study, the fluorescence quenching caused by the interaction between BSA and typical soybean isoflavones, daidzein and daizin, was utilized for visualization, and we developed a technique to visualize the distribution of isoflavone secreted from soybean roots using sheets with BSA immobilized on glass microfiber filters. Soybean root exudates were transferred onto the sheets, and fluorescence quenching was visualized. A standard solution of daidzein was dropped onto the sheets with immobilized BSA, and fluorescence quenching was shown to be concentration-dependent Soybean roots were placed in contact with the sheets and root exudates were transferred to the sheets. The total amount of soybean isoflavone extracted from the sheets and the quenched fluorescence intensity of each of the sheets showed a high positive correlation of 0.781 ($p<0.01$). Transcription and image processing of soybean root exudates showed that quenching was strong at the base of the taproot and the tips of the lateral roots, suggesting that large amounts of soybean isoflavone may be secreted in these parts. This study is the first in which the secretory distribution of soybean isoflavone was visualized. Our technique could facilitate the elucidation of rhizospheric compounds for further studies to achieve stable crop production. We plan to improve the loading method for transferring root exudates to sheets with immobilized BSA and to obtain the distribution of isoflavone secretion at different growth temperatures and growth periods. We are also developing a simple and rapid immunosensor for daidzein concentration, which can contribute to yield stabilization in actual fields.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that may influence the work reported in this paper.

Credit authorship statement

Takeshi Onodera: Conceptualization, Methodology, Formal analysis, Supervision, Writing - Original Draft, Writing - Review and Editing
Haruna Miyazaki: Methodology, Investigation, Formal analysis, Writing - Original Draft
Xinzhu Li: Methodology, Investigation, Writing - Original Draft
Jin Wang: Methodology, Writing - Review and Editing
Masaru Nakayasu: Investigation, Writing - Original Draft, Writing - Review and Editing
Rui Yatabe: Methodology, Writing - Review and Editing
Yusuke Tahara: Methodology, Writing - Review and Editing
Ai Hosoki: Methodology, Writing - Review and Editing
Nozomu Sakurai: Conceptualization, Writing - Review and Editing
Akifumi Sugiyama: Conceptualization, Resources, Funding acquisition, Supervision, Writing - Review and Editing

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Figure captions

Fig.1. Fluorescence measurement system.

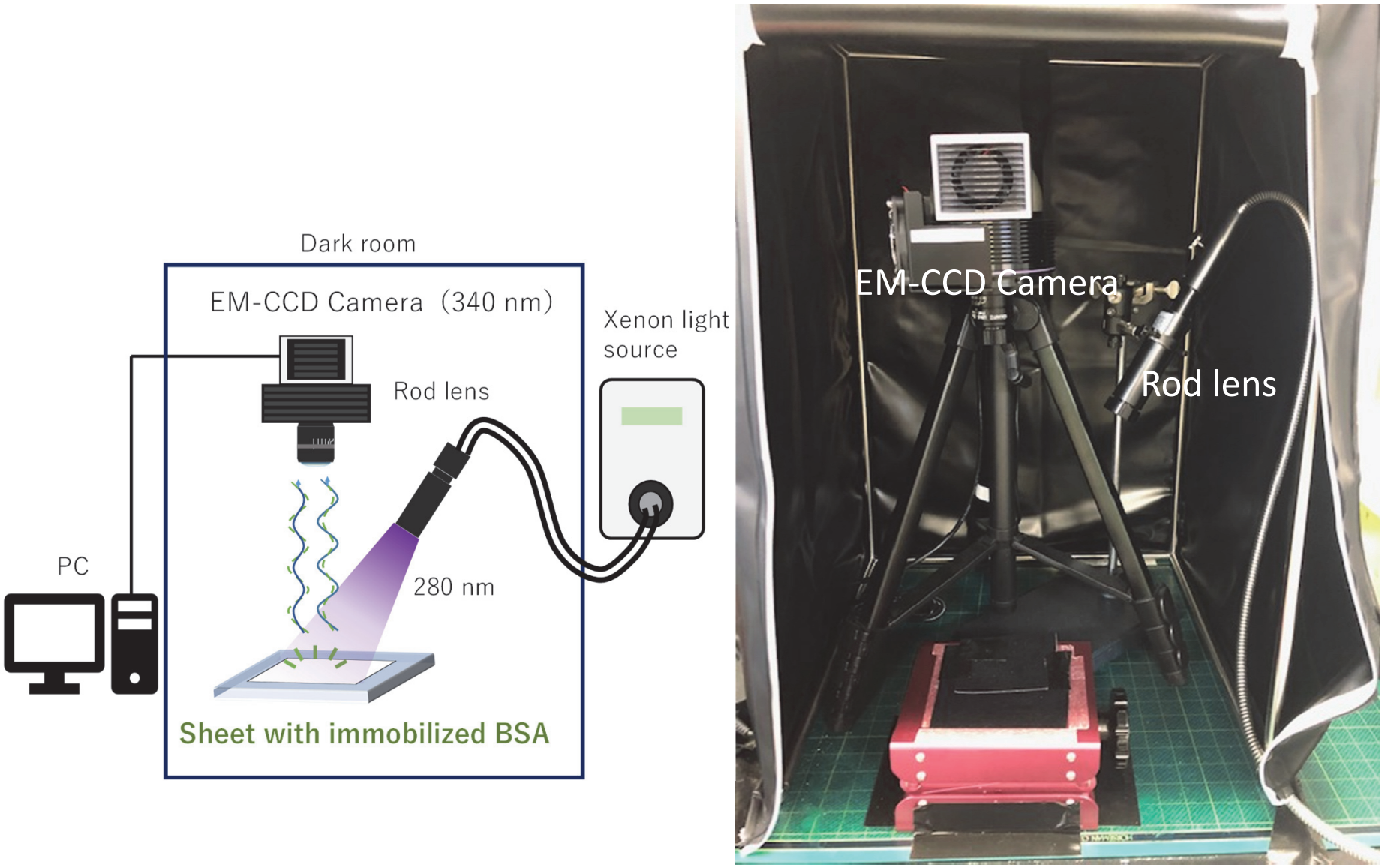
Fig. 2. Two-dimensional images of root exudates transferred from soybean roots grown for 7 days. (a) Before roots come into contact with sheet with immobilized BSA, (b) during transfer of root exudates, (c) after roots come into contact with the sheet, (d) daidzein solution spotted in (c).

Fig. 3. Daidzein solution spotted on sheets with immobilized BSA. (a) 1×10^{-2} M, (b) 1×10^{-3} M, (c) 1×10^{-4} M, (d) quenched fluorescence intensity vs daidzein concentration.

Fig. 4. Fluorescence images of sheets with immobilized BSA. (a) Before placing soybean roots, (b) during transfer of root exudates from soybean roots, (c) after placing soybean root, (d) pseudocolors applied to analyzed images from (a) and (c).

Fig. 5. Transferring root exudates from roots to sheets with immobilized BSA. (a) soybean seedling, 7 days after sowing, (b) during transfer of root exudates from soybean roots to the sheet, (c) (b) with superimposed pseudocolor-processed quenched fluorescence images, (d) pumpkin seedling 7 days after sowing, (e) during transfer of root exudates from pumpkin roots to the sheet, (f) (e) with superimposed pseudocolor-processed quenched fluorescence images.

Fig. 6. Total amount of isoflavones recovered from sheets with immobilized BSA vs quenched fluorescence intensity of sheets.



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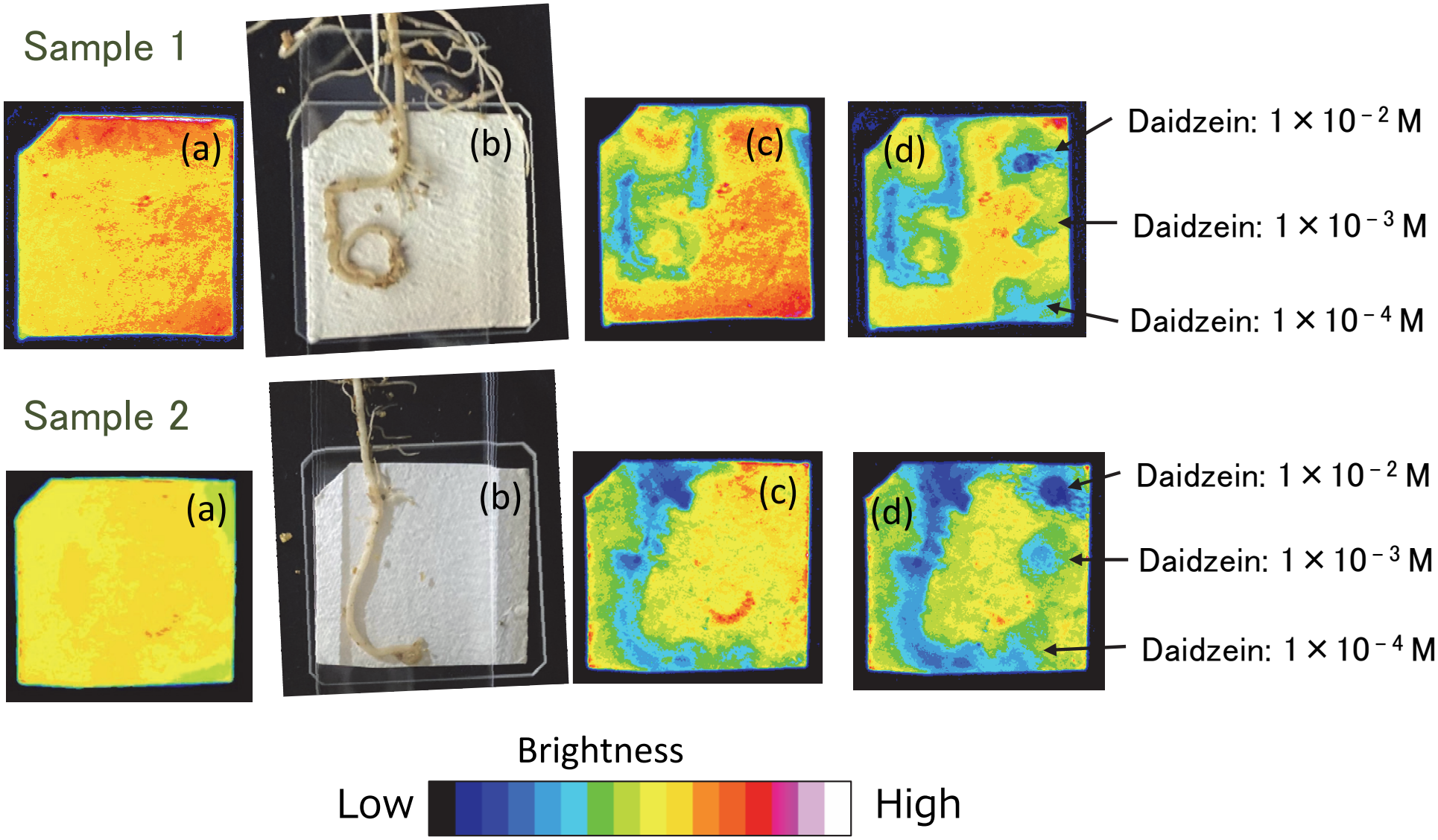

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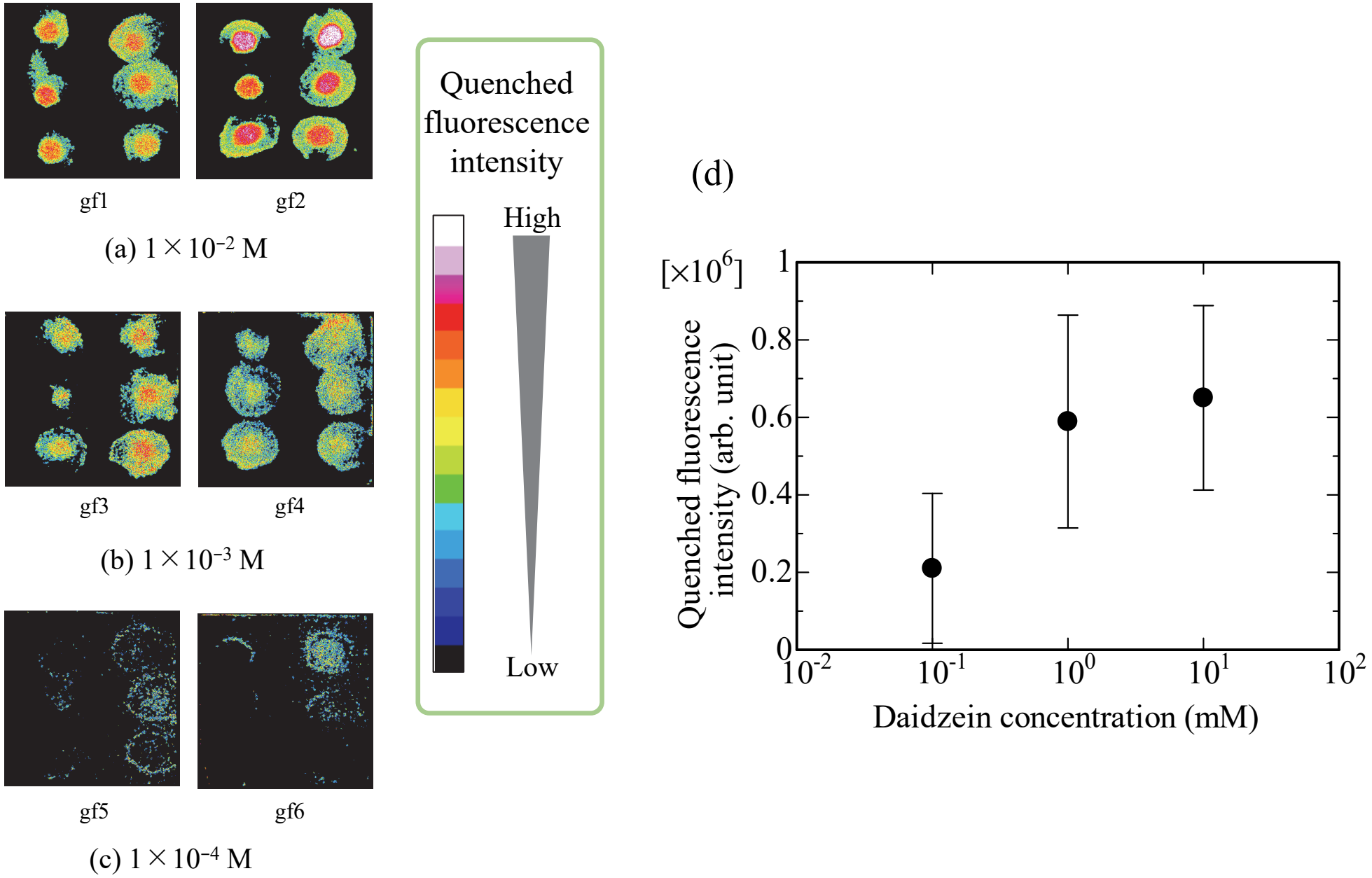

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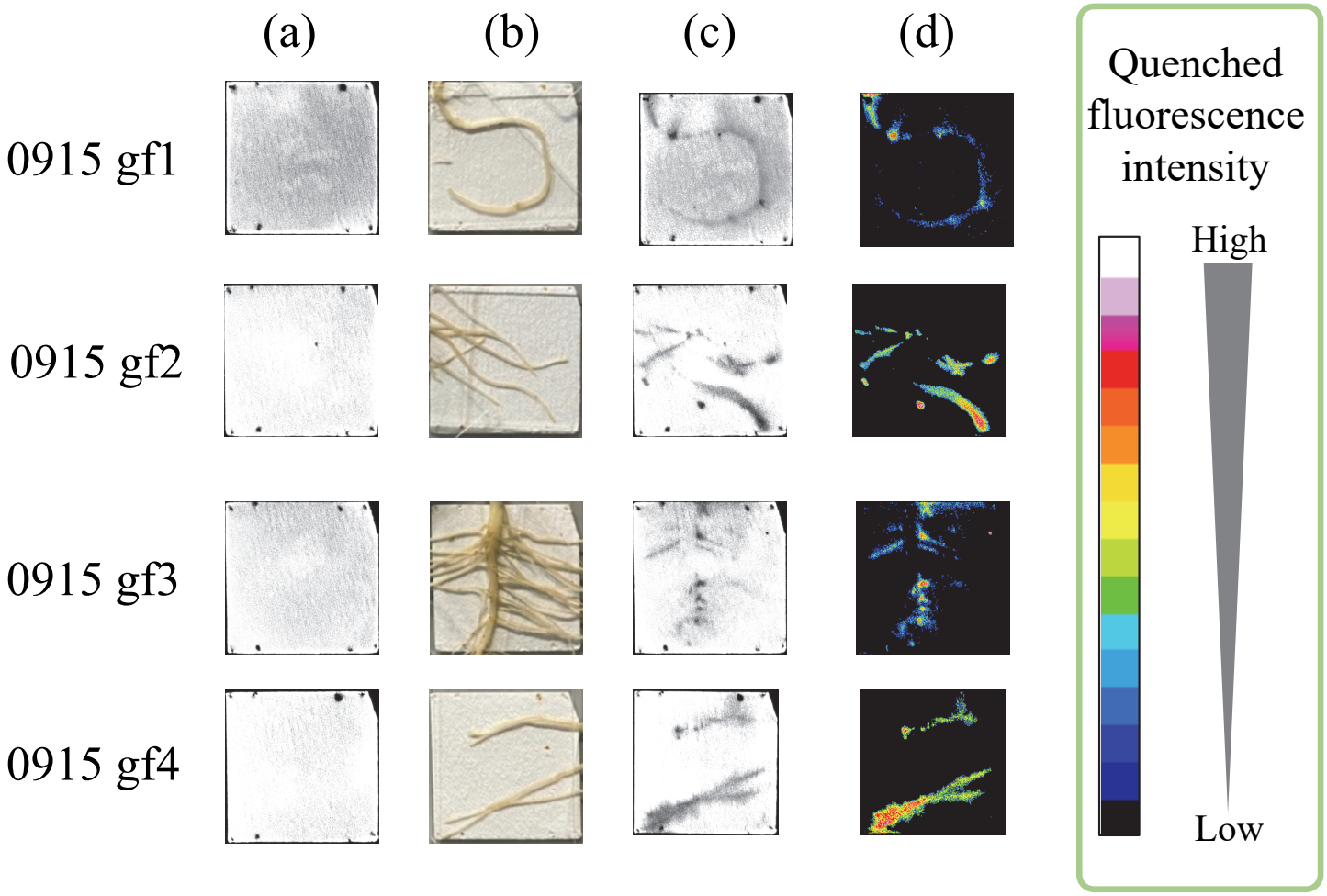

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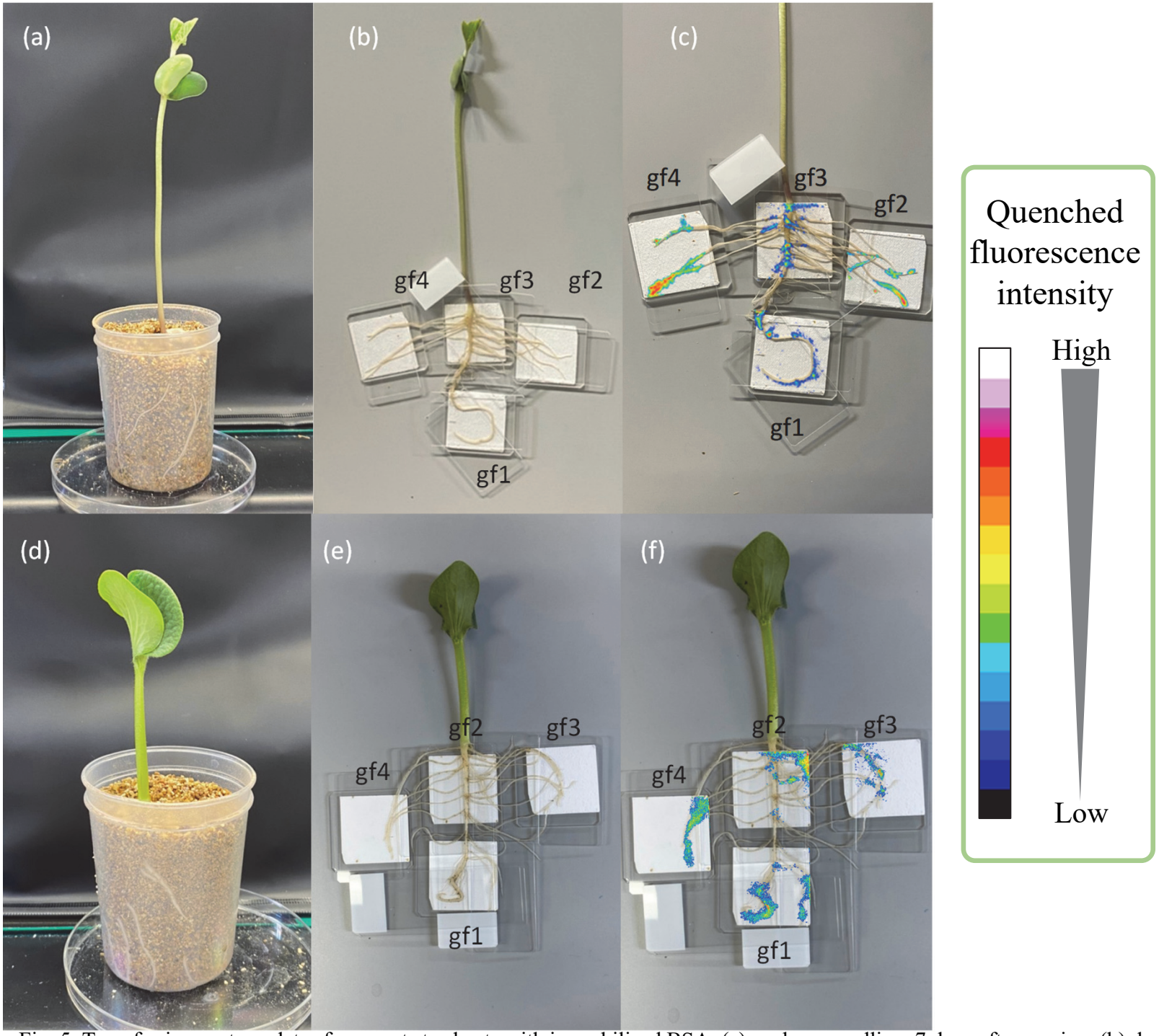

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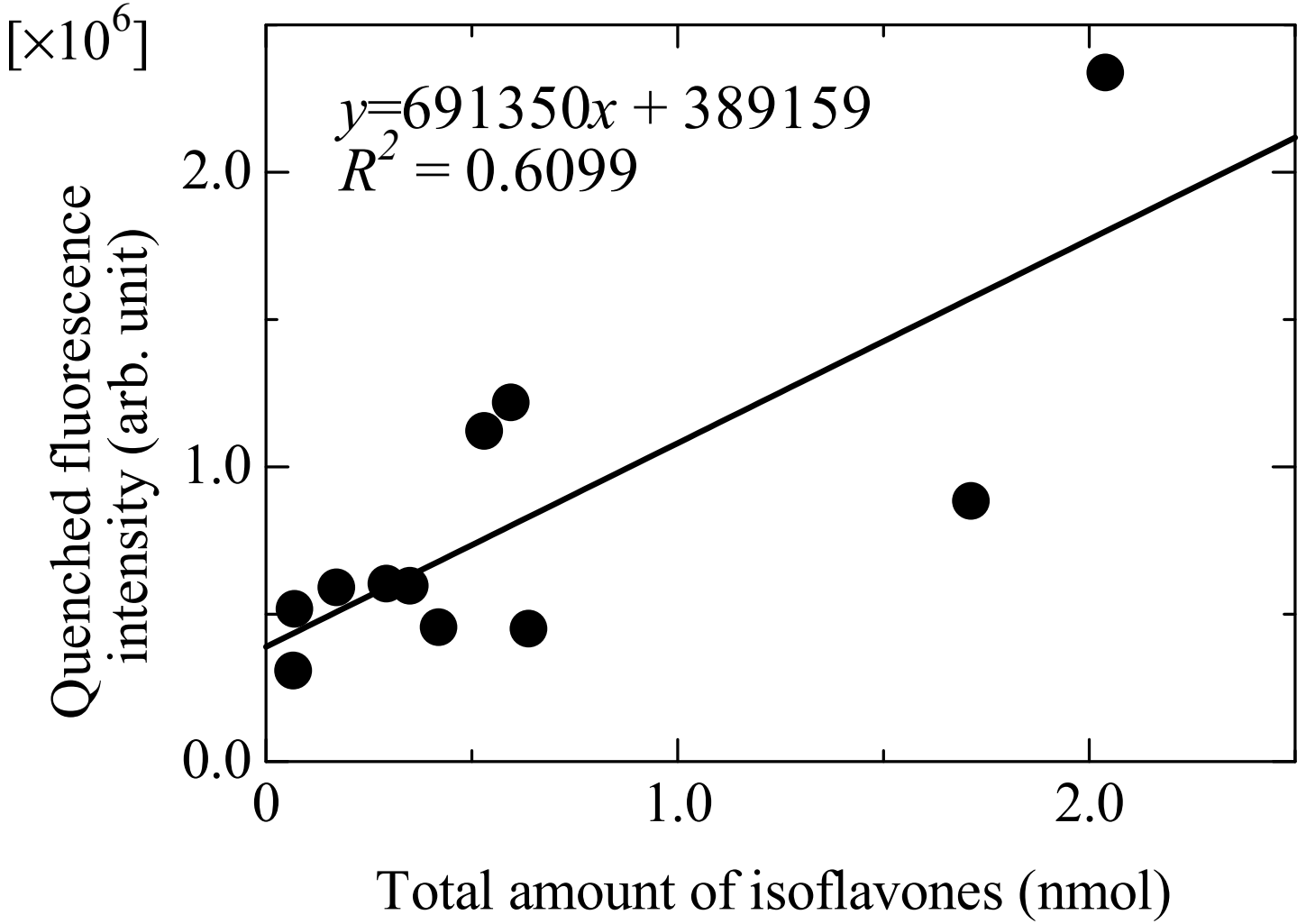

Fig. 6. Total amount of isoflavones recovered from sheets with immobilized BSA vs quenched fluorescence intensity of sheets.

Electronic Supplementary Information for Biosensors and Bioelectronics

Development of two-dimensional qualitative visualization method for isoflavones secreted from soybean roots using sheets with immobilized bovine serum albumin

Takeshi Onodera[a]*, Haruna Miyazaki[b], Xinzhu Li[b], Jin Wang[c], Masaru Nakayasu[d], Rui Yatabe[a], Yusuke Tahara[e], Ai Hosoki[f], Nozomu Sakurai[f], Akifumi Sugiyama[d]

[a]Faculty of Information Science and Electrical Engineering, Kyushu University, 744 Motooka, Fukuoka 819-0395, Japan

[b]Graduate School of Information Science and Electrical Engineering, Kyushu University, 744 Motooka, Fukuoka 819-0395, Japan

[c]Graduate School of Interdisciplinary Science and Engineering in Health Systems, Okayama University, 3-1-1 Tsushimanaka, Kitaku, Okayama 700-8530, Japan

[d]Research Institute for Sustainable Humanosphere (RISH), Kyoto University, Uji, Kyoto 611-0011, Japan

[e]Faculty of Textile Science and Technology, Shinshu University, 3-15-1 Tokida, Ueda City, Nagano 386-8567, Japan

[f]Bioinformation and DDBJ Center, National Institute of Genetics, 1111 Yata, Mishima, Shizuoka 411-8540, Japan

*Corresponding author. 744-W2-457, Motooka, Fukuoka 819-0395, Japan

E-mail address: onodera@ed.kyushu-u.ac.jp (T. Onodera)

1. Measurement of interaction between BSA and plant-related substances such as isoflavones by fluorescence spectrophotometry

Daidzein, genistein, daidzin, dibutyl phthalate, coumarin (Tokyo Chemical Industry), genistin (Nagara Science), malonyldaidzin, malonylgenistin, lauric acid, soyasaponin, campher (FUJIFILM Wako Pure Chemicals), and dimethyl sulfoxide (DMSO) (FUJIFILM Wako Pure Chemical Industries) were used. Daidzein, genistein, daidzin, genistin, malonyl daidzein, and malonyl genistin were used as soybean isoflavones, and dibutyl phthalate, lauric acid, soyasaponin, and coumarin were used as other plant-related substances (Massalha et al. 2017) to measure their interactions with BSA. The results of this study were as follows.

The experiments were performed using a fluorescence spectrophotometer (SHIMADZU RF-6000) at room temperature of 25 °C. A BSA solution shows the maximum fluorescence intensity at 340 nm when excited by UV light at 285 nm. Therefore, we measured the fluorescence spectra under the following conditions: excitation wavelength, 285 nm; excitation side bandwidth, 3.0 nm; fluorescence side bandwidth, 3.0 nm; and fluorescence wavelength range, 300–450 nm. First, 3 mL of 1×10^{-5} M BSA solution was placed in a quartz cell for fluorescence spectrophotometry, and the fluorescence spectrum was measured at a quencher concentration of 0 M. Next, 15 μL of 0.5×10^{-5} M quencher solution was added, mixed well, and allowed to stand for 3 min to measure the fluorescence spectrum at a quencher concentration of 0.5×10^{-5} M. The above process of dropping the quencher solution and measuring the fluorescence spectrum was repeated seven times until the quencher concentration reached 3.5×10^{-5} M.

Using the obtained data, we calculated the quenching constant $k_{\mathrm{SV}}[\mathrm{M}^{-1}]$, and quenching rate constant $k_{\mathrm{q}}[\mathrm{M}^{-1}\cdot\mathrm{s}^{-1}]$ as follows (Shang and Li 2010):

$$\frac{F}{F_0} = 1 + k_{\mathrm{SV}}[quencher] \qquad \text{(S1)}$$

$$k_{\mathrm{q}} = \frac{k_{\mathrm{SV}}}{\tau_0} \quad , \qquad \text{(S2)}$$

where F_0 and F are the fluorescence intensities at 340 nm in the absence and presence of the quencher, respectively, [quencher] is the concentration of the quencher, and τ_0 is the lifetime of the phosphor in the absence of the quencher ($\tau_0=10^{-8}$ s). From equation (S1), k_{SV} was calculated from the curve of $\frac{F}{F_0}$ versus [quencher] (Stern–Volmer curve). The results are shown in Table S1.

Table S1. Quenching constant and quenching rate constant of BSA for isoflavones and plant-related substances.

Compound	**Stern—Volmer quenching constant k_{SV} [M^{-1}]**	**Quenching rate constant k_q [$M^{-1} \cdot s^{-1}$]**
Daidzein	8.72×10^{4}	8.72×10^{12}
Genistein	1.09×10^{5}	1.09×10^{13}
Daidzin	1.78×10^{4}	1.78×10^{12}
Genistin	4.95×10^{4}	4.95×10^{12}
Malonyldaidzin	3.64×10^{4}	3.64×10^{12}
Malonylgenistin	2.24×10^{4}	2.24×10^{12}
Dibutyl phthalate	4.06×10^{4}	4.06×10^{12}
Lauric acid	1.13×10^{4}	1.13×10^{12}
Camphor	-	-
Coumarin	2.11×10^{4}	2.11×10^{12}
Soyasaponin B	7.87×10^{3}	7.87×10^{11}

2. Preparation of sheets with immobilized BSA

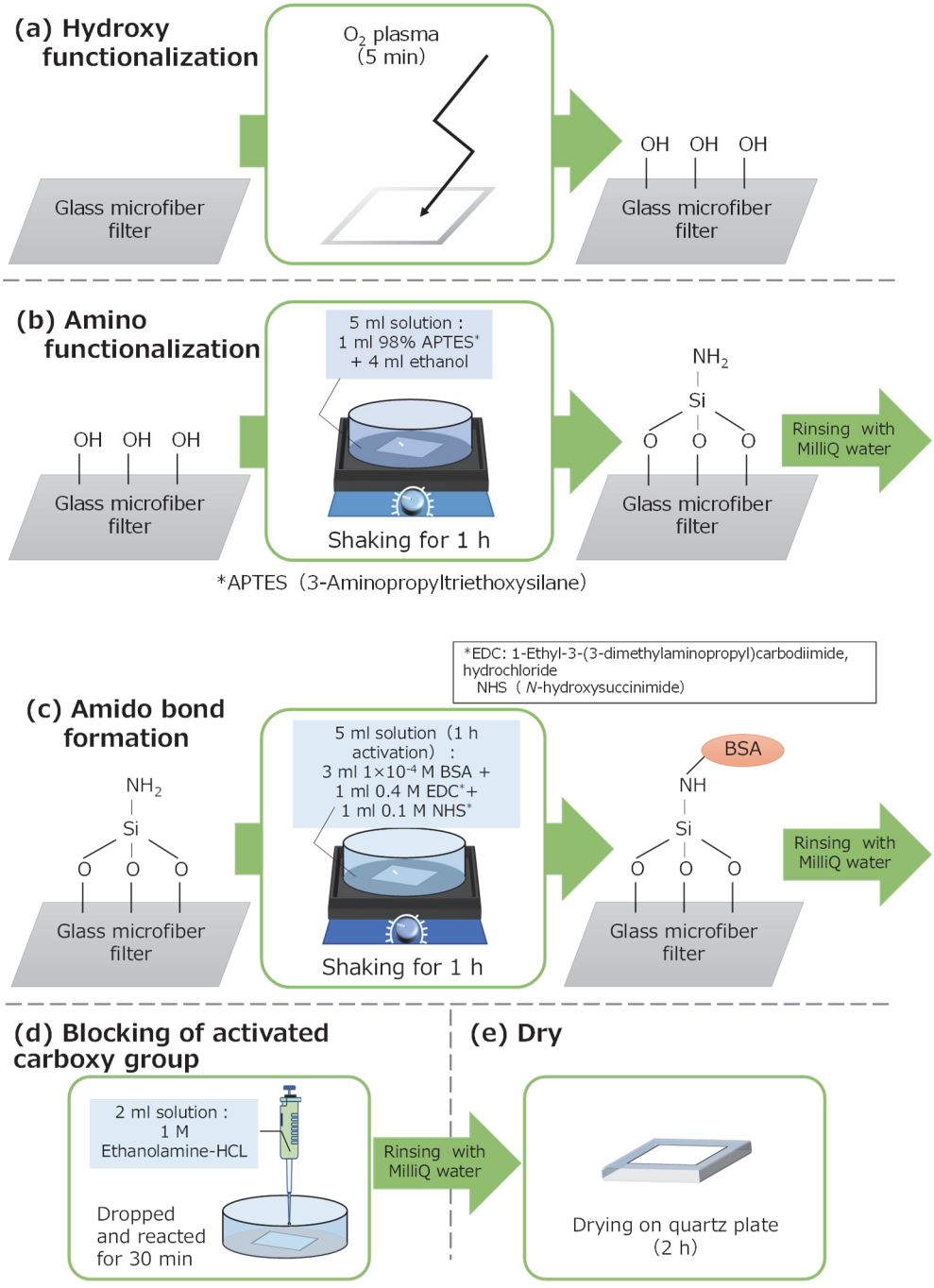

Fig. S1. Fabrication of sheets with immobilized BSA.

3. Comparison of physical adsorption and chemical bonding of BSA immobilized on glass microfiber filter

A glass microfiber filter was cut into pieces (about 35 mm × 35 mm) sufficiently large to be placed on a quartz plate (36 mm × 36 mm) for fluorescence measurement. BSA solution (1×10^{-4} M) was poured into a Petri dish and the filter was immersed in the solution for 10 min, after which the filter was transferred to a quartz plate for dried and fluorescence measurement.

Images of the prepared sheets with BSA immobilized by physical adsorption were taken with an EM-CCD camera. The original black-and-white images were converted to the TIFF format and 16-pseudocolored images using the look up table of ImageJ without further image processing. The result is shown in Fig. S2(a). The center of the sheet is brighter than the edges. It is possible that the BSA accumulated at the edges of the glass microfiber filter during drying. In actual applications, the sheet with immobilized BSA needs to be in contact with the soybean roots. To simulate this, two cotton threads were immersed in 1×10^{-2} M daidzein solution and Milli-Q water for 30 min each. The threads were removed from the solution and water, and after lightly wiping off the solution and water dripping from the thread surface, they were placed in contact with the sheet with BSA immobilized by physical adsorption, as shown in Fig. S2(b). After 30 min, the threads were removed and the image of the sheet was taken. Fig. 2S(c) shows the result after the thread was placed in contact with the sheet. In the case of physical adsorption, the fluorescence intensity decreased at the area where two threads were placed; BSA may have been adsorbed on the area of contact of cotton threads and desorbed from the glass microfiber filter. The periphery with originally low fluorescence intensity (blue or dark blue) showed high intensity (green or light green), suggesting that BSA was spread by the solution and increased the fluorescence intensity at the periphery.

On the other hand, the fluorescence distribution of BSA immobilized in the sheet by chemical bonding in Fig. S2(d) is not completely uniform; however, it is more uniform than that in Fig. S2(a). The sheets with BSA immobilized by chemically bonding were rinsed with Milli-Q water to remove unreacted reagents and nonspecifically adsorbed BSA. To verify this, cotton threads were immersed in Milli-Q water for 30 min as shown in Fig. S2 (e). They were placed in contact with the chemically bonded sheets with immobilized BSA in the same manner as in the case of sheets with BSA immobilized by physically adsorption. After 30 minutes, the thread was removed and the image of the sheet was taken. Fig. S2(f) shows the image of the sheet after the thread had been placed in contact with it. As a result, no quenching was observed in the region where the threads were in contact. Therefore, the sheets with BSA immobilized by chemical bonding were selected to be more suitable than those with physically adsorbed BSA for the detection of isoflavones secreted from soybean roots.

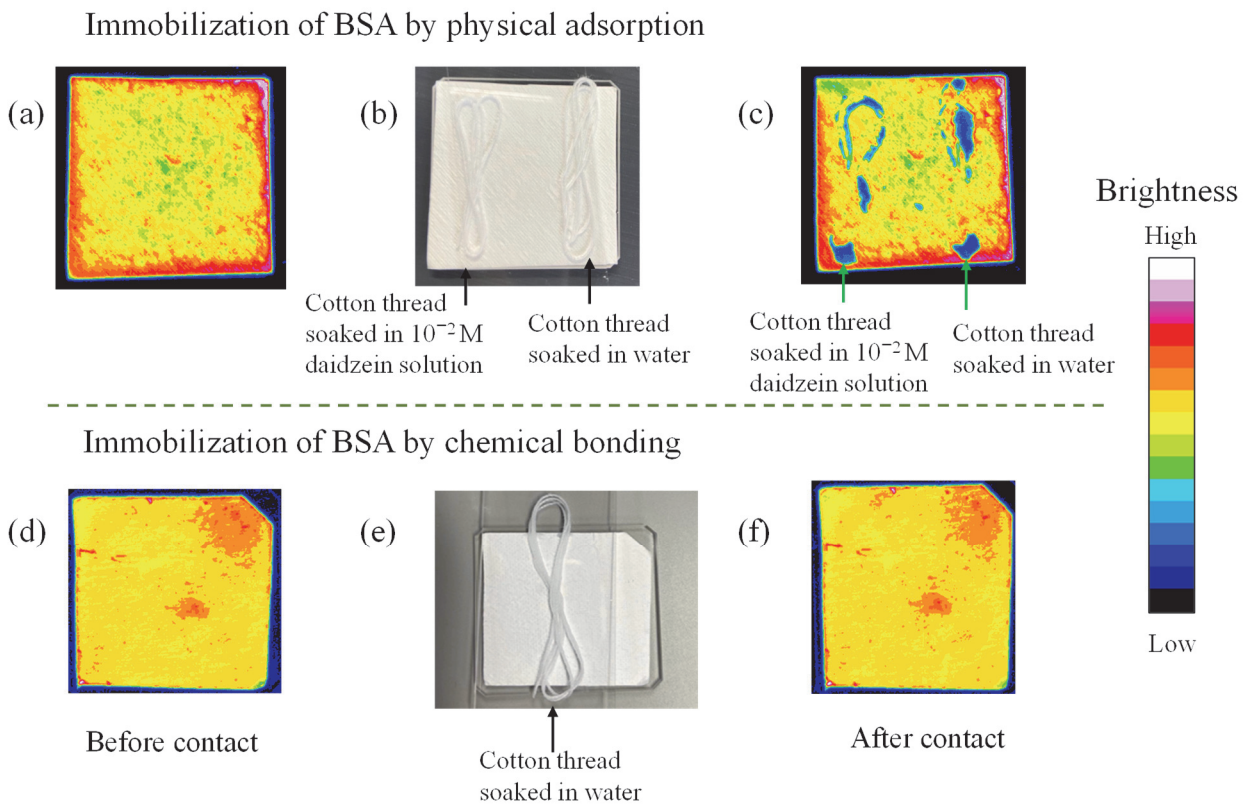

Fig. S2. BSA immobilization by two methods.

4. Analysis of fluorescence quenching by spotting of daidzein solutions with six concentrations

An additional experiment for fluorescence quenching by spotting of daidzein solution was carried out. The measurement conditions are the same as those in 2.3 in the paper except for the intensity of excitation light. The intensity was set to 10.

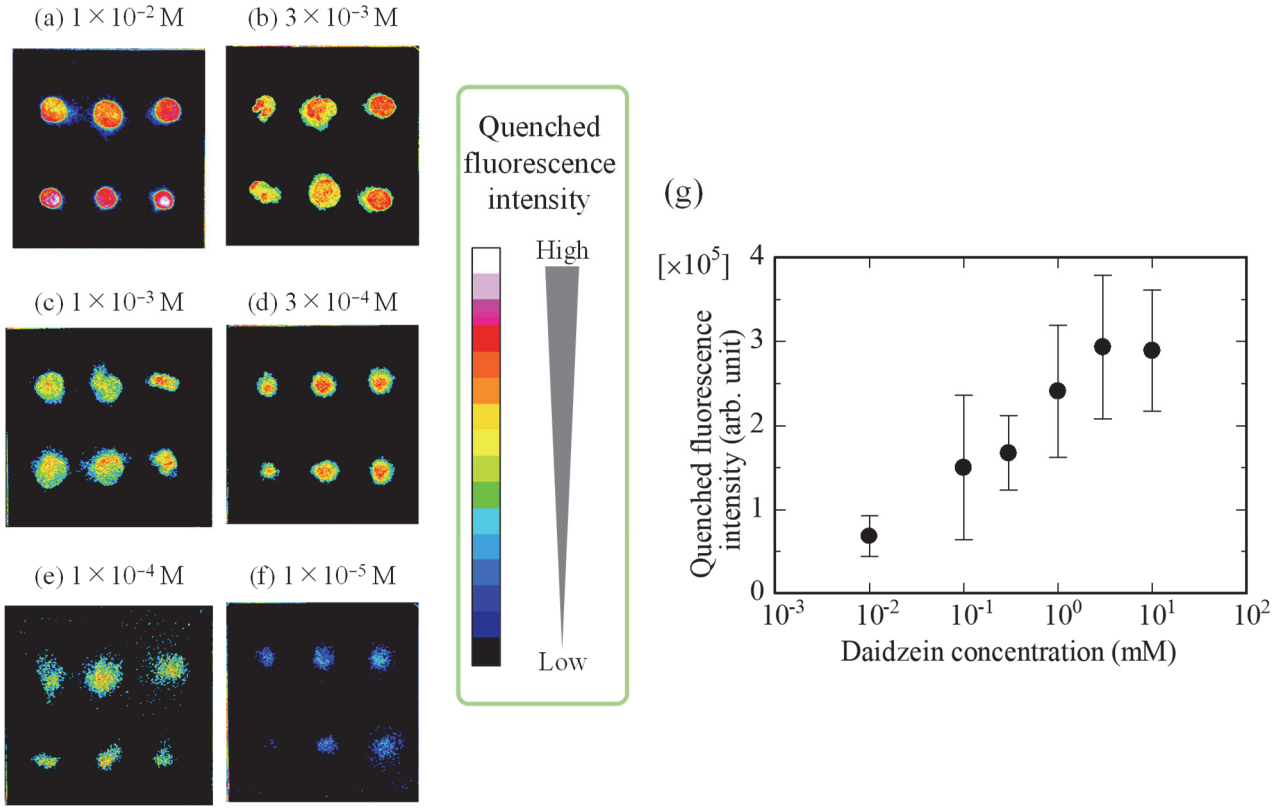

Fig. S3 Daidzein solution spotted on sheets with immobilized BSA. (a) 1×10^{-2} M, (b) 3×10^{-3} M, (c) 1×10^{-3} M, (d) 3×10^{-4} M, (e) 1×10^{-4} M, (f) 1×10^{-5} M, (g) quenched fluorescence intensity vs daidzein concentration.

5. Fluorescence image analysis

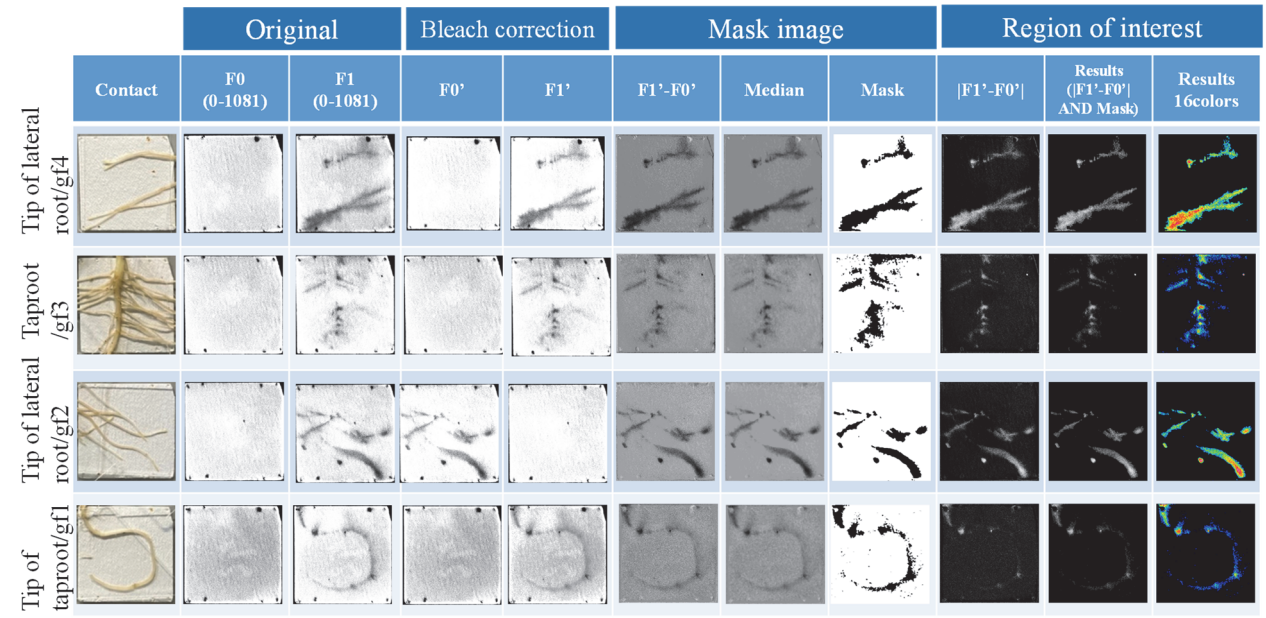

Fig. S4. Analysis of fluorescence images of soybean roots.

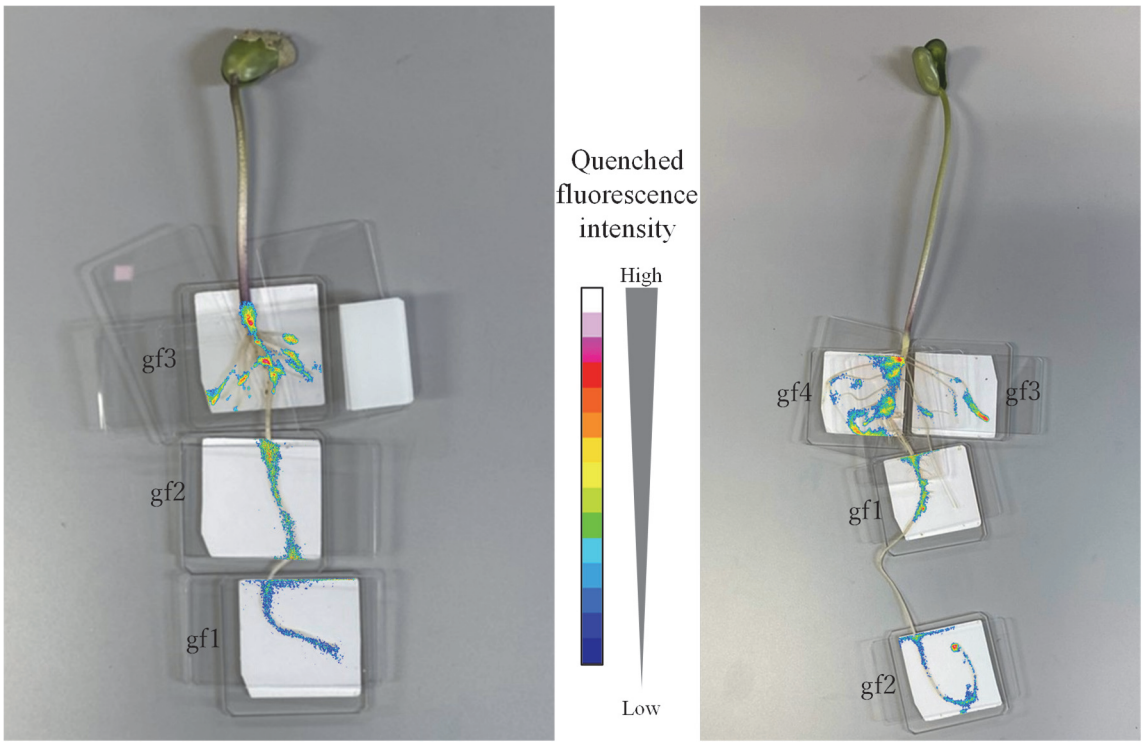

Fig. S5. Analyzed fluorescence images overlayed on photographs of soybean seedings.

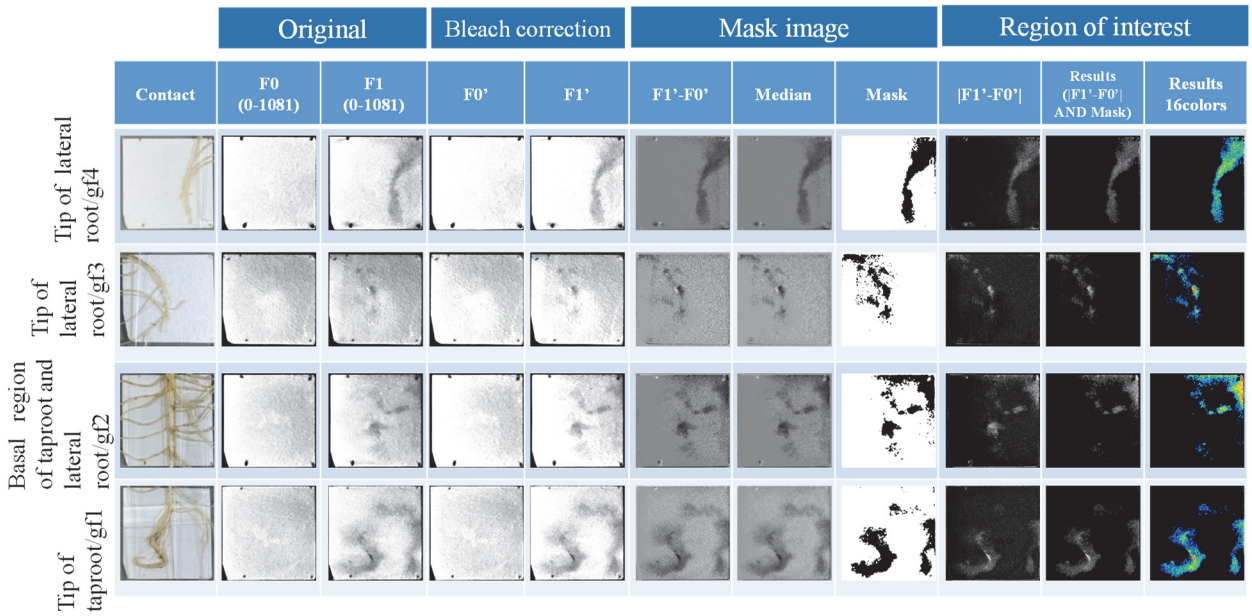

Fig. S6. Analysis of fluorescence images of pumpkin roots.

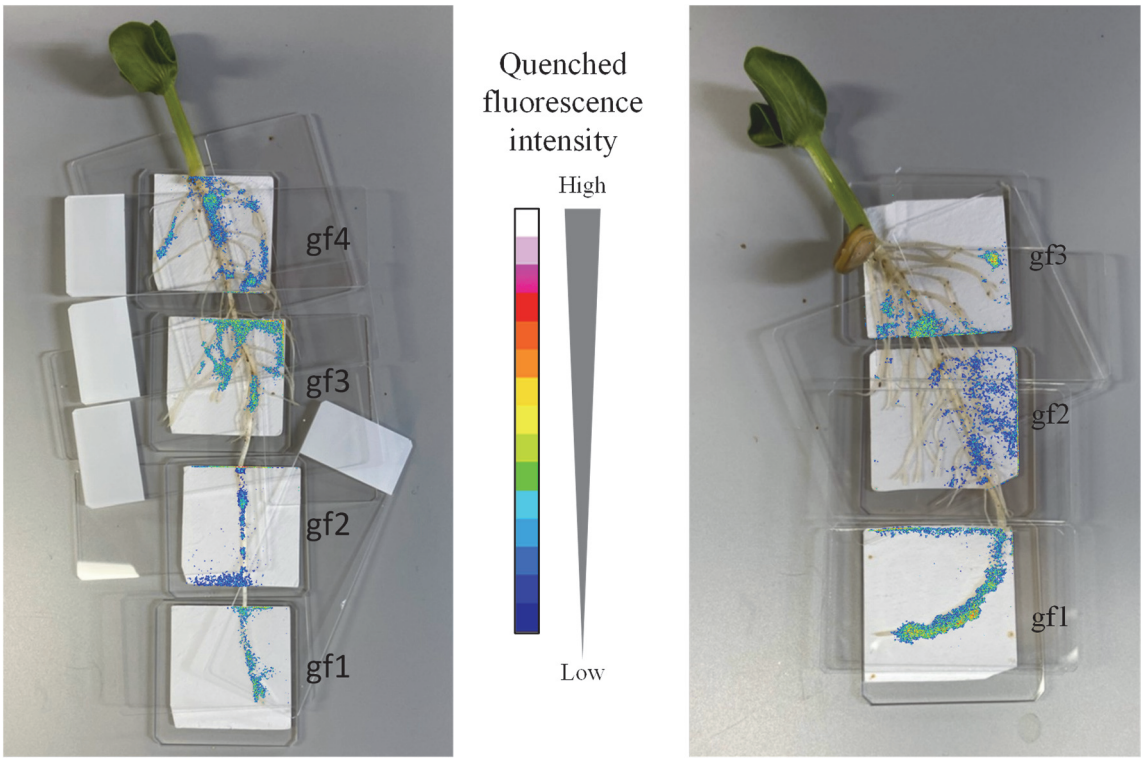

Fig. S7. Analyzed fluorescence images overlayed on photographs of pumpkin seedings.

6. Quantification of isoflavones recovered from sheets with immobilized BSA

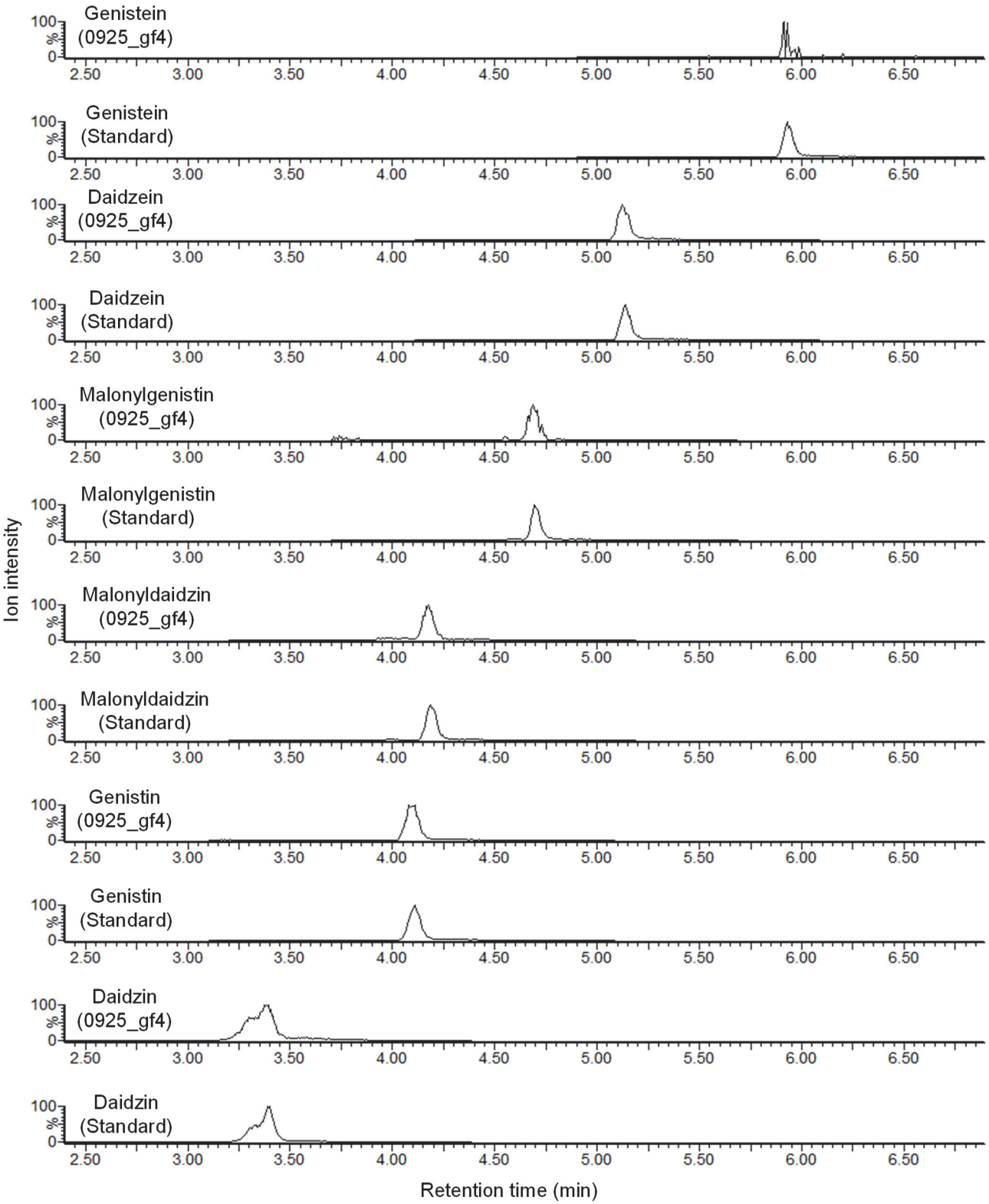

Fig. S8. LC-MS analysis of the authentic standards of isoflavones and extracts from sheets with immobilized BSA (0925_gf4). Chromatograms were obtained by multiple reaction monitoring modes.

Table S2. Quantification of isoflavones recovered from sheets with immobilized BSA

	nmol/sheet						
Sheet	Daidzein	Genistein	Daidzin	Genistin	Malonyldaidzin	Malonylgenistin	Total
0915_gf1	0.227	ND*	0.402	0.010	0.001*	0.000*	0.640
0915_gf2	0.531	ND*	1.150	0.034	0.000*	0.000*	1.715
0915_gf3	0.132	ND*	0.159	0.004	0.000*	0.000*	0.295
0915_gf4	0.176	ND*	0.413	0.008	0.000*	ND*	0.597
0925_gf1	0.092	ND*	0.080	0.002*	0.000*	ND*	0.174
0925_gf2	0.090	ND*	0.256	0.006	0.000*	0.000*	0.352
0925_gf3	0.138	ND*	0.277	0.007	ND*	ND*	0.421
0925_gf4	0.360	ND*	1.561	0.059	0.057	0.0048	2.041
0929_gf1	0.054	ND*	0.014	0.001*	ND*	ND*	0.068
0929_gf2	0.070	ND*	0.002*	0.000*	ND*	ND*	0.072
0929_gf3	0.201	ND*	0.316	0.015	0.000*	ND*	0.533

*below quantification limit

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