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The distribution and chemical form of selenium in the tissue of ferns were visualized using a synchrotron radiation X-ray microbeam.

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Abstract

In this study, *Pteris vittata* L. was cultivated *via* hydroponics by incorporating inorganic selenate (selenium concentration of 5 mg/kg or 50 mg/kg). The growth of the plants was not impaired when they were grown for three weeks with a selenium concentration of 5 ppm. Furthermore, selenium accumulated in the roots (389 mg/kg), stems (85 mg/kg), and leaves (166 mg/kg). Selenium accumulation and metabolism were observed in the roots. As the cultivation period increased, the proportion of Se(-II) in the above-ground parts increased. After three weeks, more than 80 % of the accumulated selenium had been metabolized into Se(-II) compounds. This phenomenon was particularly pronounced under conditions of low selenium concentration, with 5 mg/kg added. Moreover, an analysis conducted using a synchrotron radiation X-ray microbeam demonstrated that the metabolized selenium was present in specific tissues, including the root epidermis and central cylinder. Conversely, the extraction of selenium compounds from each tissue revealed the presence of *Se*-methyl selenocysteine and selenomethionine as soluble components among the selenium compounds produced by metabolism. It is hypothesized that these methylated selenium compounds accumulate in the plant body as low-toxicity chemical species. The extraction rate of selenium in the above-ground parts, such as stems and leaves, was relatively low, and it is postulated that it was metabolized into chemical species, such as selenium-containing proteins.

Keywords: selenate, fern, chemical speciation, X-ray absorption spectrometry, accumulation, extraction

Statements about COI: The authors have no conflicts of interest associated with this manuscript to declare.

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Introduction

The environmental remediation technology that uses plants is called phytoremediation [1]. Phytoremediation is a process that removes contaminants from soil by using uptake by plant roots, transport to above-ground parts, and accumulation of pollutants. Phytoremediation is attracting attention as a new environmentally friendly remediation technology because it uses little energy for decontamination and has little impact on the ecosystem. Phytoremediation is applied to plants that

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hyperaccumulate heavy metals and metalloids [1, 2].

Pteris vittata L. is a fern reported to hyperaccumulate arsenic in 2001 [3] and attracted attention. When cultivated for 6 weeks in soil containing 1,500 mg/kg (ppm) arsenic, it accumulated approximately 22,000 mg/kg (dry weight) arsenic in the aerial parts. In addition, it has been reported in recent years that selenium has a beneficial effect on the accumulation of arsenic in *P. vittata* [4, 5].

Selenium is an essential trace element for humans and animals [6]. Selenium is found in proteins as an element of the amino acid selenocysteine (SeCys) [7]. In contrast, there is debate about whether selenium is essential for plants, as no known plant enzymes require selenium. However, it is known that trace amounts of selenium promote plant growth, and it is positioned as a beneficial element in plants. Low doses of selenium are thought to protect plants from stresses such as cold, drought, and metal stress [8, 9]. However, the mechanism is complex and not fully understood.

Selenium is a rare metal used in various applications, including electronic materials such as photoreceptors, glass colorants and decolorants, and chemical catalysts. It is released into the environment in wastewater and waste from these industrial activities. In general, it is present in the soil at concentrations ranging from sub-ppm to a few ppm, mainly in the form of inorganic selenate and selenite and, to a lesser extent, in organic selenomethionine (SeMet) [10]. The main area where selenium has a significant impact on the environment is in the aquatic environment. Because inorganic selenium exhibits chronic and acute toxicity to living organisms, Japan has set a National effluent standard of 0.1 mg/L or less for selenium and selenium compounds [11]. The World Health Organization (WHO) has recommended 10 μg/L as the lower intake limit of Se in drinking water [12]. There are almost no selenium-contaminated areas in Japan. Still, the effects of selenium contamination on livestock and humans have been reported in some regions of China, India, the United States, etc. [13, 14].

Plants known to accumulate high levels of selenium include *Brassica pinnata*, which is native to North America, and wheat (*Triticum aestivum*) and Indian mustard (*Brassica juncea*), which are found in the Punjab region of India. There is a tendency for plants in the Brassica family to accumulate high levels of selenium [10, 15]. When the soil selenium concentration was 6 ppm, the amount of selenium accumulated in the leaves of these plants was extremely high, about 3,000 ppm. In wheat (*T. aestivum*), when the soil selenium concentration was 13.1 ppm, the selenium accumulation in the leaves was about 390 ppm, and in mustard (*B. juncea*), when the soil selenium concentration was 6.8 ppm, the selenium accumulation in the leaves was about 935 ppm [16, 17].

These plants take up the highly toxic inorganic ion of selenium and metabolize them to less toxic organic selenium compounds in the plant body [14]. In wheat and Indian mustard, selenate is added and metabolized *via* the intermediate SeCys to *Se*-methylselenocysteine (MeSeCys). It is characteristic of plants that accumulate high levels of selenium that MeSeCys is further metabolized to γ-glutamyl-methyl-selenocysteine (γ-GluMeSeCys), which is the final metabolite of selenium. Another pathway has also been proposed in which SeCys is metabolized to selenocystathionine, SeCys, SeMet, selenohomolanthionine (SeHLan), and other compounds [18-20].

There are several methods to study the chemical form of selenium in plants, and high-performance liquid chromatography (HPLC) combined with inductively coupled plasma mass spectrometry (ICP-MS) is commonly used [21, 22]. The sample is a solution extracted from plant tissue. On the other hand, X-ray absorption spectroscopy using synchrotron radiation X-rays is also helpful in studying the chemical form of plant elements [23]. Regardless of the state of the sample, non-destructive analysis is possible, whether the sample is liquid or solid (powder).

We applied X-ray absorption spectroscopy to *P. vittata* L. growing in arsenic-contaminated soil while alive in a pot. Although the arsenic in the soil was pentavalent, As(+V) and As(+III) coexisted in the stem, and almost all of the arsenic in the leaves was trivalent [24]. In addition, when the chemical form of arsenic taken up by the roots was examined, it was found that trivalent arsenic was more abundant at the root tips, and pentavalent arsenic became dominant as one moved toward the base of the roots [25, 26]. These results suggest that arsenic is actively reduced at the root tips.

The fern of *P. vittata* L. is well-known for its ability to hyperaccumulate arsenic, but it has also been found to take up and accumulate other elements [27, 28]. When grown with added Cr(+VI), it accumulated to high concentrations

in the roots, around 40,000 ppm, while when grown with added Cr(+III), it accumulated to high concentrations in the roots, around 20,000 ppm. With Cr(+III), almost none are collected in the above-ground parts, and nearly all are collected in the roots. In addition, using synchrotron radiation X-ray absorption spectroscopy, it was found that the accumulated Cr was in the trivalent chemical form, regardless of whether Cr(+VI) or Cr(+III) was added [28].

To carry out phytoremediation effectively, it is necessary to elucidate the accumulation mechanism of metals and metalloids in hyperaccumulator plants. Therefore, in this study, we added selenate to *P. vittata* and aimed to clarify the chemical form in which selenium accumulates and where it accumulates in the roots, stems, and leaves. We also extracted soluble components from each part of the fern and attempted to identify the chemical species. We focused on the changes in chemical form to determine what chemical form the selenium taken up by the fern moves to the aboveground part.

Experimental

Reagents

Pteris vittata L. was provided by Fujita Corporation (Japan). The sodium selenate (Na_2SeO_4) and potassium selenite (K_2SeO_3) were procured from Kanto Chemical Co. The cellulose powder, with a particle size of 38 μm (400 mesh), was procured from Wako Pure Chemical Industries, Ltd. Tris(hydroxymethyl)aminomethane and MeSeCys were procured from Tokyo Chemical Industry Co. Boron nitride (BN), Driselase® from *Basidiomycetes* sp., and Protease from bovine pancreas were obtained from Sigma-Aldrich Japan.

Sodium selenate was diluted with cellulose as required, and tablets with a diameter of 5 mm were formed, which were employed as samples for the calibration curve when quantifying selenium using X-ray fluorescence (XRF) spectroscopy. Na_2SeO_4, K_2SeO_3, and *Se*-methyl selenocysteine were each mixed with BN as necessary, and tablets were formed. These were utilized as reference materials for selenium compounds with known valence in chemical speciation analysis of selenium by X-ray absorption spectroscopy.

Tris(hydroxymethyl)aminomethane was dissolved in pure water to a concentration of 30 mmol/L to prepare a buffer solution (Tris-HCl buffer) with a pH of 7.2 using 0.1 mol/L hydrochloric acid (HCl). This solution was employed to extract soluble selenium compounds from fern plants.

The authentic standards for HPLC-ICP-MS were obtained as below. Sodium selenite, SeMet, and MeSeCys were purchased from Nacalai Tesque (Kyoto, Japan). Sodium selenate was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). γ-GluMeSeCys was synthesized in our laboratory [29]. Ammonium acetate was purchased from Sigma Aldrich (St. Louis, MO, USA).

Cultivation of *Pteris vittata* L. and addition of selenate

The sporophytes of the fern, cultivated in soil, were removed from the soil and transferred to a culture solution for acclimatization. The culture solution was a 10-fold dilution of the culture solution described in **Table S1** (modified Hoagland nutrient solution [30]). The fern with culture solution was agitated in an incubator, and the culture solution was replaced every two days for two weeks. Sodium selenate was dissolved in the culture solution to prepare the selenium-added solution. The concentration of selenium added to the culture solution was set at 5 ppm (63.3 μmol/L), based on the selenium concentration observed in soil from polluted areas such as China [31]. A higher concentration of 50 ppm (633 μmol/L) Se was also prepared. In the selenium addition experiment, the fern plant bodies were cultured in an incubator for either three weeks using the 5 ppm solution or two weeks using the 50 ppm solution. During incubation, the selenium addition solution was replaced with a new one every two days.

Sample preparation of slice cross-sections for XRF imaging and X-ray absorption spectrometry using X-ray microbeam

The roots, stems, and leaves of the *P. vittata* L, which had been treated with selenate, were embedded in OCT compound (Sakura® Finetek) and 60 μm-thick sections were cut perpendicular to the elongation direction using a cryomicrotome (LEICA CM1900). Subsequently, the sections underwent a freeze-drying process, producing freeze-

dried sections [32, 33]. The section samples were mounted in a 40 mm square acrylic holder using double-sided tape [34] and subjected to XRF imaging and X-ray absorption spectrometry for selenium.

Sample preparation as bulk for determination of selenium concentration and chemical speciation of selenium

After adding selenate, the plant bodies were separated into three parts: roots, stems, and leaves. Each part was freeze-dried, ground, and then homogenized. 0.1 g of the resulting powdered samples were weighed out and formed into tablets with a diameter of 5 mm, which were subsequently utilized for the quantification and chemical form analysis of selenium.

Extraction procedure of soluble selenium compounds accumulated in various parts of ferns

The extraction of soluble selenium compounds from the freeze-dried powder of the roots, stems, and leaves of plants to which selenate had been added was conducted in the following manner [18-20]. Two hundred milligrams of powder were added to 5 mL of Tris-HCl buffer solution and homogenized by ultrasonic crushing for 5 minutes, with 30/30 pulses and 50 % output power. Subsequently, 30 mg of Driselase was added and allowed to stand for 24 hours at 37 °C, followed by 30 mg of Protease and another 24 hours of standing at the same temperature. This was done to break down the cell walls and proteins. The supernatant was then filtered through a 0.45 μm mesh filter, and the soluble selenium extract was prepared for each part.

Meanwhile, the residue resulting from the extraction process was subjected to a washing, freeze-drying, and molding procedure to create tablets for utilization in XRF and XAFS analyses, respectively, to determine the selenium concentration present in the extraction residue and investigate the chemical form of the selenium.

Determination of Se concentrations in various parts of ferns using XRF analysis

The concentration of selenium in various parts of ferns cultivated with the addition of selenate was quantified by XRF analysis. The analysis was carried out using the energy-dispersive X-ray fluorescence spectrometer (Epsilon 5, PANalytical) equipped with a three-dimensional polarization optical system. The instrument has the Cartesian arrangement of the X-ray tube, secondary target, sample, and detector. Selecting a secondary target material appropriate for the target element makes it possible to perform a more sensitive analysis than conventional X-ray fluorescence spectrometers [35]. Zirconium was selected as the secondary target material, and the measurement time per sample was 600 seconds. A calibration curve for selenium was prepared and quantified using it.

Chemical speciation of selenium in various parts of ferns by X-ray absorption spectroscopy

X-ray absorption near edge structure (XANES) spectra of the Se K-edge were obtained at BL-12C of the Photon Factory (PF) of the High Energy Accelerator Research Organization (Tsukuba, Ibaraki). Following the monochromatization of the synchrotron radiation emitted from the bending magnet using a Si (111) double-crystal monochromator, the beam was focused using a curved cylindrical mirror, resulting in an X-ray beam with a diameter of approximately 1 mm (vertical direction, V) × 1 mm (horizontal direction, H) [36]. In the transmission method, the ionization chambers I_0 and I were filled with 100% N_2 and 15 % Ar + 85% N_2 gases, respectively, and employed as X-ray detectors. In the fluorescence method, a 19-elements germanium semiconductor detector was utilized, and an arsenic filter and a latticed soller slit were incorporated to mitigate the background interference caused by scattered X-rays from the sample. The maximum value of the first derivative curve of the XANES spectrum of Na_2SeO_4 was employed as the energy calibration for the K absorption edge of selenium at 12654.5 eV (8.9886 deg). The REX (2000) ver. 2.57 software (RIGAKU) was utilized to analyze the spectra obtained from the measurements.

The valence ratio of selenium in the samples was calculated using the XANES spectra of reference materials with known chemical forms (oxidation states). Specifically, three reference materials were employed: sodium selenate, potassium selenite, and MeSeCys. Pattern fitting analysis was conducted for the energy range (12620 eV – 12680 eV). The relative abundance of each oxidation state of selenium (Se(+VI), Se(+IV), Se(-II)) was determined.

XRF imaging and X-ray absorption spectroscopy using X-ray microbeams

The micro-XRF imaging and micro-XANES spectra of selenium were obtained using BL-4A at PF. A monochromatic synchrotron X-ray beam, which was monochromatized by a Si(111) double crystal monochromator, was focused to a 5.0 μm (vertical, V) × 5.0 μm (horizontal, H) spot [37]. In XRF imaging, the X-ray energy was set to 13.5 keV, which has an optimal excitation efficiency for selenium and is minimally affected by Compton scattering. The fluorescent X-rays emitted from the sample were detected using a Silicon Drift Detector (SDD). The sample was scanned in two dimensions using a pulse motor control, and a two-dimensional elemental map was created from the XRF intensity of each element at each position.

Subsequently, energy scans were conducted at designated locations on the cross-section to obtain μ-XANES spectra of selenium, thereby facilitating the analysis of its chemical form.

Identification of soluble selenium compounds in each part of the fern

To identify soluble selenium compounds, we analyzed the extracts from each part using a combination of HPLC and ICP-MS/MS (Agilent 8800, Agilent Technologies, Hachiouji, Japan). HPLC-ICP-MS was used to analyze the extracts from each part [18-20]. The multi-mode size exclusion column (Shodex Asahipak GS-320HQ, 7.5 i.d. × 300 mm with a guard column, 7.5 i.d. × 75, Showa Denko, Tokyo) was used in this study. This column was eluted with 50 mM ammonium acetate, pH 6.5, at a 0.6 mL/min flow rate. The ICP-MS/MS was equipped with an octapole reaction cell (ORC), which serves to mitigate the impact of multi-atomic ion interference. For detecting selenium, the ion was selected at *m/z* 80 in Q1, and was reacted with oxygen in Q2, and then, the reacted ion was detected at *m/z* 96 in Q3. Additionally, for the identification of selenium compounds, authentic standards, including selenate, selenite, γ-GluMeSeCys, SeMeCys, and SeMet, were employed. The sample introduction volume was 20 μL for each.

Results and discussion

Quantitative results of selenium concentration accumulated in each part of the fern

Table 1 illustrates the selenium concentration in the various parts of the fern plant cultivated with the addition of selenate (at selenium concentrations of 5 ppm or 50 ppm). The table presents the mean values of the results obtained from the repeated independent experiments conducted on the fern plant body. While there is considerable

Table 1. Changes in selenium concentration over time in the roots, stems and leaves of the *P. vittata* L. cultivated with the addition of selenate

Addition conditions	Concentration of selenium (mg/kg DW)		
	Roots	Stems	Leaves
5 mg/kg Se			
7 days	280 ± 123	22 ± 4	38 ± 5
14 days	392 ± 48	63 ± 17	92 ± 31
21 days	389 ± 135	85 ± 25	166 ± 25
50 mg/kg Se			
1 day	226 ± 206	24 ± 22	24 ± 20
3 days	372 ± 71	266 ± 139	109 ± 109
7 days	978 ± 661	188 ± 28	415 ± 177
14 days	1299 ± 656	889 ± 31	396 ± 176

Average of n = 3 samples

variation overall, the general trend can nonetheless be discerned. When the concentration of added selenium was 5 ppm, the selenium concentration in the roots reached 280 ppm after 7 days and approximately 400 ppm after 14 and 21 days. Furthermore, a gradual accumulation of selenium concentration was observed in the above-ground parts until 21 days after addition. In the above-ground parts, a more significant selenium accumulation was observed in the leaves than in the stems. After 21 days of addition, the selenium concentration in the leaves reached 166 ppm. Consequently, when 5 ppm of Se was added, the roots reached saturation after 14 days, while the concentration in the above-ground parts continued to increase, indicating that the concentration in the above-ground parts continued to increase.

Conversely, when 50 ppm of selenium was added for one day, the roots accumulated 226 ppm of selenium, while the above-ground parts (stems and leaves) accumulated 24 ppm. Subsequently, the concentration in the above-ground parts gradually increased over 14 days. Additionally, the roots demonstrated the highest selenium accumulation, reaching approximately 1,300 ppm after 14 days. The selenium concentration in the leaves decreased slightly after 21 days compared to 14 days. The decrease in selenium concentration in the stems and leaves due to long-term addition of 50 ppm selenium (**Table 1**) may be due to multiple reasons, including: 1) plant damage, 2) a decrease in selenium concentration due to an increase in biomass, and 3) the loss of selenium from the plant body due to its conversion into volatile compounds. Indeed, when 50 ppm of selenium was administered for a period of 14 days, some of the leaf veins exhibited a slight discoloration. However, no such changes were observed at 5 ppm addition.

Plants have been classified as hyperaccumulators, secondary accumulators, and non-accumulators depending upon Se accumulation inside their cells [14, 38]. Hyperaccumulators accumulate higher amounts of Se in their cells, i.e., > 1,000 mg Se/kg DW, and thrive well in Se-rich regions of the world. They have methylated forms of SeCys and SeMet, which confer the Se tolerance of these plants and can be vaporized further as dimethyldiselenide (DMDSe). Secondary accumulators accumulate Se and show no signs of toxicity up to 100-1,000 mg Se/kg DW, for e.g., *Brassica juncea*, *Brassica napus*, Broccoli, etc. These Se concentrations in **Table 1** indicate that *P. vittata* is classified as a secondary-hyperaccumulator plant of Se.

In contrast, *Pteris vittata* L. has been identified as a plant species that accumulates significant levels of arsenic. When cultivated in soil contaminated with arsenic, high concentrations accumulate in the plant's above-ground parts [3]. The pentavalent arsenic absorbed by the roots is rapidly transported to the plant's above-ground parts, accumulating in high concentrations as trivalent arsenic [24, 39]. The addition of 50 ppm chromium to the hydroponic culture resulted in the accumulation of this element in the roots at concentrations of tens of thousands of ppm [28], which is the opposite of what was observed with arsenic. Concurrently, the accumulation in the above-ground parts was constrained to a few parts per million. In other words, the transport of chromium from the roots to the above-ground parts was impeded. From these findings, it has been postulated that biological processes regulate chromium uptake. The findings of this study indicate that, despite the addition of selenium, the concentration of selenium accumulated in the roots exceeded that observed in the above-ground parts, suggesting that, in contrast to arsenic, the transport of selenium from the roots to the above-ground parts is restricted. Selenium is assumed to be taken into cells as selenate *via* sulfur transporters on the root cell membrane [40]. When the concentration added was 5 ppm, the leaf concentration was higher than in the stems, indicating that selenium migrated to the leaves and accumulated there.

Results in chemical speciation of selenium in various parts of fern plants cultivated with the addition of selenate

The results of K-edge XANES spectrum measurements of selenium in the roots, stems, and leaves of ferns to which selenate (selenium concentration: 5 ppm) was added are presented in **Figure 1A**. In contrast, the results for the fern plants that were cultivated with the addition of selenate (selenium concentration: 50 ppm) are presented in **Figure S1**. The chemical forms of sodium selenate, potassium selenite, and MeSeCys are offered as reference samples. The oxidation state of selenium can be estimated from the peak position of the spectrum. The spectrum of

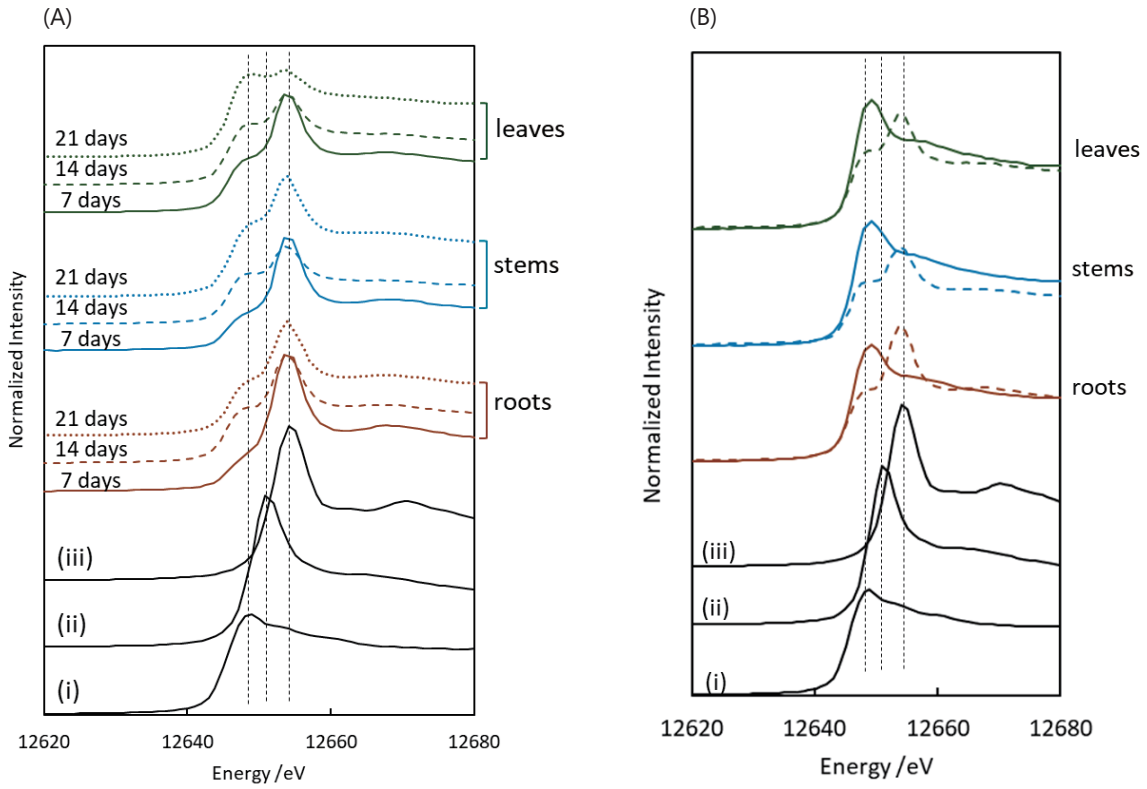

Figure 1. **Selenium K-edge XANES spectra obtained from various parts of the *P. vittata* L.**
Addition conditions: selenium was added as selenate (selenium concentration; 5 ppm) for a certain time
(A) Spectra for the roots, stems, and leaves of the fern over time
(B) Spectra of the roots, stems, and leaves after 14 days of addition (dotted line) and spectra obtained from the residue of each part after extracting the soluble components (solid line)
Reference materials; (i) *Se*-methylseleno cysteine (MeSeCys), (ii) potassium selenite (K_2SeO_3), (iii) sodium selenate (Na_2SeO_4).

the culture medium containing dissolved selenate is not shown here. Still, the addition of selenate was confirmed to result in forming Se(+VI) in the culture medium.

The spectrum of the selenate (selenium concentration 5 ppm) added to the roots after 7 days (illustrated in **Figure 1A**) is nearly identical to Se(+VI) selenate. However, when the addition was prolonged to 14 and 21 days, a substantial shift in the spectrum was observed. Specifically, the intensity of the Se(+VI)-derived peak decreased, while the Se(-II)-derived peak increased, indicating a shift in selenate metabolism. This shift suggests that some of the added selenate (Se(+VI)) was reduced, and the Se(-II) metabolites SeMet and SeHLan were produced. The toxicity of organic selenium compounds, such as SeMet (a selenium-containing amino acid found in plants), is considerably lower than inorganic selenium compounds [6, 7, 17]. This suggests that the fern metabolizes selenate (Se(+VI)) to convert it to organic selenium compounds (Se(-II)), detoxifying and accumulating selenium.

As the XANES spectrum is additive, it is possible to calculate the percentage of each compound present when multiple compounds are mixed using the spectrum of a reference material [41]. Therefore, the ratio of each component of selenium in each part of the fern was calculated using the spectra of reference materials for selenate (Se(+VI)), selenite (Se(+IV)), and organic selenium compounds (Se(-II)) as shown in **Figure 2**.

Following seven days, approximately 70 % of the selenium taken up by the roots remained in the form of selenate (Se(+VI)). However, after 14 days, the proportion of Se(+VI) had decreased to 44 %, with around 40 % of the selenium forming the reduced chemical species as selenium (Se(-II)). As observed in the stems and leaves, the Se(-II) ratio increased over time. Approximately 85 % of the accumulated selenium was Se(-II) in the leaves

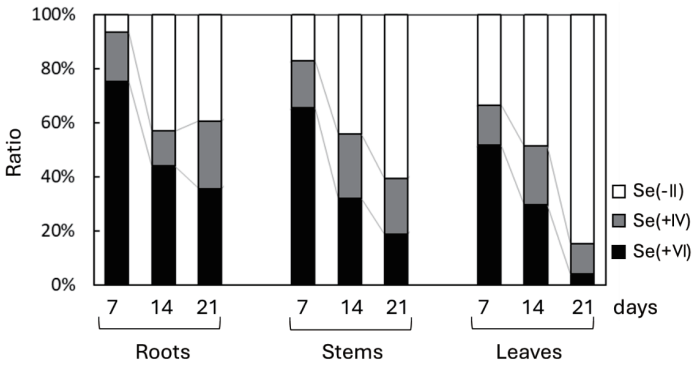

Figure 2. Results in chemical speciation of selenium accumulated in each part of the fern
Addition conditions: Selenate (selenium concentration: 5 ppm) was added for a certain time
The values were calculated from the pattern-fitting spectra analysis in **Figure 1A**.

after 21 days of addition.

In contrast, the XANES spectra for each part and the proportion of the respective oxidation states when 50 ppm of selenium were added are presented in **Figure S2**. Approximately 30-40% of Se(-II) was produced in the roots, whereas, in the stem, which serves as the primary conduit for transporting selenium from the roots to the leaves, predominant selenium existed as Se(+VI). This is consistent with the understanding that the homologous element sulfur is taken up by the plant body as a sulfate ion and transported within the plant [14, 40]. It is postulated that the added selenate is transported in the state of Se(+VI). As illustrated in **Figure 1A**, adding 5 ppm of selenium resulted in a high proportion of Se(-II) in the stems and leaves. However, when the concentration of the additive was increased to 50 ppm, as demonstrated in **Figure S2**, the proportion of Se(-II) reduced was low. This suggests the reduction reaction may not keep pace with the selenium uptake. It is postulated that this is due to the reduced stress on the plant when 5 ppm of selenium is added, facilitating the reduction and metabolism processes within the body.

Results in selenium distribution and selenium chemical form obtained using X-ray microbeams

Elemental distribution measurements were performed on root, stem, and leaf section samples of *P. vittata* after adding selenium at a concentration of 5 ppm for 14 days. The obtained μ-XRF imaging results are shown in **Figure 3** (**I**). The results obtained by adding 50 ppm selenium are shown in **Figure S3**. As shown in **Figure 3**(**a**) and **Figures S3**(**a**) (**b**), selenium accumulated in the epidermis, cortex, and vascular bundles with the central cylinder of the root. The cell shape was visible in these figures, and selenium was highly concentrated in the cell wall.

There are two pathways for water and mineral transport in roots: apoplast (outside the cell membrane) and symplast (inside the cell membrane) [42]. Substances moving from the soil by diffusion through the extracellular (apoplastic) pathway are blocked by the Caspary strip in the root endodermis. The transporters transport substances from the non-specifically blocked substances in a substrate-specific manner, allowing the plant to take the necessary substances (nutrients) into the plant body. Both transporters and barriers enable plants to take up only specific nutrients.

The distribution of selenium in roots suggests that the apoplast mainly transports it to the vascular bundles of the central cylinder since its concentration is high in the cell wall. In the aboveground stem, selenium distribution was also observed in the vascular bundles (which appear U-shaped in this section) and throughout the tissue. In the leaf cross-section, Se was detected in the vascular bundles around the central veins and the leaf flesh.

The results of the μ-XANES analysis at points A-E in **Figure 3** (**I**) are shown in **Figure 3** (**II**), together with the spectra (bulk) data obtained from the tablets shown in **Figure 1A**. In the XANES spectrum of the root cortex layer (point A), the Se(-II) peak can be seen in **Figure 3**(**II**), indicating that the selenium taken up by the root has already been reduced in the cortex layer. The XANES spectrum of selenium in the root tablets (**Figure 1A**) represents the average state of the bulk sample. In contrast, points A and B in **Figure 3** (**II**) show the local chemical speciation of selenium in the points of high selenium concentration (A cortex layer and B central cylinder) in the root section.

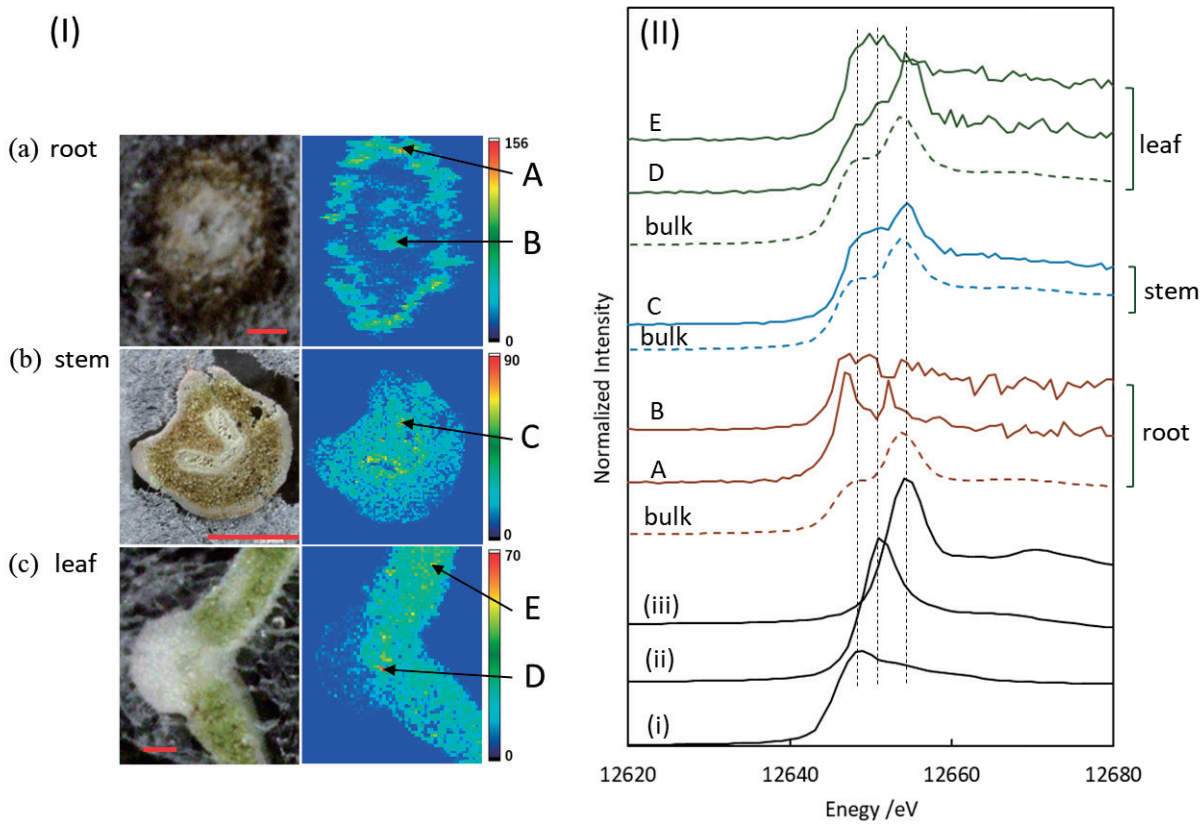

Figure 3. Distribution and chemical form of selenium obtained by X-ray microbeam analysis
Addition conditions: Selenate (selenium concentration: 5 ppm) was added for 14 days
X-ray beam size; 5 μm (H) × 5 μm (V)
(I) Distribution of selenium in fern root, stem and leaf sections
Measurement area and step size; 355 μm (H) × 405 μm (V), step size 5 μm for root section
1620 μm (H) × 1720 μm (V), step size 20 μm for stem section
560 μm (H) × 660 μm (V), step size 10 μm for leaf section
Scale bar; 10 μm in (a) and (c), and 1000 μm in (b)
(II) Micro XANES spectra of the selenium K-edge obtained from the measurement points in the sections
Reference materials; (i) *Se*-Methylseleno cysteine (MeSeCys), (ii) potassium selenite (K_2SeO_3), (iii) sodium selenate (Na_2SeO_4).

It was shown that there are localized points of high selenium intensity in the root tissue, such as the cortex layer and the central cylinder, where a high percentage of Se(-II) is present.

At point C in the stem section, Se(+VI) and Se(-II) coexisted in the spectrum, like the bulk spectrum obtained from the tablets. At point C, which is the vascular bundle of the stem, the percentage of Se(-II) was 56%, indicating a slightly higher rate of Se(-II) than in the bulk (44%).

Point D, near the central vein in the leaf section, shows a spectrum like the stem vascular bundles (Point C). On the other hand, at point E in the mesophyll of the leaf section, Se(-II) and Se(+IV) were 62% and 42%, respectively, while Se(+VI) was negligible. Thus, although the bulk analysis of the whole leaf did not reveal it, the Se(-II) content differed significantly between the central vein and the mesophyll, indicating that reduced chemical species, i.e., metabolized selenium compounds, accumulate in the mesophyll.

Identification of soluble selenium compounds extracted from *P. vittata* L.

Figure 4A illustrates the HPLC chromatograms of the extracts prepared from the samples' roots, stems, and leaves with 5 ppm selenium for 14 days. **Figure 4B** has been enlarged slightly to visualize the minor peaks. The reference materials employed were selenate (i), γ-GluMeSeCys (ii), selenite (iii), MeSeCys (iv), and SeMet (v). The respective retention times were 14.5 minutes (i), 15.8 minutes (ii), 16.7 minutes (iii), 19.3 minutes (iv), and 20.3 minutes (v), respectively. The selenate peak was unambiguously identified in all extracts of roots, stems, and leaves. In contrast, MeSeCys (iv) and SeMet (v) were identified in the roots, albeit at an intensity of less than a few tenths of that observed for the selenate peak. Additionally, a minor peak was observed at a retention time of 21.8 minutes; however, it was not identified due to a lack of correspondence with the retention time of the reference substance. Furthermore, in addition to selenate (i), a selenite (iii) peak was detected in the stem and leaf extracts.

The same HPLC-ICP-MS analysis of the sample with 50 ppm selenium added for 7 days demonstrated that the extracts from the roots, stems, and leaves parts exhibited predominantly selenate peaks. It can be inferred that almost selenate was extracted from the roots, stems, and leaves. The results suggest that Se(-II) accumulated in the roots, stems, and leaves are not easily extracted.

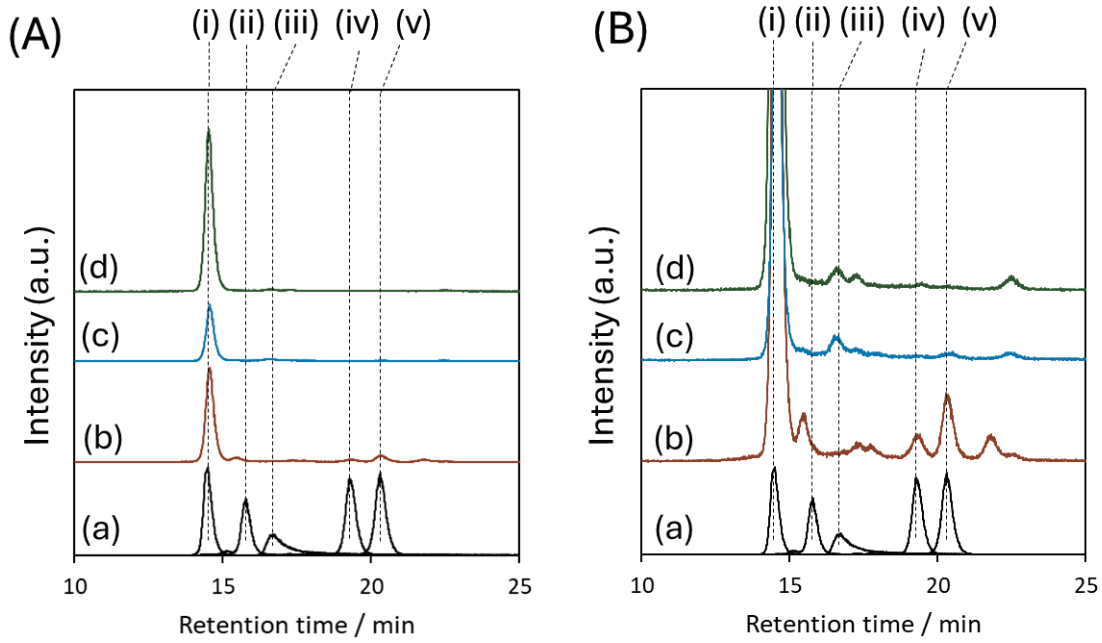

Figure 4. Elution profiles of Se in the extracts from each part of the fern cultivated with the addition of selenate.
Cultivation conditions: selenate (the concentration of selenium 5 ppm) for 14 days.
A 20 μL aliquot of the extract was subjected to HPLC coupled with ICP-MS.
The profiles for (b)-(d) in **Fig 4(B)** are a 10-fold magnification of the vertical axis in **Fig. 4(A)**.
(a) authentic standards; (i) selenate (14.5 min), (ii) γ-glutamyl-methyl-selenocysteine (γ-GluMeSeCys) (15.8 min), (iii) selenite (16.7 min), (iv) *Se*-methylselenocysteine (MeSeCys) (19.3 min), (v) selenomethionine (SeMet) (20.3 min).
(b) root extract, (c) stem extract, (d) leaf extract.

Results in Chemical Speciation of Selenium in Extraction Residues

Following a 14-day period during which selenate (5 ppm selenium concentration) was added, soluble constituents were extracted from the roots, stems, and leaves of *P. vittata* L. **Table 2** illustrates the concentrations of selenium present in the pre-extraction sample and the residue obtained following the extraction process. By employing

these selenium concentrations ($C_{pre\text{-}extraction}$ and $C_{residue}$) and the dry weights of the sample before and following the extraction ($W_{pre\text{-}extraction}$ and $W_{residue}$), respectively, the percentage of selenium extracted from each part was calculated using the following equation:

$$Extraction\ efficiency\ (\%) = \left(1 - \frac{C_{residue} \times W_{residue}}{C_{pre-extraction} \times W_{pre-extraction}}\right) \times 100$$

The highest selenium extraction rate was approximately 51 % for the roots, indicating that approximately half of the accumulated selenium was extracted. In contrast, the selenium extraction rate in the above-ground parts (i.e., stems and leaves) was approximately 30 %, with more than half of the remaining unextracted. Our initial hypothesis was that most selenium would be extracted from the root, stem, and leaf samples of *P. vittata*, as previously described in the literature [18-20]. However, the observed extraction rate was lower than anticipated.

Figure 1B illustrates the K-edge XANES spectra of selenium in the roots, stems, and leaves before and after extraction. Dotted lines represent the spectra before extraction, while the spectra of the residue following extraction are shown as solid lines. Before extraction, the spectra of the roots, stems, and leaves exhibited a coexistence of Se(+VI) and Se(-II). However, the peaks shifted to the low energy following extraction, indicating the presence of Se(-II) in significant quantities. Furthermore, the ratios of the components Se(+VI), Se(+IV), and Se(-II) were calculated for the XANES spectra of the extraction residue. Consequently, the proportion of Se(-II) in the residue of each component was 78.8% for the roots, 92.3 % for the stems, and 84.3 % for the leaves. The proportion of Se(+VI) was minimal in all cases, indicating that Se(+VI) was effectively extracted and that only a minimal amount remained in the residue. The results of the extracted soluble components (**Figure 4**) correspond with the finding that the main component was selenate.

As can be observed, the extraction process was successful in extracting selenate (Se(+VI)) as a soluble component, but less effective in removing organic selenium compounds (Se(-II)). The inability to extract organic compounds can be attributed to two primary factors: their strong binding within plant tissue and the ineffectiveness of the enzymes utilized for their extraction. Further clarification is necessary to elucidate the underlying mechanisms. By integrating extraction operations with X-ray absorption spectroscopy, as demonstrated in this study, we gained insights into the behavior of selenium accumulated within plants.

Table 2. Selenium concentrations before and after extraction for each part of the fern and the selenium extraction efficiencies calculated from these values

Parts	Se concentration (mg/kg DW)		Se extraction efficiency (%)
	Pre-extraction	Residue	
Roots	184.4	88.7	51.9
Stems	26.7	19.1	28.5
Leaves	25.0	16.4	34.4

The following equation calculated selenium extraction efficiency.

Extraction efficiency (%) = $\left(1 - \frac{C_{residue} \times W_{residue}}{C_{pre-extraction} \times W_{pre-extraction}}\right) \times 100$

$C_{pre\text{-}extraction}$ and $C_{residue}$ were Se concentrations (μg/g) before and after extraction, respectively. $W_{pre\text{-}extraction}$ and $W_{residue}$ were sample dry weights (g) before and after extraction.

Conclusions

The addition of low concentrations of selenate (5 ppm selenium) resulted in the uptake of selenium by the fern through its roots, with subsequent transport to the above-ground parts of the plant. This process was observed to persist for 21 days following the initial addition. Adding a high concentration of selenate solution (50 ppm selenium) resulted in the transport of selenium to the plant's above-ground parts. However, most of the selenium accumulated in the roots, indicating limited transport to the above-ground parts. In previous research, it was reported that adding arsenic to *P. vittata* resulted in rapid transport to the plant's above-ground parts, with concentrations reaching tens of thousands of ppm [43]. Furthermore, adding hexavalent chromium resulted in its accumulation in the roots at tens of thousands of ppm [28]. In comparison, the concentration of selenium accumulation in the fern plant was relatively low, and it is postulated that there is a defense mechanism against selenium uptake.

In this study, adding inorganic selenate to *P. vittata* resulted in the uptake and metabolism of selenium in the roots. As the cultivation period was extended, the proportion of Se(-II) in the above-ground parts increased. This phenomenon was particularly evident under conditions of low selenium concentration. Moreover, synchrotron radiation X-ray microbeams demonstrated that metabolized selenium was present in specific tissues, including the root epidermis and central cylinder. Among the selenium compounds produced by metabolism, MeSeCys and SeMet were identified as soluble components. It is hypothesized that these methylated selenium compounds accumulate in the plant body as low-toxicity chemical species. The extraction rate of selenium in the above-ground parts, such as the stems and leaves, was relatively low. It is hypothesized that this is due to selenium being metabolized into more significant selenium compounds, such as proteins. This study employed protease as the most prevalent method for extracting organic selenium compounds. However, by examining the extraction conditions in greater detail and identifying the selenium compounds that were not extracted on this occasion, it is anticipated that a more profound comprehension of the intricacies of the selenium metabolic pathway in *P. vittata* will be achieved.

Synchrotron radiation X-ray absorption spectroscopy is a technique that enables the acquisition of information on chemical forms without the necessity for chemical pretreatment or disruption of the sample. Although this approach has certain advantages for analysis, it is challenging to identify specific substances when multiple compounds are present. This study employed a combined extraction and HPLC-ICP-MS to measure soluble components. We anticipate that this methodology will prove valuable for investigating selenium metabolism in samples subjected to diverse selenium addition conditions and competition with the uptake of other elements, such as sulfur.

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Supplementary materials

Cultivation of fern

The plants were transferred to plastic cups containing pure water with the soil still attached and then covered with a plastic bag with two holes in the top to maintain a high-humidity environment. They were then cultivated in an incubator. The cultivation environment conditions were 12 hours of cyclic light irradiation, light intensity of approximately 10,000/400 lx day/night, humidity of 70~80 %, and room temperature of 25 ± 3 °C. After about two weeks, the soil attached to the roots was gently washed away with pure water, and the plants were transferred to a polyethylene bottle wrapped in aluminum foil to prevent light from reaching them and grown hydroponically. At this time, the plants were shaken (100 rpm) using a shaker.

The culture solution was changed once every two days.

Table S1. Composition of the culture solution by modified Hoagland nutrient solution [30]

Compound	Concentration (mg L^{-1})
KNO_3	81
$Ca(NO_3)_2 \cdot 4H_2O$	95
$MgSO_4 \cdot 7H_2O$	50
$NH_4H_2PO_4$	15.5
NaFe-EDTA	2
H_3BO_3	0.3
$MnCl_2 \cdot 4H_2O$	0.181
$CuSO_4 \cdot 5H_2O$	0.005
$Na_2MoO_4 \cdot 2H_2O$	0.002
$ZnSO_4 \cdot 7H_2O$	0.022

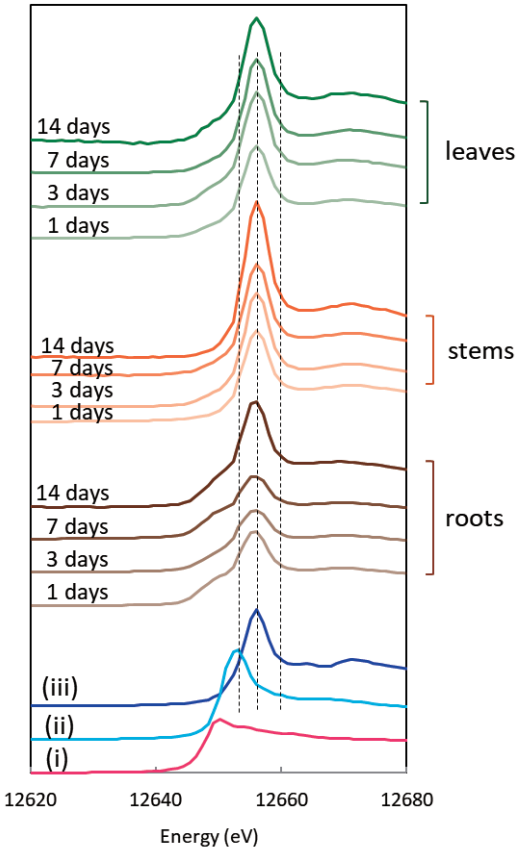

Figure S1. Selenium K-edge XANES spectra obtained from various parts of the *P. vittata* L.
Addition conditions: selenium was added as selenate (selenium concentration; 50 ppm) for a certain time
Spectra for the roots, stems, and leaves of the fern over time
Reference materials; (i) *Se*-methyl selenocysteine (MeSeCys), (ii) potassium selenite (K_2SeO_3), (iii) sodium selenate (Na_2SeO_4).

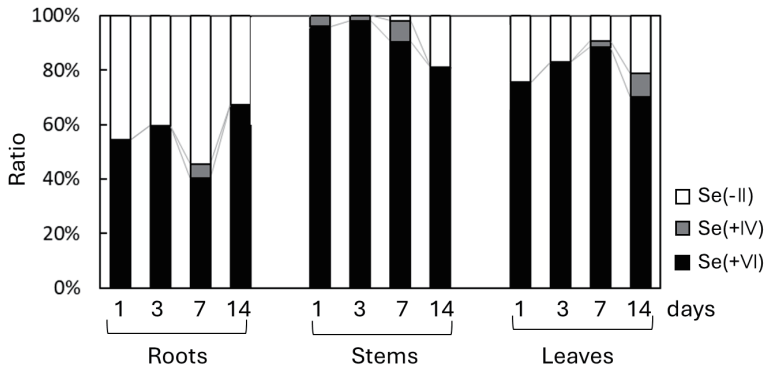

Figure S2. **Results in chemical speciation of selenium accumulated in each part of the fern**
Addition conditions: Selenate (selenium concentration: 50 ppm) was added for a certain time
The values were calculated from the pattern-fitting spectra analysis in **Figure S1**.

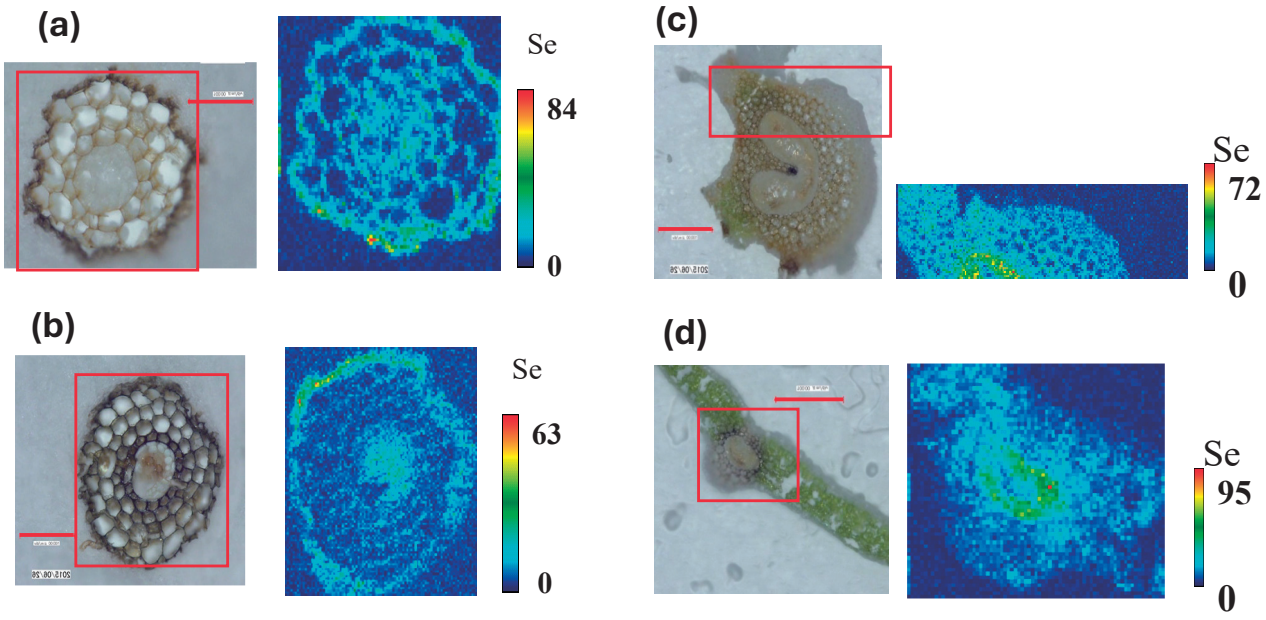

Figure S3. **Distribution of selenium in fern root, stem and leaf sections**
X-ray beam size; 5 μm (H) × 5 μm (V)
Measurement area and step size; 365 μm (H) × 355 μm (V), step size 5 μm for root section (a)
475 μm (H) × 525 μm (V), step size 5 μm for root section (b)
850 μm (H) × 240 μm (V), step size 20 μm for stem section (c)
375 μm (H) × 300 μm (V), step size 10 μm for leaf section (d)
Addition conditions: Selenate (selenium concentration: 50 ppm) was added for 7 days.
Scale bar; 100 μm