Lead and cadmium tolerance and accumulation of proanthocyanidin-deficient mutants of the fern *Athyrium yokoscense*

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Abstract

The fern *Athyrium yokoscense* often flourishes around mine sites in Japan and can tolerate and accumulate heavy metals such as lead (Pb) and cadmium (Cd). In this work, we examined whether proanthocyanidins, also called condensed tannins, were involved in the mechanisms of Pb and Cd tolerance and accumulation of *A. yokoscense* because proanthocyanidins are known to alleviate metal stress in several plant species and are present at high levels in *A. yokoscense*. For this purpose, we used mutant gametophytes deficient in proanthocyanidins, in which the relative proanthocyanidin contents were 20% of those of the wild-type gametophytes. Although the proanthocyanidin contents of the mutant were quite low, the growth of the mutant was very similar to that of the wild-type gametophytes even in the presence of 80 mg/kg Pb or 48 mg/kg Cd. Under the same conditions, the mutant gametophytes also accumulated Pb and Cd as much as the wild-type gametophytes did. These results indicate that the proanthocyanidins in *A. yokoscense* are not important for the Pb and Cd tolerance and accumulation properties.

Introduction

The fern *Athyrium yokoscense* (Fr. et Sav.) Christ, which often flourishes around mine sites, smelters and areas that are highly polluted with heavy metals in Japan, contains high concentrations of cadmium (Cd) and barium (Ba) in its fronds, and zinc (Zn), lead (Pb) and copper (Cu) in its roots.[1-3] Thus, *A. yokoscense* has unusual heavy metal tolerance and accumulation abilities.[4-6] However, the tolerance and accumulation mechanisms remain poorly understood. In a previous report by Kamachi *et al.*,[4] proanthocyanidins (PAs), also called condensed tannins, were inferred to contribute to the Pb tolerance and accumulation abilities because the PAs might precipitate with Pb in the rhizoidal cells of Pb-treated *A. yokoscense* gametophytes, as determined by analytical transmission electron microscopy.

PAs, which are ubiquitous in all land plants except for bryophytes and lycophytes,[7,8] are structurally related to flavonoids, consist of oligomers of two or more flavan-3-ols (such as catechins, epicatechins or the corresponding gallocatechins), and are produced naturally in the leaves, flowers, fruit, seeds, bark and roots of many plant species as a defense against biotic and abiotic stressors.[9,10] There are various biological roles of PAs, depending on plant species, organs, or tissues.[7,9,11] For example, PAs have been traditionally thought to deter herbivore feeding, defend against pathogens, and alleviate abiotic stresses such as UV-B, wounding, and aluminum toxicity in acidic soils.[7,12-15]

Our goal in this work was to elucidate whether PAs are involved in the mechanisms of heavy metal tolerance and accumulation in *A. yokoscense*. For this purpose, we isolated *A. yokoscense* gametophytes deficient in PAs and examined their Pb and Cd tolerance and accumulation properties compared with those of wild-type gametophytes. Gametophytes rather than sporophytes are appropriate for experiments to evaluate such properties since it is easy to control culture conditions and composition of the culture medium. Furthermore, *A. yokoscense* gametophytes have been confirmed to show the same properties regarding heavy metal tolerance and accumulation as sporophytes.[4] Therefore, we used gametophytes as experimental materials in this work.

Materials and methods

Plant materials and cultivation of gametophytes

Spores of *A. yokoscense* were harvested from the sporophytes that had grown around Kamioka Castle in Gifu Prefecture, Japan, dried in silica gel at room temperature, and stored at 4ºC. *A. yokoscense* gametophytes were cultured on 10-fold diluted Murashige and Skoog media supplemented with 0.1% (w/v) 2-morpholinoethanesulfonic acid (MES)-NaOH (pH 5.6) to enhance pH buffer action and solidified with 1% (w/v) bacto agar (BD, USA) at 25°C under continuous white light. For Pb and Cd treatments, designated amounts of $Pb(NO_3)_2$ and $CdCl_2$ were added to the agar media.

Isolation of mutants

The wild-type spores were mutagenized with ethylmethanesulfonate (EMS) according to the methods of Banks[16], with slight modifications. Five milligrams of *A. yokoscense* spores were incubated with 10 mL of 45 mM EMS dissolved in 0.1 M phosphate buffer (pH 7.0) for 24 h at 25°C in the dark, washed 5 times in ultrapure water, sterilized according to the procedure reported by Kamachi *et al.*[4], and then germinated in 50 mL of 10-fold diluted Murashige and Skoog media under continuous white light. After 10 days, germinated spores were inoculated on 10 agar plates and further cultured. After 1–2 months of culture, every single gametophyte that grew to approximately 5 mm was stained with a 4-dimethylaminocinnamaldehyde (DMACA) solution that consisted of 1% (w/v) DMACA, 50% (v/v) methanol and 3 M HCl to screen PA-deficient mutants.[17] Since stained gametophytes die, only a part of them were cut out and stained to check the presence of PA. The remaining portions

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were further cultured, and then restained to confirm the mutation.

Determination of PA and total phenolic contents

The methods were basically performed as described by Waterman and Mole,[18] with slight modifications. For PA determination, 1 mg of the wild-type gametophytes or 3 mg of the mutant gametophytes dried at 60°C for two days were added to a solution consisting of 1 mL of 50% (w/v) ethanol and 4 mL of the butanol-HCl and incubated for 2 h at 95°C. The absorbance at 550 nm was then measured and quantified with cyanidin chloride (Extrasynthese, France) as a standard.

For total phenolic determination, 10 mg of the wild-type gametophytes or 20 mg of the mutant gametophytes dried at 60°C for two days were extracted in 1.5 mL of 80% (v/v) acetone with a glass homogenizer, the precipitates were removed by centrifugation at 8,000 rpm for 5 min, and the total phenolic contents were then determined with the Folin-Ciocalteu method. One hundred microliters of the extract were added to 900 µL of H_2O, and an additional 1 mL of 2-fold diluted Folin-Ciocalteu reagent (Fujifilm Wako Pure Chemical Corporation, Japan) was added. After a 2-min incubation period, 1 mL of 10% (w/v) Na_2CO_3 was added to the mixture and then incubated for 2 h. The absorbance at 750 nm was then measured and quantified, with (+)-catechin hydrate (Sigma-Aldrich Co., USA) as a standard.

Determination of Pb and Cd contents

Gametophytes were dried at 60°C for 2 days, and 40 mg of the dried material was digested in 2 mL of concentrated HNO_3 at 140°C for 5 h. The digested solutions were then diluted to 10 mL with 0.1 M HNO_3, filtered through filter paper (type No. 3, Advantec, Japan), and analyzed via inductively coupled plasma atomic emission spectrometry (ICP-AES) with an Optima 7300DV device (Perkin-Elmer, USA).

Results

We could isolate only one PA-deficient mutant from approximately 60,000 spores treated with EMS and named it *Athyrium yokoscense proanthocyanidin deficient 1* (*Aypad1*). Table 1 shows the contents of PAs and total phenolic compounds in the wild-type and *Aypad1* gametophytes. The relative PA contents of *Aypad1* were 20%, based on those of the wild type, and the relative total phenolic compounds were 19%. These results indicate that the contents of the PA and total phenolic compounds in the mutants were obviously low, although they were not completely absent.

Next, we examined the effects of Pb and Cd on the growth of the *Aypad1* gametophytes. Figure 1 shows the gametophytes regenerated from small fragments (~5 mm^2) of the wild-type and *Aypad1* gametophytes in the presence and absence of Pb or Cd. Even in the presence of 80 mg/kg Pb or 48 mg/kg Cd, the mutant gametophytes grew normally, like the wild-type gametophytes did. The dry weight of the mutant gametophytes also revealed that Pb and Cd did not significantly influence the biomass compared to that of the wild-type gametophytes (Figure 2).

To determine the accumulation properties of the mutant gametophytes, we analyzed the Pb and Cd contents of the gametophytes used in the experiments whose results are shown in Figures 1 and 2 (Figure 3). The results showed that the *Aypad1* gametophytes accumulated these heavy metals as much as the wild-type gametophytes did, indicating that the mutant gametophytes have the ability to accumulate Pb and Cd.

To investigate the heavy metal tolerance of the mutant gametophytes under field conditions, the wild-type and *Aypad1* gametophytes were grown outside in pots filled with soil collected from the Kamegai mine tailings area in Toyama Prefecture, Japan, where high concentrations of Pb, Cd, and arsenic (As) are present.[1] Regardless of the long cultivation from Oct. 21, 2019, to Apr. 7, 2020, both gametophytes were alive, although some regions of the gametophytes were dead compared to those on the first day of cultivation (Figure 4). These results indicate that the heavy metal tolerance of the *Aypad1* gametophytes is very similar to that of the wild-type gametophytes.

Discussion

We isolated PA-deficient mutant gametophytes of *A. yokoscense*, whose PAs were reduced by 80% compared to those of the wild-type gametophytes. At present, we have no genetic data about which gene(s) were mutated in this mutant. In the model plant species *Arabidopsis thaliana*, mutations in the genes encoding enzymes or transcription factors involved in the flavonoid biosynthesis pathway and a vac-

Table 1. Contents of proanthocyanidins and total phenolics in wild-type and *Aypad1* gametophytes.

Gametophytes	Proanthocyanidins (mg/kg)	Total phenolic compounds (mg/kg)
Wild-type	8730±210	48700±2220
Aypad1	1720±40	9020±230

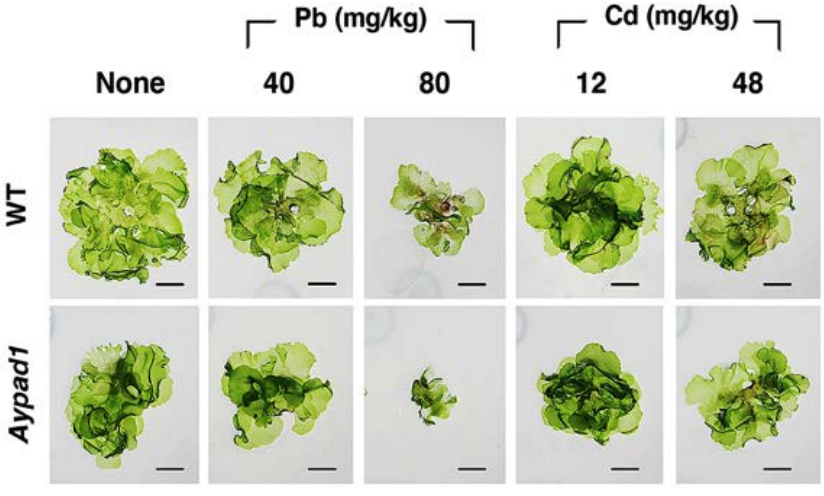

Figure 1. Wild-type (WT) and *Aypad1* gametophytes regenerated on agar media that included Pb or Cd. Small fragments (~5 mm^2) of the gametophytes were precultured for 10 days and then cultured at the indicated Pb or Cd concentrations. After 33 days of cultivation, typical plants were imaged. *Bar* = 3 mm

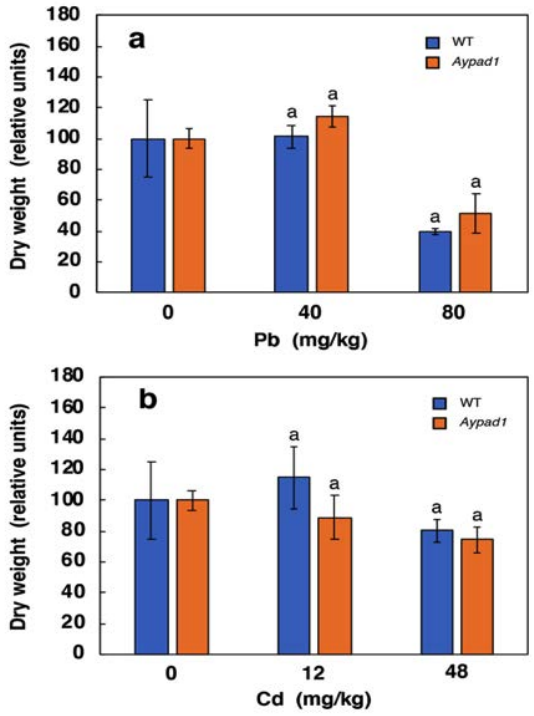

Figure 2. Influences of Pb (a) and Cd (b) on the growth of wild-type (WT) and *Aypad1* gametophytes. Small fragments of the gametophytes were precultured for 10 days and then cultured at the indicated Pb or Cd concentrations. After 33 days of cultivation, the gametophytes were dried and weighed. The data are shown as relative units based on the values without Pb and Cd. The different letters indicate significant differences determined by the Student's t-test (p≤0.05) within the designated days. The *error bars* indicate the SEs (*n*=3–5).

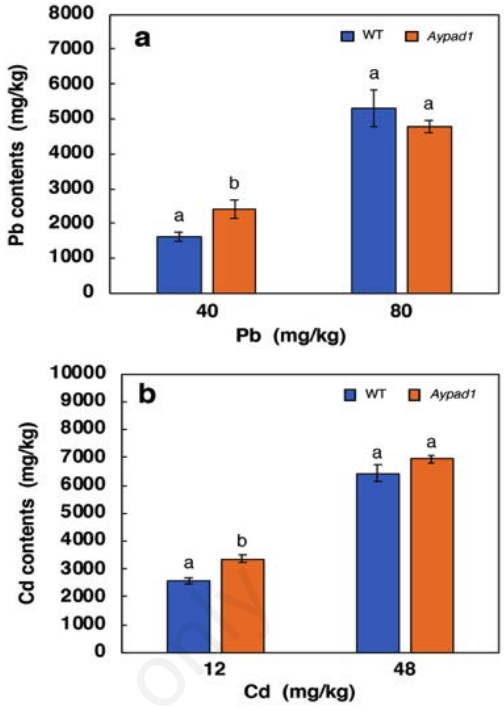

Figure 3. Accumulation of Pb (a) and Cd (b) in wild-type (WT) and *Aypad1* gametophytes. After 33 days of cultivation, the gametophytes were dried, digested with HNO_3, and subjected to ICP-AES. The different letters indicate significant differences determined by the Student's t-test (p≤0.05) within the designated metal concentrations. The *error bars* indicate the SEs (*n*=3–4).

uolar flavonoid/H^+-antiporter involved in the vacuolar accumulation of PA precursors resulted in PA deficiency.[19-21]

The Pb and Cd tolerance of the *Aypad1* gametophytes was almost the same as that of the wild-type gametophytes, indicating that the *Aypad1* gametophytes could be highly tolerant to the heavy metals compared with ordinary plants. Indeed, the growth of the fern *Pteris vittata* and *Ceratopteris richardii* gametophytes was severely negatively affected in the presence of 40 mg/kg Pb or 12 mg/kg Cd (data not shown). The results were further confirmed by an experiment where actual contaminated soil and field conditions were adopted as the culture conditions (Figure 4). In addition, the Pb and Cd accumulation properties were very similar between the wild-type and *Aypad1* gametophytes, indicating that the PAs were not involved in the mechanisms of Pb and Cd accumulation in *A. yokoscense*.

Recently, Ukai *et al.*[22] performed an RNA-seq analysis of *A. yokoscense* sporophytes exposed or not to Cd and found few significant changes in the *A. yokoscense*

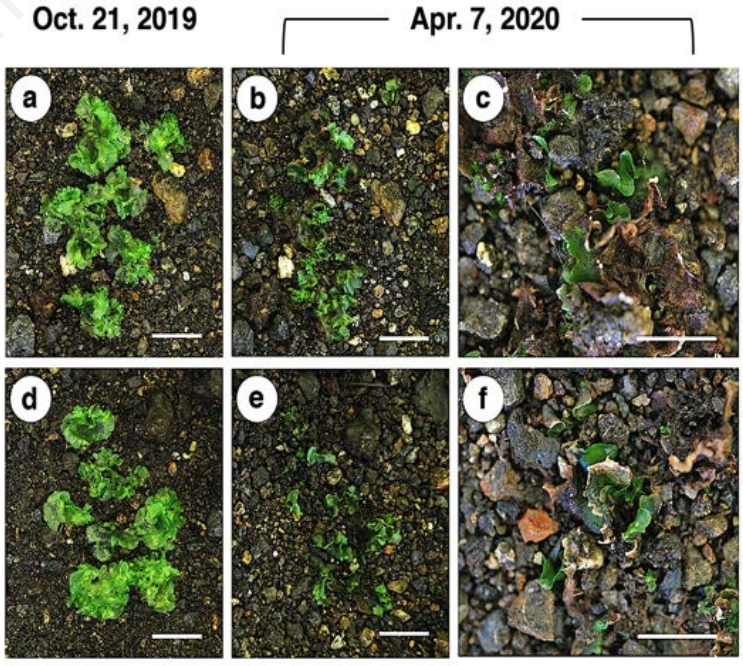

Figure 4. Wild-type (a–c) and *Aypad1* (d–f) gametophytes grown on soil collected from mine tailings areas where *A. yokoscense* had grown in colonies. Bar = 5 mm

transcriptome, even under high Cd conditions. These results imply that most genes involved in Cd tolerance and accumulation may be constitutively expressed in *A. yokoscense*, regardless of the presence or absence of Cd, like the case of *Arabidopsis halleri*, a facultative Cd and Zn hyperaccumulator.[23] Interestingly, however, Ukai *et al*.[22] also found that the expression level of an *NPF* ortholog, which is a putative nitrate transporter gene, doubled in response to Cd stress in the roots. For plants, owing to its low availability, nitrogen is the critical limiting element. In addition, heavy metals severely hinder nitrogen metabolism by reducing nitrate uptake and altering the enzyme activity involved in the nitrogen assimilation process.[24,25] Thus, it seems that the nitrate uptake or distribution between roots and shoots might have been regulated by Cd exposure in *A. yokoscense*. In *A. thaliana*, *NRT1.8*, a member of the nitrate transporter (*NRT1*) family, was suggested to remove nitrate from xylem vessels and play an important role in Cd tolerance by regulating nitrate distribution.[26]

Given that heavy metals inhibit nitrogen uptake, nitrogen uptake could be a problem as well as detoxification of heavy metal ions for plants living in heavy metal-contaminated soils. Furthermore, such heavy metal-contaminated sites are usually infertile because the concentrations of soil nitrogen are low depending on the concentrations of heavy metals such as Cd, Cu, Pb and Zn.[27] Thus, the uptake of nitrogen from such soils is likely problematic for *A. yokoscense*. Given the role of the PAs in *A. yokoscense*, it is worth considering whether the PAs help *A. yokoscense* recycle its nitrogen from dead fronds. The biological roles of PAs have been studied and discussed over the past decades, especially in the field of forest ecology, probably due to the richness of PAs in woody plant species rather than herbaceous plants and thier importance in ecosystem function.[7,9,11] Various kinds of functions of PAs have been proposed in terms of involvement in ecological processes: herbivore defense, nutrient cycling, antioxidative ability, metal complexation, wound sealing, structural support, and drought resistance.[28] In general, PA contents in foliage could affect decomposition rates, and the lower decomposition rates with high PA contents may improve synchrony between mineralization and uptake of mineral nutrients, especially in highly acidic and infertile soils.[28,29] For *A. yokoscense*, which is a summer-green perennial fern species, PAs may play an important role in nutrient recycling from dead fronds or roots through mineralization. Indeed, a large amount of dead fronds remain around these plant without detaching from the rhizomes. Further consideration will be given to this hypothesis in future work.

Conclusions

A proanthocyanidin-deficient mutant could be isolated from the metalliferous fern *A. yokoscense*. In the mutant gametophytes, the Pb and Cd tolerance and accumulation properties were almost the same as those of the wild-type gametophytes. From these results, we conclude that PAs do not play important roles in the heavy metal tolerance and accumulation properties of *A. yokoscense*. Given that heavy metals inhibit nitrogen uptake, further research should focus on the nutrient recycling as the role of PAs.

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