

De novo* transcriptome analysis reveals an unperturbed transcriptome under high cadmium conditions in the Cd-hypertolerant fern *Athyrium yokoscense

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Athyrium yokoscense shows strong tolerance to cadmium exposure, even at levels that are many times greater than the toxic levels in ordinary plants. To determine the mechanism of Cd tolerance in *A. yokoscense*, we grew these plants under high Cd conditions and observed the tissue-specific accumulation of Cd and generation of reactive oxygen species, which is one of the major physiological responses to Cd stress. Fuchsin staining indicated the existence of a casparian strip in *A. yokoscense* roots, which may participate in Cd hypertolerance in *A. yokoscense*. Moreover, we performed RNA-seq of RNA samples from *A. yokoscense* plants treated with or without Cd exposure and obtained comprehensive RNA sequences as well as the Cd-responsive expression patterns of individual genes. Through *de novo* transcriptome assembly and gene expression analysis, we found that *A. yokoscense* showed normal features with no significant change in the expression levels of any transporter genes, even under high Cd exposure conditions. Our results demonstrate that *A. yokoscense* has an unusual mechanism that allows the invading Cd to partition into the distal roots, thus avoiding translocation of Cd into the xylem.

Key words: *Athyrium yokoscense*, cadmium tolerance, casparian strip, fern genome, transcriptome analysis, transporter gene

INTRODUCTION

Cadmium is very toxic to most plants when it is inadvertently taken up and translocated (Clemens,

2006). For example, Cd toxicity strongly affects nontolerant plants when their leaf concentration reaches 5–10 µg/g dry weight (DW) (White and Brown, 2010). Tobacco (*Nicotiana tabacum*) is sensitive to Cd stress, resulting in a significant reduction in plant weight along with severe inhibition of root elongation due to exposure to 100 µM Cd (Misra and Gedamu, 1989). In contrast, other plant species have acquired tolerance to high Cd concentrations and can grow after accumulating more than 100 µg/g DW Cd (Baker et al., 2000). One such species, *Athyrium yokoscense*, shows normal growth fea-

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tures under culture conditions containing 1,000 μM Cd and accumulates 1.5 to 3.5 mg/g DW Cd in the plant body over six weeks (Yoshihara et al., 2005). *Athyrium yokoscense* is a fern indigenous to eastern Asia including Japan (Fig. 1A) (Van et al., 2006). In addition to Cd tolerance, which reaches hundreds of times that of ordinary plants, *A. yokoscense* also displays tolerance to other heavy metal species (e.g., Zn, Cd, Pb and Cu) (Morishita and Boratynski, 1992). Thus, identifying the mechanism of Cd hypertolerance could advance our total recognition and understanding of metal tolerance and accumulation in plants.

Cd is often incorrectly absorbed into plant cells via specific transporters in the cell membrane that are ordinarily involved in the absorption of essential nutrient elements, such as Fe, Zn and Mn (Clemens, 2006). We have reported that the distal root tissues of *A. yokoscense* retained most of the absorbed Cd, but some could be translocated into the upper parts of the plant through the xylem (Yoshihara et al., 2014). This presumes one of the following two possibilities: (1) *A. yokoscense* has a specific transporter to separate essential nutrient elements from Cd, or (2) rhizome-like organs play a role in Cd tolerance in *A. yokoscense*. The rhizome is an underground organ in ferns that stores nutrients (Carlquist and Schneider, 2001). If the former is the case, little is known about the genomic information of fern species, including *A. yokoscense*. In fact, only the genome sequence of *Selaginella moellendorffii*, which belongs to the primitive fern clade Lycopodiophyta, has been determined thus far (Banks et al., 2011). If the latter is the case, it is important to know whether *A. yokoscense* contains a casparian strip among its underground parts. The casparian strip is mainly composed of lignin, fatty acid and suberin (Van Fleet, 1961), and restricts the influx of undesirable substances into the plant body (Steudle and Frensch, 1996). For example, in the roots, both water and nutrients are always transported across the casparian strip to vascular bundles (Steudle and Frensch, 1996). However, in ferns, there are few descriptions of the presence of the casparian strip (Lersten, 1997).

In this study, we observed the structure of *A. yokoscense* roots, particularly in relation to the casparian strip, and analyzed the effect of Cd and Cd-inducible stress on reactive oxygen species (ROS) in the roots. We also comprehensively examined *A. yokoscense* gene expression by *de novo* transcriptome analysis. Based on these results, we discuss fluctuations in gene expression and traits associated with hyperaccumulation of Cd in distal roots when the plants were treated with Cd.

MATERIALS AND METHODS

Plant materials and growth conditions Callus-derived homogeneous plantlets of the fern *A. yokoscense*

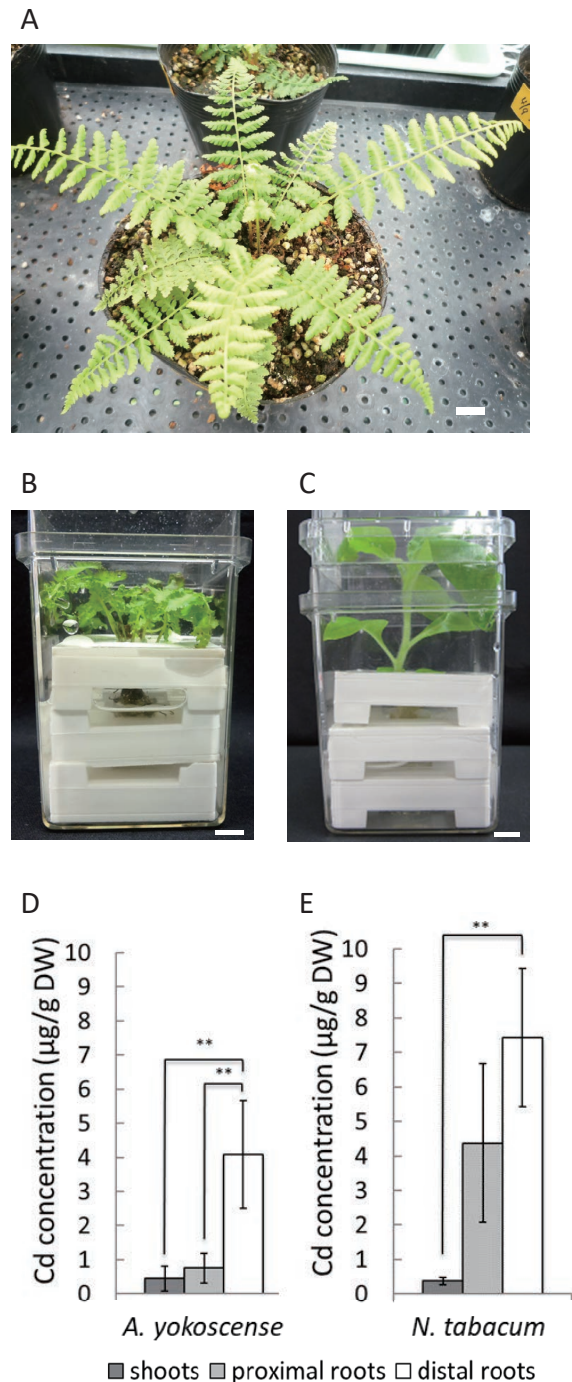


Fig. 1. (A) A sporophyte-stage *A. yokoscense* plantlet. Bar, 1 cm. (B, C) Overview of *A. yokoscense* (B) and *N. tabacum* (C) under Cd stress conditions. Three parts of the plantlet, namely the shoots, proximal roots and distal roots, were harvested. Proximal roots and distal roots correspond to the parts of roots that were undipped and dipped into the culture solution, respectively. Bars, 1 cm. (D, E) Cd concentration in the shoots, proximal roots and distal roots of *A. yokoscense* (D) and *N. tabacum* (E) exposed to Cd stress. The plantlets were grown on medium containing 0.1 μM CdCl_2 for 24 h. Each bar shows the values of the average $\pm$ SD of three independent samples. ** $P < 0.01$ differs significantly from the distal roots according to Student's paired *t*-test.

were prepared under *in vitro* conditions (Yoshihara et al., 2005). We used aseptically derived plants from the sporophyte stage that were redifferentiated from cultured cells (Fig. 1A). *Athyrium yokoscense* plantlets were grown in a magenta box containing 1/2 × Murashige and Skoog (MS) medium (1/2 diluted MS medium supplemented with 30 g/l sucrose, 0.3% gelangum, and vitamins) (Murashige and Skoog, 1962). The culture room was set at 25 °C, and plants were illuminated in a 14 h/10 h (L/D) cycle. Tobacco (*N. tabacum* L. cv. Petit Havana SR1) plantlets were used as a control, and were grown under the same conditions as the *A. yokoscense* plantlets.

Measurement of Cd content in plants *Athyrium yokoscense* was cultured for several months to the sporophyte stage, and *N. tabacum* was grown to a size similar to *A. yokoscense* (Fig. 1B, 1C). According to a previous report (Yoshihara et al., 2014), plants (*A. yokoscense* and *N. tabacum*) were transplanted onto 1/4 diluted MS medium supplemented with a low concentration (0.1 μM) of CdCl₂, and cultivated for 24 h. The plantlets were then subjected to inductively coupled plasma atomic emission spectroscopy (ICP-AES; S Optima 5300 DV, Perkin Elmer, Waltham, MA, USA) analysis at 214.440 nm. The specimens were dried at 65 °C for two days and used for Cd content analysis. The dried samples were weighed and ashed with 1.5 ml each of HNO₃ and H₂O₂ at 120 °C twice. The dried precipitate was dissolved in 10 ml of 1 N HCl and filtered.

RNA-seq of *A. yokoscense* treated with Cd exposure Plants (*A. yokoscense*) were grown without Cd stress, and transplanted onto 1/4 diluted MS medium supplemented with or without 100 μM CdCl₂. After incubation for 24 h, total RNA was extracted from fresh roots and shoots of three independent plants using an RNeasy Plant Mini kit (QIAGEN, Venlo, Netherlands). The total RNA was quantified using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and its purity was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA-seq libraries were constructed for three biological replicates using the Truseq RNA library Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. The libraries were subjected to sequence analysis using a HiSeq2000 sequencer (Illumina) to obtain 100-bp paired-end reads. The raw reads dataset was submitted to the DNA Data Bank of Japan Sequence Read Archive under accession number DRA008924.

De novo transcriptome assembly and ORF prediction The raw reads were assessed with FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and were trimmed using Trimmomatic v0.36 (Bolger et al., 2014) with the following parameter settings: LEAD-

ING, 20; TRAILING, 20; SLIDINGWINDOW, 4:15; and MINLEN, 36. The trimmed reads were assembled using Trinity (v2.4.0) (Grabherr et al., 2013) with the parameters --seqType fq --SS_lib_type RF. Trinity-based contigs were further assembled using PCAP (Huang et al., 2003) with the parameters -y 10 and -t 80. To obtain a non-redundant transcripts dataset, PCAP-based contigs were grouped using CD-HIT-EST (Fu et al., 2012) to cluster sequences that have greater than 80% overlap with the longest sequence in a transcript cluster. Using the longest sequence in the transcript cluster, potential protein coding sequence regions were predicted using TransDecoder v3.0.1 (<https://transdecoder.github.io/>) through sequence comparison with the UniProt database and Pfam-A database with a BLAST search (v2.3.0+) and the hmmscan program in the HMMER package (Johnson et al., 2010), respectively. Representative transcript sequences with coding potential were used for downstream analyses as the reference transcripts dataset. The gene-space completeness of the reference transcripts dataset was examined using BUSCO v3.0.2 (Simão et al., 2015) with -e 1e-05 -m tran and the embryophyta_odb9 database.

Transcriptome analysis The trimmed RNA-seq reads were mapped to the reference transcripts dataset using the BWA 0.7.17-r1188 program (Li and Durbin, 2010) with the parameters mem -M. The expression level of each transcript was quantified based on the reads per million mapped reads (RPM) computed from the read count data using the featureCounts program (Liao et al., 2014). Transcripts showing RPM ≥ 1 in all three samples were considered to be significantly expressed. Differentially expressed transcripts were identified using the DESeq2 program (Love et al., 2014) in R, with the thresholds as fold change ≥ 2 and adjusted q < 0.01.

Quantitative RT-PCR Total RNA for quantitative RT-PCR (qRT-PCR) was prepared as for the RNA sequence analysis. The quality and quantity of the RNA were evaluated using a NanoDrop 2000. First-strand cDNA was synthesized using 1.5 μg of each total RNA sample and random primers with ReverTra Ace (Toyobo, Osaka, Japan) according to the manufacturer's protocol. The qRT-PCR amplification mixture (20 μl) contained 50 ng template cDNA, 10 μl Thunderbird SYBR qPCR Mix (Toyobo), and 0.25 μM forward and reverse primer DNAs. Reactions were run on a QuantStudio 3 (Thermo Fisher Scientific). The cycling conditions comprised 10 min polymerase activation at 94 °C, followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s and 77 °C for 30 s. The 2^{-ΔΔCt} method was used for quantification of relative transcript abundance. The following primer sets were used for qRT-PCR: (1) NPF gene (*Athy41034c000010*), FP, 5'-CGAGGCGACACTCTACATGA and RP, 5'-AGAACCTCGTGCTTCCAAGA; (2)

catalase gene (*Athyo6890c000010*), FP, 5'-GAGATGTG-GAGGGTTCTGGA and RP, 5'-AACGGCCTCTTCAA-CAGAGA; and (3) actin gene (*Athyo3914c000010*), FP, 5'-CCATCCAGGCTGTGCTATCC and RP, 5'-CAC-GACCTGCAAGATCCAGA. Actin was used to normalize the expression level of the NPF and catalase genes. Nucleotide sequences of these genes are shown in Supplementary Fig. S1.

Prediction of orthologous genes in *A. yokoscense*

Putative orthologs of the representative transcripts were identified using OrthoMCL1.4 (--mode 4) following an all-against-all BLASTP search (-e 1e-5 -F F -m 8) (Chen et al., 2006) with the deduced proteome datasets from *Arabidopsis thaliana* (Phytozome), *Marchantia polymorpha* (<http://marchantia.info/download/>), and *S. moellendorffii* (Ensembl Plants).

Analysis of ROS and the casparian strip ROS in root sections were detected using CellROX Green reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. The resultant roots were cut to 100 µm thickness sections using a tabletop hand microtome TH (Kenith, Osaka, Japan). They were observed using an upright microscope (ECLIPSE 80i, Nikon, Tokyo, Japan). Fluorescence was observed at 485 nm/520 nm (excitation/emission) with an ultrahigh pressure mercury lamp. Fluorescence intensity was measured using ImageJ image analysis software (W. Rasband, USA; <http://rsb.info.nih.gov/ij/>).

To observe the casparian strip, distal root sections were fixed in a formalin/acetic acid/alcohol mixture (3.7% formaldehyde, 5% acetic acid, 50% ethanol) for at least 24 h. The fixed samples were sequentially immersed in a series of ethanol mixtures at 50, 70, 85, 95 and 100%. Next, the root sections were embedded in Techno-

vit 7100 (Kulzer, Hanau, Germany) and positioned. Sectioning was performed with a microtome PR-50 (Yamato Kohki Industrial, Saitama, Japan); the thickness of the paraffin ribbons was adjusted to 20 µm. The resultant ribbons were stained with 0.1% (w/v) New Fuchsin (Sigma Aldrich, St. Louis, MO, USA) and observed using an inverted microscope (PHASE CONTRAST-2 ELWD 0.3, Nikon).

RESULTS

Accumulation of Cd in roots and shoots and response to Cd stress To determine the response to Cd stress, we observed the morphology of *A. yokoscense* sporophyte-stage plants cultured under the low (0.1 µM) concentration of Cd condition and compared them with tobacco plants. There were no notable differences in appearance before and after Cd treatment in either species. In *A. yokoscense*, a large amount of Cd (4 µg/g DW) accumulated in the distal roots, whereas very low Cd accumulation (less than 1 µg/g DW) was observed in the proximal roots and shoots (Fig. 1D). In contrast, in *N. tabacum*, high levels of Cd accumulation (greater than 4 µg/g DW) were detected in both the distal and proximal roots (Fig. 1E). Thus, there was a significant difference in the Cd accumulation and its location in the tissues between *A. yokoscense* and tobacco.

De novo transcriptome assembly in *A. yokoscense*

We obtained RNA-seq reads using *A. yokoscense* sporophyte-stage plants cultured with or without 100 µM Cd. Using *de novo* assembly of these RNA-seq reads, we developed the first comprehensive nonredundant transcripts dataset of the Cd-hypertolerant fern. Via Illumina-based RNA-seq, we obtained 46 to 72 million paired-end reads for each sample (Table 1), and, in total,

Table 1. Summary statistics of RNA-sequencing analysis in *A. yokoscense*

Tissue	Condition	Biological replicate	Number of total reads	Number of mapped reads	% mapped reads	% properly paired
Root	Control	1	48,230,866	45,805,297	94.97	90.83
		2	57,181,790	54,423,271	95.18	91.44
		3	50,025,214	47,598,471	95.15	91.76
	Cadmium	1	55,842,456	52,930,001	94.78	91.33
		2	48,564,620	46,185,852	95.1	91.71
		3	47,405,502	44,597,098	94.08	89.98
Shoot	Control	1	68,156,644	64,627,722	94.82	91.08
		2	72,690,788	68,962,500	94.87	90.6
		3	55,572,558	52,693,188	94.82	91.37
	Cadmium	1	56,730,274	53,794,918	94.83	90.33
		2	54,409,480	51,606,502	94.85	91.3
		3	46,753,554	44,035,626	94.19	90.72

661 million paired-end reads were used for the subsequent transcriptome assembly. From the assembly and subsequent contig clustering, we produced a nonredundant transcripts dataset composed of 35,681 transcripts (Table 2). We found that its N50 metrics were greater than 2.6 kb (Table 2) and its transcript length distribution was largely greater than 2 kb (Fig. 2A). We also assessed its assembly completeness using BUSCO (Simão et al., 2015), and found hits for 957 (66%) full-length and 70 (5%) fragmented BUSCO proteins (Fig. 2B). All of these transcripts dataset matrices indicate high quality, which is appropriate for the expression analysis as a reference sequence dataset. We then searched for the closest orthologs of *A. yokoscense* proteins through BLASTP

searching against the NCBI nr database and found that 71.8% of the query sequences showed the highest hit to uncharacterized or hypothetical proteins (Fig. 2C), suggesting that the *A. yokoscense* genome encodes many

Table 2. Statistics of *de novo* transcriptome assembly in *A. yokoscense*

Statistics	
Number of transcripts	35,681
Mean transcript length (bp)	2,193
N50 transcript length (bp)	2,668
GC content (%)	44.86

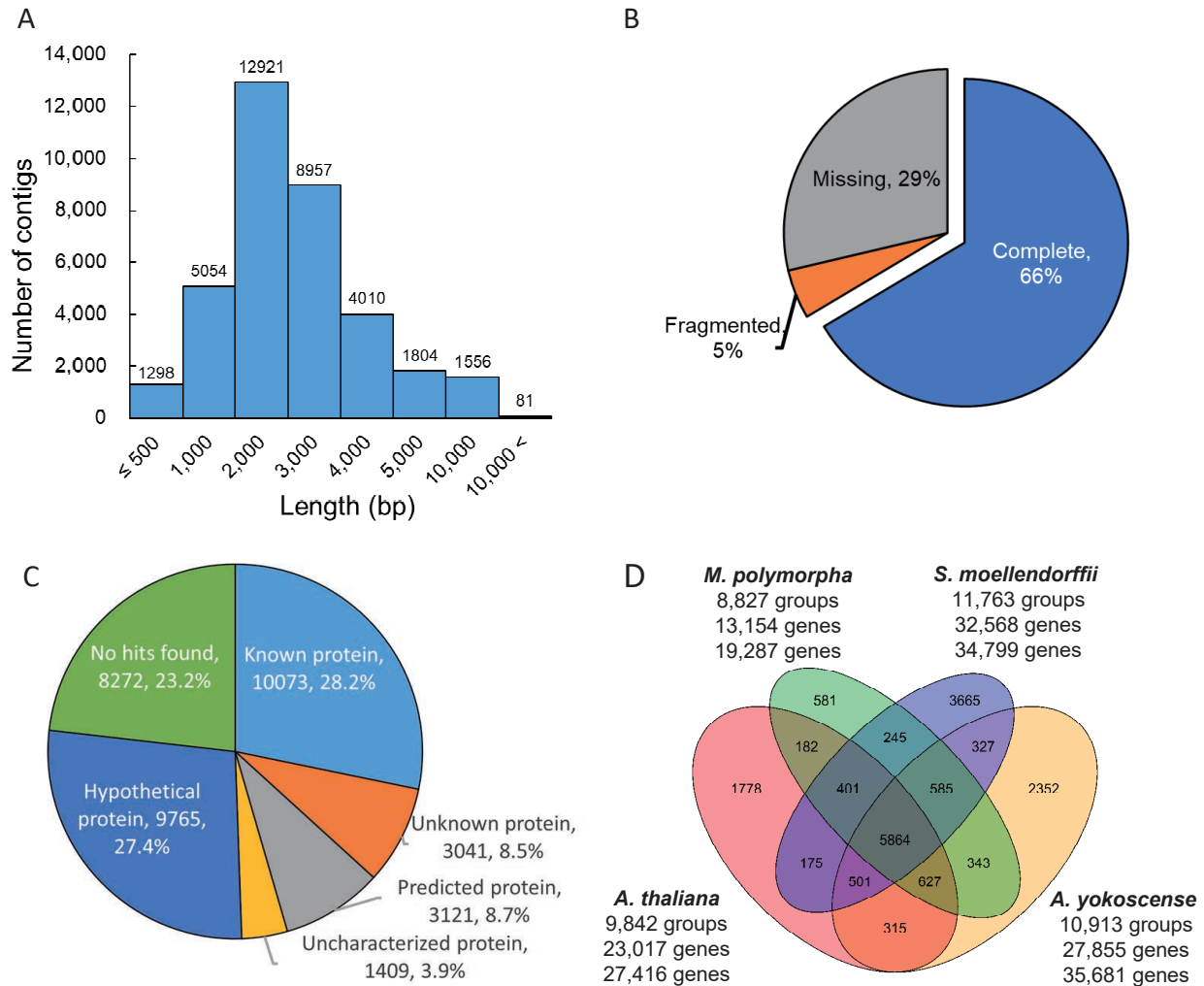


Fig. 2. Overview of expressed transcripts in *A. yokoscense* roots and shoots in response to Cd exposure. Number of expressed transcripts was counted as the sum of occurrences in either roots or shoots. (A) Distribution of contig lengths. The total number of contigs was 35,681. (B) BUSCO v3.0.2 results to assess the gene-space completeness of the reference transcripts dataset with $-e 1e-05$ -m tran using the embryophyta_odb9 database. This result is for the core gene set. (C) Proportion of expressed transcripts that showed sequence similarity with entries in the NCBI nr database. (D) OrthoMCL gene family clustering of the *A. yokoscense* genes with genes from *A. thaliana*, *M. polymorpha* and *S. moellendorffii*. The first number is the number of gene clusters, the second number is the number of genes in clusters, and the third number is the total number of genes. Numbers in the sections of the diagram indicate the numbers of clusters (gene groups), not individual genes.

functionally unknown genes. Moreover, we examined the distribution of *A. yokoscense* transcript orthologs in *S. moellendorffii*, *M. polymorpha* and *A. thaliana* using the OrthoMCL program (Chen et al., 2006), and found that 78% of *A. yokoscense* transcript orthologs are shared with at least one organism used to predict the orthologous groups (Fig. 2D).

Genes showing differential expression in culture with and without cadmium By comparing gene expression data between the samples with and without Cd exposure, we found an extremely small number of genes that were differentially expressed in response to Cd exposure (Fig. 3, Supplementary Table S1). When the analysis used the q-value as 0.00001, which was commonly employed, only four differentially expressed genes (DEGs), all without known functions, were detected in roots, and none was detected in shoots. When the q-value was set to

0.01, we found 43 and two DEGs in the roots and shoots, respectively (Table 3). Among these, all 43 transcripts increased in roots, while one gene increased and the other decreased in the shoots, under the Cd conditions (Table 3). These genes included a putative nitrate transporter (NPF family) gene (*Athyo41034c000010*) and three catalase genes (*Athyo6890c000010*, *Athyo71933c000010* and *Athyo38788000010*), whose fold changes were calculated as 2.7-, 3.9-, 3.8- and 3.7-fold, respectively, in the roots (Supplementary Table S1). In shoots, their gene expression fold change values were not significantly different between treatment with and without Cd, suggesting that no alteration occurred in the shoots. No gene encoding another transporter was predicted among the significant DEGs other than the single NPF family transporter gene (Supplementary Table S1).

We analyzed the expression levels of the NPF family gene and one of the catalase genes, because these expres-

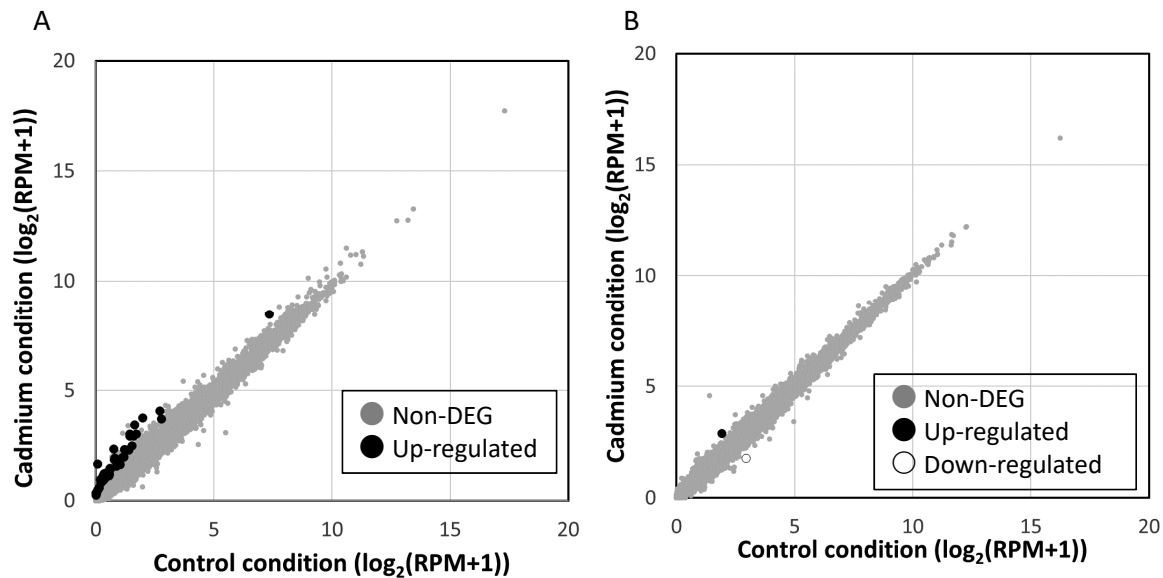


Fig. 3. Differentially expressed genes (DEGs) in response to Cd stress. In total, 35,681 nonredundant transcripts were analyzed. Scatter plots of pairwise combinations with selected DEGs in roots (A) and shoots (B). Up-regulated and down-regulated DEGs are shown by black and white circles, respectively. DEGs were selected using DESeq2 with Fisher's exact test (fold change ≥ 2 and adjusted $q < 0.01$).

Table 3. Number of DEGs in *A. yokoscense* compared with other plants

Plants	Cd [μ M]	Organ	Number of DEGs		Reference
			Up-regulated	Down-regulated	
<i>Athyrium yokoscense</i>	100	Shoot	1	1	this work
<i>Athyrium yokoscense</i>	100	Root	43	0	this work
<i>Solanum nigrum</i>	50	Root	233	126	Xu et al., 2012
<i>Solanum torvum</i>	50	Root	318	316	Xu et al., 2012
<i>Oryza sativa</i>	10	Root	214	22	He et al., 2015
<i>Oryza sativa</i>	100	Root	914	248	He et al., 2015

sion levels increased in presence of Cd stress and showed greater than a 2.0-fold change in RPM. Quantitative RT-PCR results showed a slight increase in the amount of the NPF transcript, but no significant change in expression of the selected catalase gene, which showed the larg-

est DEG value of the three (Fig. 4, Supplementary Table S1).

The *A. yokoscense* transcripts dataset contained expressed gene sequences orthologous to genes reported to be involved in Cd transport (Supplementary Table S2), such as *NPF*, *AtIRT1*, *OsNramp5*, *OsHMA3*, *Hmt1*, *OsACA6* and *AhCAX1* (Ortiz et al., 1992; Connolly et al., 2002; Li et al., 2010; Miyadate et al., 2011; Ishikawa et al., 2012; Shukla et al., 2014; Ahmadi et al., 2018), whereas no ortholog for *TaLCT1* (Clemens et al., 1998) was identified from this dataset using the BLASTP program. No significant changes were observed for the amounts of the following transcripts due to Cd stress: *IRT1* (*Athyo10186c000010*), *Nramp5* (*Athyo2574c000010*), *HMA3* (*Athyo6577c000010*), *Hmt1* (*Athyo90534c000010*), *ACA6* (*Athyo28924c000010*), *CAX1* (*Athyo1380c000010*) and another *NPF* gene (*Athyo44698c000010*) (Table 4). Other orthologs were found, but they also showed no significant changes in expression levels (Supplementary Table S2).

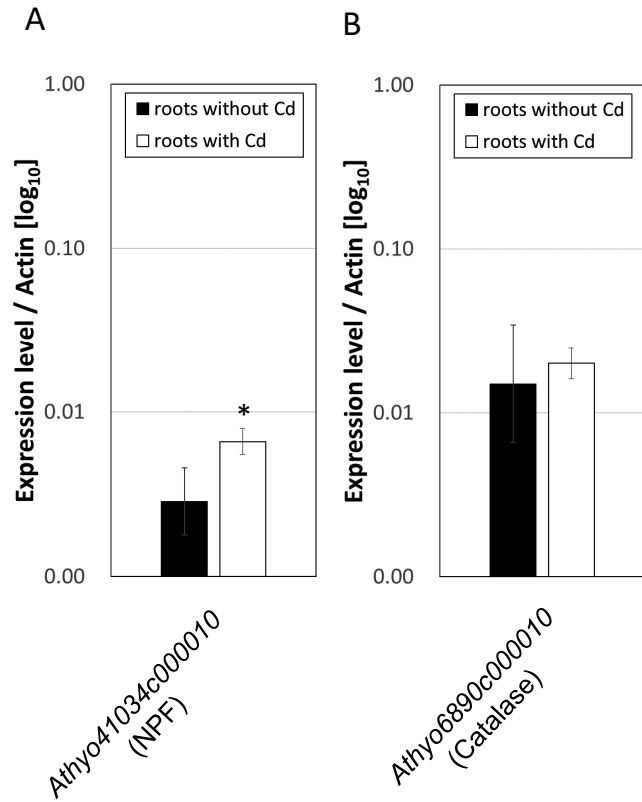


Fig. 4. NPF and catalase gene transcript levels in *A. yokoscense* roots with and without Cd stress. Quantitative RT-PCR results are shown for the *Athyo41034c000010* gene encoding an NPF (A) and the *Athyo6890c000010* gene encoding a representative catalase (B). Values are indicated as the relative amount of each transcript normalized to that of the actin gene. Each bar shows the average $\pm$ SD of three independent samples. * $P < 0.05$ differs significantly from the control condition according to Student's paired *t*-test.

***Athyrium yokoscense* generates few ROS in response to Cd stress and possesses a casparian strip** Because exposure to heavy metals often causes strong stresses to plants via the generation of ROS (Andresen and Küpper, 2013), we analyzed ROS generation in *A. yokoscense* cells in the roots. ROS signals were detected in the central pillars and endothelial cells in the roots, but no marked difference was observed in the fluorescence intensity in cells with or without Cd exposure (Fig. 5A–5D).

Moreover, to understand the structural differentiation of *A. yokoscense* roots, sectioned roots were observed. *Athyrium yokoscense* roots have specific tissues corresponding to the epidermal cells and vascular bundles. We detected tissues stained with Fuchsin around the central pillars in root sections, in which large amounts of lignin were contained (Fig. 5E). Fuchsin is known to bind lignin and shows staining with red color (Barlow, 1969), and it can detect the presence of a casparian strip (Vermeer et al., 2014). These results indicate that there is a caspar-

Table 4. Similarity search results and fold change values in roots

Gene name	Accession No.	BLASTP		DESeq2 in roots		Reference
		% identity	E-value	Fold change value	q-value	
<i>NPF</i>	Athyo44698c000010	46.8	1.47E-171	1.3	7.35E-01	Athyo41034c000010
<i>IRT1</i>	Athyo10186c000010	41.3	5.00E-92	1.0	9.98E-01	<i>AtIRT1</i> ; NP_567590.3
<i>Nramp5</i>	Athyo2574c000010	59.7	0	1.2	9.07E-01	<i>OsNramp</i> ; Q8H4H5.1
<i>HMA3</i>	Athyo6577c000010	47.7	0	1.9	7.65E-01	<i>OsHMA3</i> ; Q8H384.1
<i>Hmt1</i>	Athyo90534c000010	36.1	1.18E-129	0.9	9.85E-01	<i>Hmt1</i> ; CAA20865.2
<i>ACA6</i>	Athyo28924c000010	58.1	0	1.6	4.97E-01	<i>OsACA6</i> ; AGW24530.1
<i>CAX1</i>	Athyo1380c000010	48.7	2.23E-113	1.1	9.98E-01	<i>AhCAX1</i> ; AKS03561.1

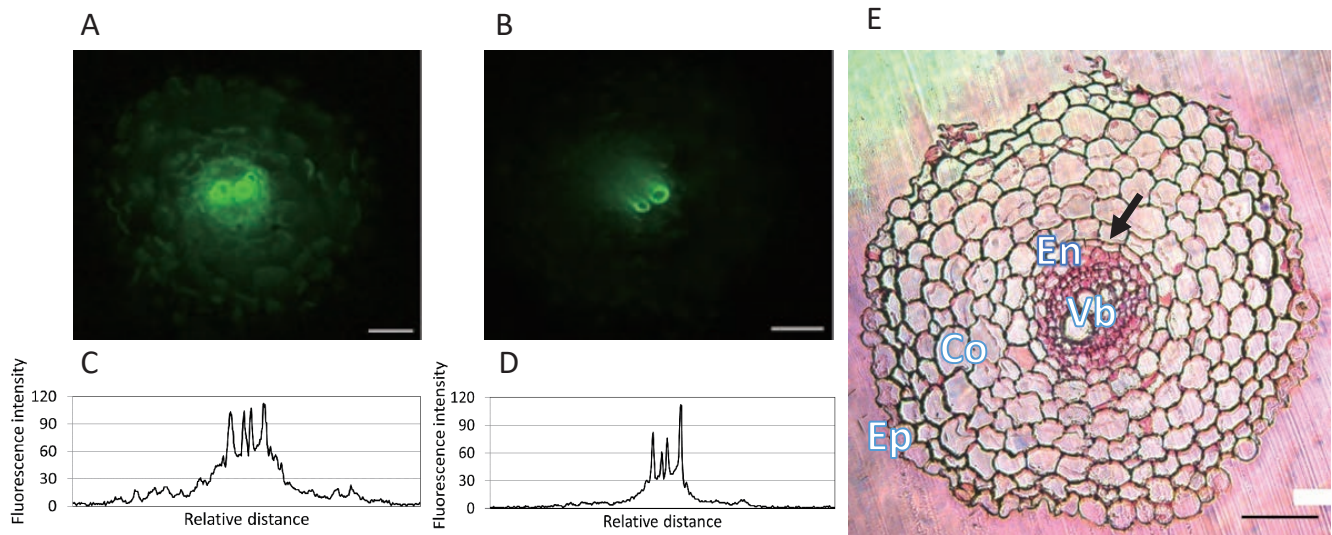


Fig. 5. Detection of ROS and the casparian strip. (A, B) Root sections stained with the CellROX Green reagent. The roots were placed in medium with (A) and without (B) 100 μM Cd for 24 h. Bars, 100 μm. (C, D) Graphical representation of the fluorescence intensity in (A) and (B), respectively. Each graph represents an arbitrary line through the center of the root. The fluorescence intensity was observed at 485 nm/520 nm (excitation/emission) using an ultrahigh pressure mercury lamp. (E) Root section stained by Fuchsin. For this observation, we used a plant grown without Cd stress. Bar, 100 μm. Arrow, casparian strip; Ep, epidermal cells; Co, cortex; Vb, vascular bundles; En, endothelium.

ian strip in the endothelium.

DISCUSSION

In *A. yokoscense*, Cd mainly accumulated in the distal roots (Fig. 1D, 1E). In contrast, a small amount of Cd is transported to the proximal roots and shoots. We attempted to elucidate the molecular mechanism of tolerance to Cd in *A. yokoscense* cells. Since few data have been reported on *A. yokoscense* genes, we constructed an expressed sequence dataset through RNA-seq-based *de novo* transcriptome analysis of *A. yokoscense* cells with and without Cd exposure. We determined the RNA sequence details and expression patterns of individual genes in *A. yokoscense*. Our dataset was considered to have sufficient quality (Tables 1 and 2, Fig. 2). BUSCO analysis showed that our dataset contained contigs corresponding to complete cDNAs, and an Orthmcl analysis suggested that many predicted cDNAs in an *A. yokoscense* ortho group were shared with other organisms (Fig. 2). These results showed that this dataset is a representative database for cDNA sequences corresponding to mRNAs in common fern species.

Using these data, we analyzed the expression patterns that changed in response to Cd stress. Contrary to previously observed gene expression changes in normal plant species under Cd stress conditions, we found no significant changes in the *A. yokoscense* transcriptome, even under high Cd conditions (Fig. 3). It has been reported that expression of a large number of genes was altered in response to Cd stress in other plant cells. In the case of

S. nigrum and *S. torvum*, the expression levels of 359 and 634 genes, respectively, were largely influenced when they were exposed to 50 μM Cd in the medium for 24 h (Xu et al., 2012). Similarly, a large number of DEGs have been reported in *Oryza sativa* under Cd stress: expression of 236 and 1,162 genes was changed by medium containing 10 μM and 100 μM Cd, respectively (He et al., 2015).

In contrast to these findings, we found that *A. yokoscense* showed no notable alterations in the expression levels of almost all genes in response to Cd stress (Fig. 3). We detected few genes showing differences in expression levels when the q-value was set to the commonly used value of 0.00001. Even when the q-value was set to a high value (0.01), we only found 43 and 2 DEGs in the *A. yokoscense* root and shoot transcriptome between plants with and without Cd stress (Table 4), indicating that the regulation of *A. yokoscense* genes was essentially unperturbed in response to Cd stress. Cd mainly moves into cells via transporters/channels, which show broad-range specificities for Fe, Ca and Zn ions (Clemens, 2006). To date, an IRT transporter belonging to the ZIP (ZRT-IRT-like proteins) family, an ortholog of the wheat LCT1 transporter, a cation (calcium) channel and Nramp have been reported to be involved in absorption of Cd into cells (Clemens et al., 1998; Connolly et al., 2002; White and Broadley, 2003; Ishikawa et al., 2012). We identified transcripts corresponding to orthologs of these transporter/channels in *A. yokoscense*, except for LCT1 (Table 3). These findings suggest that *A. yokoscense* has a similar ion transport system to other plants.

The Arabidopsis *NPF*, *AtNPF1.8* (*At4g21680.1*), has

been shown to be up-regulated by Cd exposure (Li et al., 2010). In *A. yokoscense*, the expression level of an *NPF* ortholog (*Athyo41034c000010*) doubled in response to Cd stress (Fig. 4, and Supplementary Table S1). No significant changes were detected in the transcript levels of genes orthologous to other transporters/channels (Table 3). These observations imply that these genes are not involved in Cd absorption in *A. yokoscense*.

Catalase is an enzyme that catalyzes the reduction of H_2O_2 to O_2 , and may be responsible for the removal of excess ROS during stress (Mittler, 2002). Oxidative stress is often induced by Cd exposure and causes severe toxicity (Andresen and Küpper, 2013). It has been reported that transgenic tobacco harboring a catalase gene, *OsACA6*, showed acquired tolerance to Cd stress (Shukla et al., 2014). Significant suppression of Cd-induced ROS is observed in an *Arabidopsis helleri* transformant overexpressing *AhCAX1* (Ahmadi et al., 2018). In *A. yokoscense* roots, a slight elevation in the expression levels of catalase genes (*Athyo6890c000010*, *Athyo71933c000010* and *Athyo38788000010*) was detected under the Cd stress condition by DEG analysis (Supplementary Table S1). However, no significant change was detected by qRT-PCR (Fig. 4B). DEGs of catalase genes showed less than 4-fold change (Supplementary Table S1); they were detected by analysis using a high q-value. Therefore, it seemed that catalase gene expression was little influenced by the Cd stress.

Balestri et al. (2014) reported that *Pteris vittata* is tolerant to 60 μM Cd conditions, but its root structure is damaged when it is exposed to 100 μM Cd. In contrast, even when exposed to 100 μM Cd, *A. yokoscense* showed no visual symptoms (Yoshihara et al., 2005) and no significant ROS signals (Fig. 5A–5D). We detected lower Cd concentrations in the proximal roots from *A. yokoscense* than in the distal roots (Fig. 1D, 1E). It has been reported that ROS are generated when Cd causes a deficiency in transition elements, such as iron, and reduces the role of glutathione working as an antioxidant (Andresen and Küpper, 2013). However, *A. yokoscense* generated little ROS under Cd exposure conditions (Fig. 5), whereas a large amount of ROS was generated in tobacco cells under Cd stress (Shukla et al., 2014). This suggests that Cd exposure causes low induction of ROS in *A. yokoscense* cells, implying that *A. yokoscense* has an unusual mechanism to avoid the translocation of Cd into the xylem.

Cross-sectional observation of the *A. yokoscense* roots showed the differentiation of tissues corresponding to epidermal cells and vascular bundles, as well as the existence of a casparian strip (Fig. 5E). A similar structure has been observed in the differentiated roots of *P. vittata*, another fern species, which has a casparian strip (Balestri et al., 2014). Interestingly, the casparian strip in *A. yokoscense* was stained by Fuchsin, suggesting that

it possesses a lignified cell wall (Fig. 5E). In maize, it has been reported that strong lignification occurred in the pericycle and inner cortical cells under exposure to Cd stress (Lux et al., 2011). It is thus possible that lignification of the casparian strip is involved in tolerance to Cd stress in *A. yokoscense*.

Even under strong stress condition with a high concentration of Cd exposure, *A. yokoscense* maintains its normal features without a substantial change in transporter and channel gene expression levels. It has been shown that these genes are strongly influenced by Cd stress in other plant species (Xu et al., 2012; He et al., 2015). Nishizono et al. (1987) have reported that tolerance to heavy metals is largely due to the role of the root cell wall in *A. yokoscense*, as 70–90% of metal elements (Cd, Cu and Zn) are localized in the cell wall. Our results strongly support their observation and may explain why few genes showed marked expression changes due to Cd exposure.

AUTHOR CONTRIBUTIONS

Y. U. performed the major experimental work, analyzed the results and wrote the manuscript. H. S. conceived and planned the project and supervised the research. H. S., K. M. and T. Y. designed the experiments, discussed the results and drafted the manuscript. K. I. performed the bioinformatics analysis. M. K. and S. Y. performed the plant analysis under Cd stress. H. T., K. K., K. S. and F. G. contributed to writing the manuscript, discussing the results and revising the paper. All authors read the manuscript and agree with its contents.

REFERENCES

- Ahmadi, H., Corso, M., Weber, M., Verbruggen, N., and Clemens, S. (2018) CAX1 suppressed Cd-induced generation of reactive oxygen species in *Arabidopsis helleri*. *Plant Cell Environ.* **41**, 2435–2448.
- Andresen, E., and Küpper, H. (2013) Cadmium toxicity in plants. In: *Cadmium: from Toxicity to Essentiality* (eds.: Sigel, A., Sigel, H., and Sigel, R. K. O.), pp. 395–413. Springer, Dordrecht.
- Baker, A. J. M., McGrath, S. P., Reeves, R. D., and Smith, J. A. C. (2000) Metal hyperaccumulator plants: a review of the ecology and physiology of a biochemical resource for phytoremediation of metal-polluted soils. In: *Phytoremediation of Contaminated Soil and Water* (eds.: Terry, N., and Bañuelos, G.), pp. 85–107. Lewis, Boca Raton, FL.
- Balestri, M., Ceccarini, A., Forino, L. M. C., Zelko, I., Martinka, M., Lux, A., and Castiglione, M. R. (2014) Cadmium uptake, localization and stress-induced morphogenic response in the fern *Pteris vittata*. *Planta* **239**, 1055–1064.
- Banks, J. A., Nishiyama, T., Hasebe, M., Bowman, J. L., Gribskov, M., dePamphilis, C., Albert, V. A., Aono, N., Aoyama, T., Ambrose, B. A., et al. (2011) The Selaginella genome identifies genetic changes associated with the evolution of vascular plants. *Science* **332**, 960–963.
- Barlow, P. W. (1969) Differences in response to colchicine by differentiating xylem cells in roots of *Pisum*. *Protoplasma* **68**, 79–83.
- Bolger, A. M., Lohse, M., and Usadel, B. (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformat-*

- ics **30**, 2114–2120.
- Carlquist, S., and Schneider, E. L. (2001) Vessels in ferns: structural, ecological, and evolutionary significance. *Am. J. Bot.* **88**, 1–13.
- Chen, F., Mackey, A. J., Stoeckert, C. J., Jr., and Roos, D. S. (2006) OrthoMCL-DB: querying a comprehensive multi-species collection of ortholog groups. *Nucleic Acids Res.* **34**, D363–D368.
- Clemens, S., Antosiewicz, D. M., Ward, J. M., Schachtman, D. P., and Schroeder, J. I. (1998) The plant cDNA *LCT1* mediates the uptake of calcium and cadmium in yeast. *Proc. Natl. Acad. Sci. USA* **95**, 12043–12048.
- Clemens, S. (2006) Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. *Biochimie* **88**, 1707–1719.
- Connolly, E. L., Fett, J. P., and Guerinot, M. L. (2002) Expression of the IRT1 metal transporter is controlled by metals at the levels of transcript and protein accumulation. *Plant Cell* **14**, 1347–1357.
- Fu, L., Niu, B., Zhu, Z., Wu, S., and Li, W. (2012) CD-HIT: accelerated for clustering the next-generation sequencing data. *Bioinformatics* **28**, 3150–3152.
- Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., et al. (2013) Trinity: reconstructing a full-length transcriptome without a genome from RNA-seq data. *Nat. Biotechnol.* **29**, 644–652.
- He, F., Liu, Q., Zheng, L., Cui, Y., Shen, Z., and Zheng, L. (2015) RNA-seq analysis of rice roots reveals the involvement of post-transcriptional regulation in response to cadmium stress. *Front. Plant Sci.* **6**, 1136.
- Huang, X., Wang, J., Aluru, S., Yang, S.-P., and Hillier, L. (2003) PCAP: a whole-genome assembly program. *Genome Res.* **13**, 2164–2170.
- Ishikawa, L. S., Ishimaru, Y., Igura, M., Kuramata, M., Abe, T., Senoura, T., Hase, Y., Arao, T., Nishizawa, N. K., and Nakanishi, H. (2012) Ion-beam irradiation, gene identification, and marker-assisted breeding in the development of low-cadmium rice. *Proc. Natl. Acad. Sci. USA* **109**, 19166–19171.
- Johnson, S., Eddy, S. R., and Portugaly, E. (2010) Hidden Markov model speed heuristic and iterative HMM search procedure. *BMC Bioinformatics* **11**, 431.
- Lersten, N. R. (1997) Occurrence of endodermis with a casparian strip in stem and leaf. *Bot. Rev.* **63**, 265–272.
- Li, H., and Durbin, R. (2010) Fast and accurate long-read alignment with Burrows–Wheeler transform. *Bioinformatics* **26**, 589–595.
- Li, J.-Y., Fu, Y.-L., Pike, S. M., Bao, J., Tian, W., Zhang, Y., Chen, C.-Z., Zhang, Y., Li, H.-M., Huang, J., et al. (2010) The *Arabidopsis* nitrate transporter NRT1.8 functions in nitrate removal from the xylem sap and mediates cadmium tolerance. *Plant Cell* **22**, 1633–1646.
- Liao, Y., Smyth, G. K., and Shi, W. (2014) featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **30**, 923–930.
- Love, M. I., Huber, W., and Anders, S. (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550.
- Lux, A., Martinka, M., Vaculík, M., and White, P. J. (2011) Root responses to cadmium in the rhizosphere: a review. *J. Exp. Bot.* **62**, 21–37.
- Misra, S., and Gedamu, L. (1989) Heavy metal tolerant transgenic *Brassica napus* L. and *Nicotiana tabacum* L. plants. *Theor. Appl. Genet.* **78**, 161–168.
- Mittler, R. (2002) Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.* **7**, 405–410.
- Miyadate, H., Adachi, S., Hiraizumi, A., Tezuka, K., Nakazawa, N., Kawamoto, T., Katou, K., Kodama, I., Sakurai, K., Takahashi, H., et al. (2011) OsHMA3, a P_{1B}-type of ATPase affects root-to-shoot cadmium translocation in rice by mediating efflux into vacuoles. *New Phytol.* **189**, 190–199.
- Morishita, T., and Boratynski, J. K. (1992) Accumulation of cadmium and other metals in organs of plants growing around metal smelters in Japan. *Soil Sci. Plant Nutri.* **38**, 781–785.
- Murashige, T., and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–497.
- Nishizono, H., Ichikawa, H., Suzuki, S., and Ishii, F. (1987) The role of the root cell wall in the heavy metal tolerance of *Athyrium yokoscense*. *Plant Soil* **101**, 15–20.
- Ortiz, D. F., Kreppel, L., Speiser, D. M., Scheel, G., McDonald, G., and Ow, D. W. (1992) Heavy metal tolerance in the fission yeast requires an ATP-binding cassette-type vacuolar membrane transporter. *EMBO J.* **11**, 3491–3499.
- Shukla, D., Huda, K. M. K., Banu, M. S. A., Gill, S. S., Tuteja, R., and Tuteja, N. (2014) OsACA6, a P-type 2B Ca²⁺ ATPase functions in cadmium stress tolerance in tobacco by reducing the oxidative stress load. *Planta* **240**, 809–824.
- Simão, F. A., Waterhouse, R. M., Ioannidis, P., Kriventseva, E. V., and Zdobnov, E. M. (2015) BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics* **31**, 3210–3212.
- Steudle, E., and Frensch, J. (1996) Water transport in plants: role of the apoplast. *Plant Soil* **187**, 67–79.
- Van Fleet, D. S. (1961) Histochemistry and function of the endodermis. *Bot. Rev.* **27**, 165–220.
- Van, T. K., Kang, Y., Fukui, T., Sakurai, K., Iwasaki, K., Aikawa, Y., and Phuong, M. N. (2006) Arsenic and heavy metal accumulation by *Athyrium yokoscense* from contaminated soil. *Soil Sci. Plant Nutri.* **52**, 701–710.
- Vermeer, J. E. M., von Wangenheim, D., Barberon, M., Lee, Y., Stelzer, E. H. K., Maizel, A., and Geldner, N. (2014) A spatial accommodation by neighboring cells is required for organ initiation in *Arabidopsis*. *Science* **343**, 178–183.
- White, P. J., and Broadley, M. R. (2003) Calcium in plants. *Ann. Bot.* **92**, 487–511.
- White, P. J., and Brown, P. H. (2010) Plant nutrition for sustainable development and global health. *Ann. Bot.* **105**, 1073–1080.
- Xu, J., Sun, J., Du, L., and Liu, X. (2012) Comparative transcriptome analysis of cadmium responses in *Solanum nigrum* and *Solanum torvum*. *New Phytol.* **196**, 110–124.
- Yoshihara, T., Suzui, N., Ishii, S., Kitazaki, M., Yamazaki, H., Kitazaki, K., Kawachi, N., Yin, Y.-G., Ito-Tanabata, S., Hashida, S., et al. (2014) A kinetic analysis of cadmium accumulation in a Cd hyper-accumulator fern, *Athyrium yokoscense* and tobacco plants. *Plant Cell Environ.* **37**, 1086–1096.
- Yoshihara, T., Tsunokawa, K., Miyano, Y., Arashima, Y., Hodoshima, H., Shoji, K., Shimada, H., and Goto, F. (2005) Induction of callus from a metal hypertolerant fern, *Athyrium yokoscense*, and evaluation of its Cd tolerance and accumulation capacity. *Plant Cell Rep.* **23**, 579–585.