

## Aberystwyth University

### *Expression of FIHMA3, a P<sub>1B2</sub>-ATPase from Festulolium loliaceum, correlates with response to cadmium stress*

Guo, Qiang; Meng, Lin; Humphreys, Mike W.; Scullion, John; Mur, Luis A.J.

*Published in:*

Plant Physiology and Biochemistry

*DOI:*

[10.1016/j.plaphy.2017.01.013](https://doi.org/10.1016/j.plaphy.2017.01.013)

*Publication date:*

2017

*Citation for published version (APA):*

Guo, Q., Meng, L., Humphreys, M. W., Scullion, J., & Mur, L. A. J. (2017). Expression of FIHMA3, a P<sub>1B2</sub>-ATPase from Festulolium loliaceum, correlates with response to cadmium stress. *Plant Physiology and Biochemistry*, 112, 270-277. <https://doi.org/10.1016/j.plaphy.2017.01.013>

#### Document License

CC BY-NC-ND

#### General rights

Copyright and moral rights for the publications made accessible in the Aberystwyth Research Portal (the Institutional Repository) are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the Aberystwyth Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the Aberystwyth Research Portal

#### Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

tel: +44 1970 62 2400

email: [is@aber.ac.uk](mailto:is@aber.ac.uk)

# Accepted Manuscript

Expression of *FIHMA3*, a vacuolar P<sub>1</sub>B<sub>2</sub>-ATPase from *Festulolium loliaceum*, correlates with response to cadmium stress

Qiang Guo, Lin Meng, Mike W. Humphreys, John Scullion, Luis A.J. Mur



PII: S0981-9428(17)30021-9

DOI: [10.1016/j.plaphy.2017.01.013](https://doi.org/10.1016/j.plaphy.2017.01.013)

Reference: PLAPHY 4781

To appear in: *Plant Physiology and Biochemistry*

Received Date: 18 October 2016

Revised Date: 12 January 2017

Accepted Date: 12 January 2017

Please cite this article as: Q. Guo, L. Meng, M.W. Humphreys, J. Scullion, L.A.J. Mur, Expression of *FIHMA3*, a vacuolar P<sub>1</sub>B<sub>2</sub>-ATPase from *Festulolium loliaceum*, correlates with response to cadmium stress, *Plant Physiology et Biochemistry* (2017), doi: 10.1016/j.plaphy.2017.01.013.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1 **Title:** Expression of *FIHMA3*, a vacuolar P<sub>1B2</sub>-ATPase from *Festulolium loliaceum*,  
2 correlates with response to cadmium stress

3 **Authors:** Qiang Guo<sup>a</sup>, Lin Meng<sup>a\*</sup>, Mike W. Humphreys<sup>b</sup>, John Scullion<sup>b</sup>, Luis A. J.  
4 Mur<sup>b</sup>

5 **Institutional addresses:** <sup>a</sup> Beijing Research and Development Center for Grass and  
6 Environment, Beijing Academy of Agriculture and Forestry Science, Beijing 100097, P.  
7 R. China; <sup>b</sup> Institute of Biological, Environmental and Rural Sciences, Aberystwyth  
8 University, Aberystwyth SY23 3DA, UK

9 **\*Corresponding author:** Lin Meng; E-mail: menglin9599@sina.com

10 **Tel:** +86-10-51503345

11 **Fax:** +86-10-51503297

12

13

14

15

16

17

18

19

20

21

22

**Abstract**

Heavy metal ATPase 3 (HMA3), a P<sub>1B2</sub>-ATPase, is a key tonoplast transporter involved in mediating the vacuolar sequestration of cadmium (Cd) to detoxify the intake of this element by plants. HMA3 expression in response to Cd stress has not been previously examined in the grass hybrid species *Festulolium loliaceum* (Huds.) P. Fourn. In this study, *FIHMA3* isolated from *F. loliaceum* was found to comprise 833 amino acid residues with 77% homology to the rice *OsHMA3*. Transient expression of *FIHMA3* fused to enhanced green fluorescent protein in *Arabidopsis* protoplasts suggested its localization to vacuolar membranes. Quantitative real-time RT-PCR analysis of *F. loliaceum* revealed that *FIHMA3* is expressed predominantly within roots and up-regulated by excess Cd. Over the 168 h treatment, Cd content of *F. loliaceum* roots was significantly higher than that of shoots, regardless of external CdCl<sub>2</sub> concentrations. A significant positive correlation was found between *FIHMA3* expression and Cd accumulation in roots of *F. loliaceum* seedlings subjected to 10–100 mg L<sup>-1</sup> CdCl<sub>2</sub> for 168 h or, in a separate experiment, to 25 or 100 mg L<sup>-1</sup> CdCl<sub>2</sub> for the same duration. These findings provide evidence that *FIHMA3* encodes a vacuolar P<sub>1B2</sub>-ATPase that may play an important role in Cd<sup>2+</sup> sequestration into root cell vacuoles, thereby limiting the entry of Cd<sup>2+</sup> into the cytoplasm and reducing Cd<sup>2+</sup> toxicity.

**Keywords:** cadmium, *Festulolium loliaceum*, *FIHMA3*, phytomediation, vacuole sequestration

22

## 1 **1. Introduction**

2 An expanding global population and the limited availability of agriculturally  
3 suitable land have stimulated interest in the agricultural potential of more marginal  
4 areas and contaminated “brown-field” locations on former industrial sites. Of the  
5 estimated 30,000 t of Cd released annually into the environment, 13,000 t are due to  
6 human activity (Gallego et al., 2012). A non-essential heavy metal with high toxicity  
7 to plants, Cd interferes with the homeostasis of essential elements such as zinc,  
8 calcium, and iron and initiates their displacement from proteins (Verbruggen et al.,  
9 2009; Guo et al., 2014). Cd contamination of soil has become a serious environmental  
10 concern and also threatens human health via its accumulation in the food chain  
11 (Satoh-Nagasawa et al., 2012). Crops and products derived from livestock raised on  
12 plant-based diets are important sources of heavy metals absorbed by humans  
13 (Peralta-Videa et al., 2009). Understanding the mechanism of Cd accumulation in  
14 plants and the factors affecting its deposition are crucial to reduce entry of Cd into the  
15 human food chain.

16 Plants have evolved alternative adaptation strategies to cope with Cd stress. One  
17 such mechanism involves production of phytochelatins (PCs), which are  
18 glutathione-derived peptides. PCs are synthesized in the cytosol, where they form  
19 PC-Cd complexes that are subsequently sequestered into vacuoles to reduce the  
20 deleterious effects of Cd accumulation in the cytosol (Mendoza-Cózatl et al., 2005;  
21 Clemens, 2006). This process plays an important role in heavy metal homeostasis and  
22 detoxification (Colangelo and Guerinot, 2006; Hanikenne and Nouet, 2011).  
23 Generally, vacuolar sequestration of  $\text{Cd}^{2+}$  can be mediated by heavy metal

1 transporters in plants (Hirschi et al., 2000; Song et al., 2003; Korenkov et al., 2007;  
2 Wojas et al., 2009). Among these transporters are P<sub>1B</sub>-ATPases (heavy metal  
3 P<sub>1B</sub>-ATPases, HMAs), a large group of ATP-driven pumps implicated in the transport  
4 of monovalent Cu<sup>+</sup>/Ag<sup>+</sup> (P<sub>1B1</sub>-ATPases) and divalent Zn<sup>2+</sup>/Cd<sup>2+</sup>/Co<sup>2+</sup>/Pb<sup>2+</sup>  
5 (P<sub>1B2</sub>-ATPases) heavy metal cations across plant membranes (Williams and Mills,  
6 2005). As is well known, HMAs are involved in removal of heavy metal ions from the  
7 cytosol into either the apoplast, the vacuole, or into other organelles (Hussain et al.,  
8 2004; Andrés-Colás et al., 2006; Kim et al., 2009). In *Arabidopsis thaliana*,  
9 *AtHMA1-AtHMA4* and *AtHMA5-AtHMA8* transport divalent and monovalent cations,  
10 respectively (Cobbett et al., 2003; Williams and Mills, 2005). *AtHMA3* belongs to the  
11 Zn<sup>2+</sup>/Cd<sup>2+</sup>/Co<sup>2+</sup>/Pb<sup>2+</sup> subgroup; localized in the tonoplast, it helps detoxify essential  
12 biological (Zn<sup>2+</sup>) and non-essential (Cd<sup>2+</sup>, Co<sup>2+</sup>, and Pb<sup>2+</sup>) heavy metals by  
13 participating in their vacuolar sequestration (Morel et al., 2009). *AtHMA3* is a major  
14 locus in *A. thaliana* responsible for the regulation of Cd accumulation (Chao et al.,  
15 2012). *AhHMA3* has high-level constitutive expression in *A. halleri*, a Zn  
16 hyperaccumulator and relative of *A. thaliana*, which suggests that this gene is  
17 involved in high Zn accumulation (Becher et al., 2004). Similarly, *TcHMA3* from  
18 *Thlaspi caerulescens*, a Cd hyperaccumulator, is highly expressed in leaves; it plays  
19 an important role in the detoxification of Cd by sequestering Cd into leaf vacuoles,  
20 thereby contributing to Cd hyperaccumulation and hypertolerance (Ueno et al., 2011).  
21 Among the nine *HMA* genes identified in rice is *OsHMA3*, a member of the  
22 Zn<sup>2+</sup>/Cd<sup>2+</sup>/Co<sup>2+</sup>/Pb<sup>2+</sup> subgroup (Miyadate et al., 2011; Takahashi et al., 2012).

1 *OsHMA3*, a tonoplast-localized transporter of Cd within root cells, plays a role in the  
2 sequestration of Cd<sup>2+</sup> into root cell vacuoles (Ueno et al., 2010). *OsHMA3* has been  
3 identified as the locus responsible for regulation of Cd accumulation in shoots of rice  
4 cultivars Anjana Dhan and Cho-Ko-Koku. When the function of this protein is lost,  
5 Cd<sup>2+</sup> passage through the xylem is increased, thereby leading to Cd<sup>2+</sup> accumulation in  
6 the shoots (Ueno et al., 2010; Miyadate et al., 2011). Sasaki et al. (2014) recently  
7 found that overexpression of *OsHMA3* contributed to reduced Cd<sup>2+</sup> accumulation in  
8 the grain and to enhanced Cd tolerance in rice. Taken together, the available evidence  
9 implies that *HMA3*-mediated vacuolar sequestration of heavy metals plays an  
10 important role in metal detoxification.

11 *Festulolium* grass hybrids combine many of the attributes of *Lolium* species  
12 (ryegrasses), such as high growth rates that provides high yields of nutritious,  
13 palatable fodder for livestock, and those of *Festuca* species (fescues) which provide  
14 resilience against different climatic and edaphic stresses (Humphreys et al., 2014).  
15 Natural *Festulolium* species hybrids exist, especially in undisturbed marginal  
16 grassland locations frequently exposed to stress conditions where productive *Lolium*  
17 species would be more disadvantageous (Humphreys et al., 1995). A range of  
18 synthetic *Festulolium* species hybrids are also being generated by grass breeders to  
19 achieve productive, stress-adapted varieties suitable for agriculture, with safeguards to  
20 assist withstanding various climatic conditions. The natural grass species hybrid  
21 *Festulolium loliaceum* survives in waterlogged soils prone to flooding and its  
22 synthetic varieties developed combat freezing temperatures or to mitigate incidents of

1 flooding (Macleod et al., 2013). *Festulolium loliaceum* was developed following the  
2 hybridisation of *Lolium perenne* (perennial ryegrass) with *Festuca pratensis* (meadow  
3 fescue). Eventhough *F. loliaceum* has been broadly recognized for its resilience to  
4 stress conditions, studies in this regard have been primarily confined to climatic rather  
5 than edaphic stresses; consequently, the potential use of this species for  
6 bioremediation and its tolerance to Cd-contaminated soils have not been previously  
7 explored. In this context, we isolated and characterized *FIHMA3* from *F. loliaceum*  
8 and verified its subcellular localization. We also analyzed *FIHMA3* expression in  
9 plants exposed to high Cd concentrations, which revealed patterns consistent with a  
10 role in the conferral of heavy metal tolerance. Our findings provide useful initial  
11 information on the potential future agricultural application of this grass hybrid on  
12 Cd-contaminated soils.

13



## 1 2. Materials and Methods

### 2 2.1 Plant growth conditions and treatments

3 Seeds of *Festulolium loliaceum* variety Prior ( $2n = 4x = 28$ ) were provided by the  
4 Institute of Biological, Environmental, and Rural Sciences (IBERS), Aberystwyth  
5 University. Seeds were sterilized with 5% sodium hypochlorite solution for 5 min,  
6 rinsed thoroughly with distilled water, and then germinated on moistened filter paper  
7 for 168 h at 25°C in dark. After emergence of plumules, uniform seedlings were  
8 selected and transferred into plastic containers filled with 0.6 L modified Hoagland's  
9 solution containing 2 mM KNO<sub>3</sub>, 1 mM NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 0.5 mM Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O, 0.5  
10 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 60 μM Fe-citrate, 92 μM H<sub>3</sub>BO<sub>3</sub>, 18 μM MnCl<sub>2</sub>·4H<sub>2</sub>O, 1.6 μM  
11 ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.6 μM CuSO<sub>4</sub>·5H<sub>2</sub>O, and 0.7 μM (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O for 5 weeks.  
12 The nutrient solution was renewed every 2 d. All seedlings were grown in a CE  
13 growth chamber under a 16 h/8 h day/night cycle at 25°C/18°C, a relative humidity of  
14 50% to 60%, and a light intensity of 200 μmol m<sup>-2</sup> s<sup>-1</sup>. Five-week-old plants were  
15 used for all treatments, with each treatment replicated eight times and each replicate  
16 comprising five individual plants. Containers for all treatments were arranged in a  
17 completely randomized block design. Two treatment approaches were used: (i)  
18 Hoagland's nutrient solution supplemented with 0, 10, 25, 50, and 100 mg L<sup>-1</sup> CdCl<sub>2</sub>  
19 for 168 h; and (ii) Hoagland's nutrient solution containing 25 or 100 mg L<sup>-1</sup> CdCl<sub>2</sub>,  
20 with plants removed following 0, 3, 6, 12, 24, 48, 72, 96, 120, 144, and 168 h  
21 exposure. These exposure concentrations were not selected to simulate field  
22 conditions. Rather they were determined to observe Cd uptake and stress of the plants  
23 over a short period of exposure in order to better understand plant mitigation

1 responses.

## 2 2.2. Calculation of relative growth rate

3 The relative growth rate (RGR) of whole plants was calculated using the formula  
4  $RGR = (\ln W_j - \ln W_i) / \Delta t$ , where  $W_i$  and  $W_j$  are dry weights before and after 168 h  
5 treatment, respectively, and  $\Delta t$  is elapsed time between the two measurements  
6 (Martínez et al., 2005).

## 7 2.3. Cloning of *FIHMA3*

8 Total RNA was extracted according to Guo et al. (2012) from roots of *F.*  
9 *looliaceum* seedlings exposed to 100 mg L<sup>-1</sup> CdCl<sub>2</sub> for 24 h. First-strand cDNA was  
10 synthesized from 2 µg total RNA using an oligo(dT) primer and PrimeScriptRTase  
11 (Takara). The partial cDNA fragment of *FIHMA3* was amplified by PCR using  
12 degenerate primers P1 and P2 (Table S1) designed based on the gene sequences of  
13 *BdHMA3* (*Brachypodium distachyon*, XM\_003561234), *HvHMA3* (*Hordeum vulgare*,  
14 KU212808), *OsHMA3* (*Oryza sativa*, XM\_015791882), *TaHMA3* (*Triticum aestivum*,  
15 KF683298), and *ZmHMA3* (*Zea mays*, XM\_008671782). PCR cycling conditions  
16 were as follows: 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30  
17 s, and 72°C for 40 s, with a final extension at 72°C for 10 min. The PCR product was  
18 purified from agarose gels, ligated into a pMD-19T vector (Takara), and sequenced by  
19 Sangon Biotech (China). The 5'- and 3'-ends of *FIHMA3* were obtained with 5'- and  
20 3'- Rapid Amplification of cDNA Ends kits (SMARTer RACE, Clontech) according  
21 to the manufacturer's instructions and specific primers P3 and P4, respectively (Table  
22 S1). These fragments were assembled to obtain the full-sequence of *FIHMA3* cDNA.

#### 1 2.4. Sequence analysis

2 A BLAST search was performed using the NCBI platform  
3 (<http://www.ncbi.nlm.nih.gov/BLAST>). Sequence analysis of cDNA and multiple  
4 alignments were performed with DNAMAN 8.0 software. Molecular mass and  
5 isoelectric point of the deduced protein encoded by *FIHMA3* was predicted using the  
6 ExPASy proteomics server (<http://www.expasy.org>). Hydrophobicity values were  
7 calculated using the program TMPRED available at <http://www.ch.embnet.org/>.  
8 Phylogenetic relationship of *FIHMA3* with other plant *HMA*s multiple sequence  
9 alignment was analyzed by multiple alignments using Clustal X software (Thompson  
10 et al., 1997). Then a phylogenetic tree was constructed by MEGA6.0 software using  
11 the neighbor-joining method with 1,000 bootstrap replicates (Tamura et al., 2011).

#### 12 2.5. Subcellular localization of *FIHMA3*

13 The open reading frame (ORF) of *FIHMA3* excluding the stop codon was  
14 amplified using PrimeSTAR HS DNA polymerase with primers P5 (Table S1, EcoRI  
15 restriction site underlined) and P6 (Table S1, KpnI restriction site underlined), cloned  
16 into a pMD-19T vector, and sequenced by Sangon Biotech. The amplified fragment  
17 was cut from the pMD-19T plasmid using EcoRI and KpnI restriction enzymes and  
18 cloned into a pBSHES-NL vector to generate a fusion with enhanced green  
19 fluorescent protein (EGFP) under the control of the CaMV35S promoter. The  
20 *FIHMA3*-GFP fusion construct or a non-GFP-tagged vector construct was transiently  
21 expressed in protoplasts isolated from *A. thaliana* Col-0 cell suspensions using the  
22 polyethylene glycol-mediated method (Yoo et al., 2007). Protoplasts containing the

1 plasmids were incubated at 23°C for 2–3 d in darkness. For FM4-64 staining,  
2 protoplasts were transferred into 50 µM FM4-64 in Murashige-Skoog medium  
3 containing 0.4 M mannitol for 10 min at 4°C according to the method of Ueda et al.  
4 (2001). Fluorescent signals from both GFP and FM4-64 in the protoplasts were then  
5 observed using an inverted Carl Zeiss LSM 710 confocal laser scanning microscope.  
6 GFP and FM4-64 were excited at 488 nm and 543 nm, respectively, with their  
7 corresponding fluorescence emission signals detected between 498–539 nm and  
8 580–650 nm, respectively.

### 9 2.6. Expression analysis of *FlHMA3*

10 Total RNA was extracted with Trizol kit (Takara) following the manufacturer's  
11 instructions. First-strand cDNA was synthesized from 2 µg of total RNA using an  
12 oligo(dT) primer and PrimeScriptRTase (Takara). Quantitative real-time RT-PCR  
13 (qRT-PCR) was performed using SYBR Premix Ex *Taq* II (Perfect Real Time) (Takara)  
14 on a StepOnePlus Real-Time PCR system (ABI) to monitor the amplification of each  
15 cDNA fragment. qRT-PCR amplification of *FlHMA3* was carried out with the primer  
16 pair P7 and P8 (Table S1), which yielded a 225 bp product. For use as a reference in  
17 the qRT-PCR, a 130 bp region of the *actin* gene was amplified using primers A1 and  
18 A2, which were designed according to the partial cDNA sequence of *actin* from *F.*  
19 *lioliceum* (Table S1). Primer sequences were designed with Primer 5.0 software. The  
20 amplification protocol consisted of an initial denaturation step of 95°C for 10 min,  
21 followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Gene  
22 expression data were normalized and relative expression was calculated by the  $2^{-\Delta\Delta C_t}$

1 method (Livak and Schmittgen, 2001). Each experiment included three biological  
2 replicates.

### 3 *2.7. Determination of Cd content*

4 Harvested plants were thoroughly washed with deionized water, divided into  
5 shoots and roots, and oven dried at 80°C to a constant weight. Dried shoots and roots  
6 were ground and digested in a mixture of HNO<sub>3</sub>/HClO<sub>4</sub> (5/1, v/v) for 24 h and then  
7 heated at 150–200°C to near dryness. After cooling, the residue was dissolved in  
8 distilled deionized water to a total volume of 20 mL. Cd content was determined  
9 using an atomic absorption spectrophotometer (AA-6300C, Shimadza, Kyoto, Japan).

### 10 *2.8. Statistical analysis*

11 All data were calculated as means plus standard deviation (SD). Statistical  
12 analyses, one-way analysis of variance, and Duncan's multiple range tests comparing  
13 treatment means were performed using SPSSv13.0 statistical software (SPSS Inc.,  
14 Chicago, IL, USA).

15

16

17

18

19

20

21

22

### 1 3. Results

#### 2 3.1. Characterization of *FlHMA3*

3 The full-length cDNA of *FlHMA3* isolated from roots was obtained by RT-PCR  
4 and rapid amplification of cDNA ends. The sequence, which comprised 2,957 bp,  
5 contained a 2,502 bp ORF encoding 833 amino acid residues with an estimated  
6 molecular mass of 87.58 kDa and a theoretical isoelectric point of 5.33. As shown in  
7 Fig. 1, multiple sequence alignment revealed that *FlHMA3* shares high homology  
8 with amino acid sequences of orthologs *TaHMA3* from wheat (83%) and *OsHMA3*  
9 from rice (77%) and, to a lesser extent, *AtHMA3* from *A. thaliana* (54%). This  
10 similarity indicates that *FlHMA3* is a P<sub>1B2</sub>-ATPase. A previous study has shown that  
11 *OsHMA3* encodes a divalent metal ion (Cd<sup>2+</sup>) transporter P<sub>1B2</sub>-ATPase involved in  
12 mediating the sequestration of Cd into vacuoles (Miyadate et al., 2011). Our  
13 phylogenetic analysis placed *FlHMA3* into a clade with other *HMA3* genes from  
14 closely related monocotyledonous species such as wheat (*TaHMA3*) and rice  
15 (*OsHMA3*) (Fig. S1). These results suggest that *FlHMA3* encodes a P<sub>1B2</sub>-ATPase  
16 transporter.

17 Hydrophobicity plot analysis of the deduced polypeptide showed that *FlHMA3*  
18 has eight transmembrane domains (Fig. 1, TM1 to TM8). Like all P-type ATPases, it  
19 contains the characteristic motifs for an ATP binding (GDGxNDx), a phosphorylation  
20 (DKTGTLT), and an HP locus forming a large cytoplasmic loop. Moreover, *FlHMA3*  
21 possesses a CPC ion transduction motif in TM5, and a heavy-metal-associated domain  
22 containing the motif GxCCxxE was located at the N terminus of *FlHMA3*.

#### 23 3.2. Transient expression of *FlHMA3* in mesophyll protoplasts of *Arabidopsis*

1 To determine the subcellular localization of *FIHMA3*, a GFP reporter construct  
2 was developed to express a fusion protein consisting of GFP fused to the C terminus  
3 of *FIHMA3* (FIHMA3:GFP) under the control of the CaMV35S promoter. This  
4 construct was then transfected into mesophyll cells of *A. thaliana* protoplasts using  
5 the polyethylene glycol method. Expression of the control (35S:GFP) resulted in  
6 distribution of GFP signals throughout the cytoplasm and the nucleus of mesophyll  
7 protoplasts (Fig. 2A). When FIHMA3:GFP was transfected into the protoplasts of  
8 mesophyll cells, however, the GFP signals were clearly separated, with FM 4-64  
9 signals in the area of the plasma membrane and tonoplast (Fig. 2B). This observation  
10 suggests that *FIHMA3*, in line with its presumed function, is localized specifically on  
11 the vacuolar membrane.

### 12 3.3. Expression patterns of *FIHMA3* in *F. loliaceum* exposed to Cd

13 To investigate the effect of Cd on transcription of *FIHMA3*, plants were treated  
14 with different concentrations of CdCl<sub>2</sub> for 168 h. As shown in Fig. 3A, mRNA levels  
15 of *FIHMA3* were 2.2–6.6 folds higher in roots than in shoots at external CdCl<sub>2</sub>  
16 concentrations of 10–100 mg L<sup>-1</sup>. Compared with the control, exposure to 10–100 mg  
17 L<sup>-1</sup> CdCl<sub>2</sub> led to significantly increased *FIHMA3* transcription in roots, with the  
18 highest transcription levels observed with the most concentrated solution (100 mg L<sup>-1</sup>  
19 CdCl<sub>2</sub>). The expression of *FIHMA3* was significantly lower in shoots and did not  
20 change in response to the increase in CdCl<sub>2</sub> concentration.

21 To further determine the kinetics for Cd<sup>2+</sup> induced activation of *FIHMA3* in  
22 tissues, *FIHMA3* transcript levels were examined in plants exposed to 25 and 100 mg

1  $L^{-1} CdCl_2$  over a 168 h period. When exposed to  $25 mg L^{-1} CdCl_2$ , *FIHMA3* transcript  
2 levels increased gradually in both roots and shoots from 3 to 168 h, but the magnitude  
3 was higher in roots than in shoots (Fig. 3B). In the case of  $100 mg L^{-1} CdCl_2$   
4 treatment, *FIHMA3* transcript levels increased rapidly in both shoots and roots from 3  
5 to 72 h; after peaking at 96 h, levels declined and remained constant following  
6 exposure for 120–168 h. Under this treatment, *FIHMA3* expression was always higher  
7 in roots than in shoots (Fig. 3C). Taken together, these results provide strong evidence  
8 to suggest that *FIHMA3* at least within the 168 h timeframe used in this study is  
9 expressed primarily in roots, with the extent of its transcription determined by the  
10 level of  $CdCl_2$  exposure.

#### 11 *3.4. Cd accumulation in tissues and its relationship with the expression levels of* 12 *FIHMA3*

13 As external  $CdCl_2$  concentrations were increased, Cd contents of shoots and roots  
14 increased progressively after 168 h of growth; however, Cd contents of roots were  
15 significantly higher than those of shoots at concentrations of  $10\text{--}100 mg L^{-1} CdCl_2$   
16 (Fig. 4A). Moreover, an increase in  $CdCl_2$  concentration from  $50$  to  $100 mg L^{-1}$  had  
17 no impact on Cd contents of shoots (Fig. 4A). We further observed a significant  
18 positive correlation between Cd content and *FIHMA3* expression level in roots of *F.*  
19 *loaliaceum* exposed to  $25\text{--}100 mg L^{-1} CdCl_2$  for 168 h (Fig. 4B). Despite the increased  
20 *FIHMA3* expression, however, the increase in Cd had a negative impact on plant  
21 growth rate even though roots did not appear to be necrotic or changed in overall  
22 length or branching and were therefore considered to be functional. A 14%–38%



1 reduction in relative growth rate compared with the control was recorded between the  
2 treatment extremes (10–100 mg L<sup>-1</sup> CdCl<sub>2</sub>) (Fig. S2). Despite this effect the *F.*  
3 *lohiaceum* plants survived following the treatments without further evidence of  
4 toxicity symptoms such as chlorosis or necrosis, consistent with our presumption that  
5 root functionality was maintained.

6 To monitor the differences in Cd accumulation in root and shoot tissue over time,  
7 we recorded changes in plants exposed to 25 or 100 mg L<sup>-1</sup> CdCl<sub>2</sub> over a 168 h period.  
8 The addition of either 25 or 100 mg L<sup>-1</sup> CdCl<sub>2</sub> significantly increased Cd content in  
9 both shoots and roots, but the content was always higher in roots than in shoots (Fig.  
10 5A). In plants undergone 100 mg L<sup>-1</sup> CdCl<sub>2</sub> treatment, the concentration of Cd in both  
11 shoots and roots was significantly greater than in tissues exposed to 25 mg L<sup>-1</sup> CdCl<sub>2</sub>  
12 from 3 to 168 h (Fig. 6A). In addition, we observed no significant difference in Cd  
13 accumulation by shoots in the presence of either 25 or 100 mg L<sup>-1</sup> CdCl<sub>2</sub> between 48  
14 and 168 h (Figs. 5A–6A). A significant positive correlation was found between Cd  
15 content and expression levels of *FIHMA3* in roots of *F. lohiaceum* exposed to either 25  
16 or 100 mg L<sup>-1</sup> CdCl<sub>2</sub> concentrations within the 3–168 h time frame (Figs. 5B–6B).  
17 This result provides supporting evidence to suggest an association between Cd  
18 tolerance and *FIHMA3* expression in *F. lohiaceum*.

19

20

21

22

#### 1 4. Discussion

2 P<sub>1B</sub>-ATPases are involved in heavy metal transport through biological membranes  
3 via an ATP-dependent process. P<sub>1B2</sub>-ATPases, which are unique to plants and have  
4 attracted much attention, play a critical role in controlling the translocation of Zn<sup>2+</sup> or  
5 Cd<sup>2+</sup> from roots to shoots and in sequestration of Cd<sup>2+</sup> from the cytoplasm into the  
6 vacuole (Cobbett et al., 2003; Morel et al., 2009; Mendoza-Cózatl et al., 2011).  
7 However, studies of similar proteins in non-model plants have been lacking. In this  
8 paper, we have presented the first characterization of a homologous P<sub>1B2</sub>-ATPase from  
9 a synthetic hybrid of the forage grass species *F. loliaceum*, an amphiploid species  
10 hybrid of the agricultural grasses *Lolium perenne* and *Festuca pratensis*. FIHMA3 has  
11 amino acid homologies of 54% and 77%, respectively, with AtHMA3 of *A. thaliana*  
12 and OsHMA3 of rice, both belonging to Zn<sup>2+</sup>/Cd<sup>2+</sup>/Co<sup>2+</sup>/Pb<sup>2+</sup> transporting group of  
13 P<sub>1B2</sub>-ATPases (Gravot et al., 2004; Miyadate et al., 2011). The FIHMA3 polypeptide  
14 sequence was found to possess the expected features of eight transmembrane domains  
15 and a CPC motif. The motifs DKTGTLLT, HP, and GDGxNDx shown in Fig. 1 are  
16 considered to be the domains of ion transduction, phosphorylation, and translocation  
17 of metal ions in the large cytoplasmic loop and for ATP binding. Similar motifs have  
18 been reported for AtHMA3 from *A. thaliana* (Gravot et al., 2004), GmHMA8 from  
19 soybean (Bernal et al., 2007), OsHMA3 from rice (Miyadate et al., 2011), TcHMA3  
20 from *T. caerulea* (Ueno et al., 2011), HvHMA2 from barley (Mills et al., 2012),  
21 and CsHMA3/4 from cucumber (Migocka et al., 2015). The GxCCxxE motif, which  
22 occurs in the N or C terminus of all plant P<sub>1B2</sub>-ATPases, is generally thought to be

1 associated with a heavy-metal-binding domain (Williams and Mills, 2005; Mills et al.,  
2 2010). In this study, a GxCCxxE motif was located in the N terminus of FIHMA3,  
3 thereby implying the presence of a heavy-metal-associated domain in this region (Fig.  
4 1). Overexpression of *OsHMA3* in a yeast mutant has been shown to affect sensitivity  
5 to  $\text{Cd}^{2+}$  and the ability to transport  $\text{Cd}^{2+}$  into vacuoles, which indicates that *HMA3* is  
6 responsible for sequestration of  $\text{Cd}^{2+}$  into vacuoles (Ueno et al., 2010; Miyadate et al.,  
7 2011). The methodologies we employed to demonstrate that *FIHMA3* similarly  
8 encodes a  $\text{P}_{1\text{B}2}$ -ATPases transporter involved in sequestration of  $\text{Cd}^{2+}$  into the vacuole.

9 First, we fused GFP to FIHMA3 to visualize the subcellular localization of the  
10 protein in wild-type *A. thaliana* cells. Confocal imaging revealed that FIHMA3 is  
11 specifically located on the vacuolar membrane (Fig. 2). Previous studies have found  
12 that AtHMA3 and OsHMA3 are localized on vacuolar membranes and are involved in  
13 transporting heavy metal ions from the cytoplasm into vacuoles (Gravot et al., 2004;  
14 Ueno et al., 2010). The consistency observed in localization of FIHMA3 to vacuolar  
15 membranes suggests a possible role for the protein in the transfer of  $\text{Cd}^{2+}$  from the  
16 cytoplasm into the vacuole across the vacuolar membrane. Furthermore, gene  
17 expression analysis indicated that *FIHMA3* was mainly expressed in roots of *F.*  
18 *loliaceum* exposed to  $\text{CdCl}_2$  stress (Fig. 3) as in the case of rice *OsHMA3* (Miyadate  
19 et al., 2011). An equivalent functional role may also be anticipated for *FIHMA3* as a  
20 determinant factor in the root in Cd tolerance. *FIHMA3* expression patterns in *F.*  
21 *loliaceum* do reflect a possible adaptation response to  $\text{CdCl}_2$  stress. Ueno et al. (2010)  
22 reported that OsHMA3 from a low  $\text{Cd}^{2+}$  accumulating cultivar (Nipponbare) functions

1 as a firewall by sequestering  $\text{Cd}^{2+}$  into the vacuoles of roots, thereby separating  $\text{Cd}^{2+}$   
2 from areal parts. A subsequent study confirmed that *OsHMA3* overexpression  
3 enhances tolerance to  $\text{Cd}^{2+}$  toxicity by increasing sequestration of  $\text{Cd}^{2+}$  into the  
4 vacuoles of root cells and then decreasing the translocation of toxic  $\text{Cd}^{2+}$  into shoots  
5 (Sasaki et al., 2014). One likely explanation is that only a limited amount of  $\text{Cd}^{2+}$  is  
6 loaded into the xylem from the root cells and subsequently translocated to the shoots  
7 (Miyadate et al., 2011). We note that a significant positive correlation was found  
8 between *FIHMA3* expression levels and Cd accumulation in roots of *F. loliaceum*  
9 exposed to different concentrations of Cd (10–100  $\text{mg L}^{-1}$ ) for 168 h or to 25 or 100  
10  $\text{mg L}^{-1}$  over the same time period (Figs. 4B–6B). This speculates that *FIHMA3* was  
11 detoxification of  $\text{Cd}^{2+}$  in *F. loliaceum* cells by enhancing the sequestration of  $\text{Cd}^{2+}$   
12 into the vacuole.

13 Both shoots and roots of *F. loliaceum* plants reached their highest Cd contents at  
14 the highest applied  $\text{CdCl}_2$  concentration (100  $\text{mg L}^{-1}$ ). The effect of this high  
15 concentration was morphologically reflected by a significant reduction in plant  
16 growth compared with the other Cd treatments (Figs. S2 and 4A). Most  
17 heavy-metal-accumulating plants generally exhibit slow growth rates, low biomass  
18 accumulation, and a tendency for altered root morphologies and necrosis (Clemens,  
19 2006). Although the use of *F. loliaceum* for bioremediation of polluted soils requires  
20 further scrutiny, the pilot study described herein suggests that this grass hybrid has  
21 potential in such an application. One limitation of the current study is that our Cd  
22 treatments, although extremely concentrated and highly toxic to plant growth, were

1 only applied over 168 h. Future research using the same *F. loliaceum* cultivar should  
2 include much longer exposures to Cd stress. Assuming the promising results reported  
3 here are observed over a longer time period, this grass may be used simultaneously  
4 for two functions: bioremediation of Cd-contaminated land and provision of fodder  
5 for livestock (its original agricultural role). In the latter case, the uptake of Cd<sup>2+</sup> into  
6 the shoots would have to be negligible to prevent harm to animals or subsequent entry  
7 into the food chain. An additional consideration is that other heavy metals are likely  
8 to be present in Cd-contaminated soils; the impact of these on *F. loliaceum* has yet to  
9 be assessed.

## 10 **5. Conclusion**

11 Our results demonstrate that *FIHMA3* encoding a P<sub>1B2</sub>-ATPase is a tonoplast  
12 transporter that may play important roles in the response of *F. loliaceum* to Cd stress.  
13 Further research should focus on understanding the mechanism in detail by using  
14 methods like mutation or gene silencing.

15

16

17

18

19

20

21

22

## 1 **Acknowledgements**

2 This research was financially supported by the International Cooperation Project  
3 (grant number GJHZ2015-1), the Institute for Science and Technology Innovation  
4 Team Project (grant number JNKST201604), the Scientific Innovation Ability  
5 Construction Project (grant number KJCX20140103) of the Beijing Academy of  
6 Agriculture and Forestry Sciences, and the Beijing Natural Science Foundation (grant  
7 number 6152008). Collaborations with IBERS, Aberystwyth University, were  
8 achieved through a memorandum of understanding and a material transfer agreement  
9 brought about following a Biotechnology and Biological Sciences Research Council  
10 International Partnering Award (BB/M027945/1). We are also grateful to the  
11 anonymous reviewers for their comments on the initial version of the manuscript.

12

13

14

15

16

17

18

19

20

21

22

1 **References**

- 2 Andrés-Colás, N., Sancenon, V., Rodriguez-Navarro, S., Mayo, S., Thiele, D.J., Ecker,  
3 J.R., Puig, S., Penarrubia, L., 2006. The Arabidopsis heavy metal P-type ATPase  
4 HMA5 interacts with metallochaperones and functions in copper detoxification of  
5 roots. *Plant J.* 45, 225–236.
- 6 Becher, M., Talke, I.N., Krall, L., Kramer, U., 2004. Cross-species microarray  
7 transcript profiling reveals high constitutive expression of metal homeostasis  
8 genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*. *Plant J.* 37,  
9 251–268.
- 10 Bernal, M., Testillano, P.S., Alfonso, M., Risuenõ, M.C., Picorel, R., Yruel, I., 2007.  
11 Identification and subcellular localization of the soybean copper P<sub>1B</sub>-ATPase  
12 GmHMA8 transporter. *J. Struct. Biol.* 158, 46–58.
- 13 Chao, D.Y., Silva, A., Baxter, I., Huang, Y.S., Nordborg, M., Danku, J., Lahner, B.,  
14 Yakubova, E., Salt, D.E., 2012. Genome-wide association studies identify heavy  
15 metal ATPase3 as the primary determinant of natural variation in leaf cadmium in  
16 *Arabidopsis thaliana*. *PLoS Genet.* 8, e1002923.
- 17 Clemens, S., 2006. Toxic metal accumulation, responses to exposure and mechanisms  
18 of tolerance in plants. *Biochimie* 88, 1707–1719.
- 19 Cobbett, C.S., Hussain, D., Haydon, M.J., 2003. Structural and functional  
20 relationships between type 1B heavy meta-transporting P-type ATPases in  
21 *Arabidopsis*. *New Phytol.* 159, 315–321.
- 22 Colangelo, E.P., Guerinot, M.L., 2006. Put the metal to the petal: metal uptake and

- 1 transport throughout plants. *Curr. Opin. Plant Biol.* 9, 322–330.
- 2 Gallego, S.M., Pena, L.B., Barcia, R.A., Azpilicueta, C.E., Iannone, M.F., Rosales,  
3 E.P., Zawoznik, M.S., Groppa, M.D., Benavides, M.P., 2012. Unravelling  
4 cadmium toxicity and tolerance in plants: insight into regulatory mechanisms.  
5 *Environ. Exp. Bot.* 83, 33–46.
- 6 Gravot, A., Lieutaud, A., Verret, F., Auroy, P., Vavasseau, A., Richaud, P., 2004.  
7 *AtHMA3*, a plant  $P_{1B}$ -ATPase, functions as a Cd/Pb transporter in yeast. *FEBS*  
8 *Lett.* 561, 22–28.
- 9 Guo, Q., Wang, P., Ma, Q., Zhang, J.L., Bao, A.K., Wang, S.M., 2012. Selective  
10 transport capacity for  $K^+$  over  $Na^+$  is linked to the expression levels of *PtSOS1* in  
11 halophyte *Puccinellia tenuiflora*. *Funct. Plant Biol.* 39, 1047–1057.
- 12 Guo, Q., Meng, L., Mao, P.C., Tian, X.X., 2014. An assessment of *Agropyron*  
13 *cristatum* tolerance to cadmium contaminated soil. *Biol. Plant.* 58, 174–178.
- 14 Hanikenne, M., Nouet, C., 2011. Metal hyperaccumulation and hypertolerance: a  
15 model for plant evolutionary genomics. *Curr. Opin. Plant Biol.* 14, 252–259.
- 16 Hirschi, K.D., Korenkov, V.D., Wilganowski, N.L., Wagner, G.J., 2000. Expression of  
17 *Arabidopsis CAX2* in tobacco altered metal accumulation and increased  
18 manganese tolerance. *Plant Physiol.* 124, 125–133.
- 19 Humphreys, M.W., Thomas, H.M., Morgan, W.G., Meredith, M.R., Harper, J.A.,  
20 Thomas, H., Zwierzykowski, Z., Ghesquiére, M., 1995. Discriminating the  
21 ancestral progenitors of hexaploid *Festuca arundinacea* using genomic *in-situ*  
22 hybridization. *Heredity* 75, 171–174.



- 1 Humphreys, M.W., O'Donovan, S.A., Farrell, M.S., Gay, A., Kingston-Smith, A.L.,  
2 2014. The potential of novel *Festulolium* ( $2n=4x=28$ ) hybrids as productive,  
3 nutrient-use-efficient fodder for ruminants. *Food Energy Secur.* 50, 1–13.
- 4 Hussain, D., Haydon, M.J., Wang, Y., Wong, E., Sherson, S.M., Young, J., Camakaris,  
5 J., Harper, J.F., Cobbett, C.S., 2004. P-type ATPase heavy metal transporters with  
6 roles in essential zinc homeostasis in *Arabidopsis*. *Plant Cell* 16, 1327–1339.
- 7 Kim, Y.Y., Choi, H., Segami, S., Cho, H.T., Martinoia, E., Maeshima, M., Lee, Y.,  
8 2009. AtHMA1 contributes to the detoxification of excess Zn (II) in *Arabidopsis*.  
9 *Plant J.* 58, 737–753.
- 10 Korenkov, V., Hirschi, K., CrutchWeld, J.D., Wagner, G.J., 2007. Enhancing tonoplast  
11 Cd/H antiport activity increases Cd, Zn, and Mn tolerance, and impacts root/shoot  
12 Cd partitioning in *Nicotiana tabacum* L. *Planta* 226, 1379–1387.
- 13 Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using  
14 real-time quantitative PCR and the  $2^{-\Delta\Delta CT}$  method. *Methods* 25, 402–408.
- 15 MacLeod, C.J.A., Humphreys, M.W., Whalley, R., Turner, L., Binley, A., Watts, C.W.,  
16 Skot, L., Joynes, A., Hawkins, S., King, I.P., O'Donovan, S., Haygarth, P.M.,  
17 2013. A novel grass hybrid to reduce flood generation in temperate regions. *Sci*  
18 *Rep.* 3, 1683.
- 19 Martínez, J.P., Kinet, J.M., Bajji, M., Lutts, S., 2005. NaCl alleviates polyethylene  
20 glycol-induced water stress in the halophyte species *Atriplex halimus* L. *J. Exp.*  
21 *Bot.* 419, 2430–2431.
- 22 Mendoza-Cózatl, D., Loza-Tavera, H., Hernández-Navarro, A., Moreno-Sánchez, R.,

- 1        2005. Sulfur assimilation and glutathione metabolism under cadmium stress in  
2        yeast, protists and plants. *FEMS Microbiol. Rev.* 29, 653–671.
- 3        Mendoza-Cózatl, D.G., Jobe, T.O., Hauser, F., Schroeder, J.I., 2011. Long distance  
4        transport, vacuolar sequestration, tolerance, and transcriptional responses induced  
5        by cadmium and arsenic. *Curr. Opin. Plant Biol.* 14, 554–562.
- 6        Migocka, M., Papierniak, A., Maciaszczyk-Dziubinska, E., Posyniak, E., Kosieradzka,  
7        A., 2015. Molecular and biochemical properties of two  $P_{1B2}$ -ATPases, CsHMA3  
8        and CsHMA4, from cucumber. *Plant Cell Environ.* 38, 1127–1141.
- 9        Mills, R.F., Valdes, B., Duke, M., Peaston, K.A., Lahner, B., Salt, D.E., Williams, L.E.  
10        2010. Functional significance of AtHMA4 C-terminal domain in planta. *PLoS*  
11        *One* 5, e13388.
- 12        Mills, R.F., Peaston, K.A., Runions, J., Williams, L.E., 2012. HvHMA2, a  $P_{1B}$ -ATPase  
13        from Barley, is highly conserved among cereals and functions in Zn and Cd  
14        transport. *PLoS One* 7, e42640.
- 15        Miyadate, H., Adachi, S., Hiraizumi, A., Tezuka, K., Nakazawa, N., Kawamoto, T.,  
16        Katou, K., Kodama, I., Sakurai, K., Takahashi, H., Satoh-Nagasawa, N.,  
17        Watanabe, A., Fujimura, T., Akagi, H., 2011. *OsHMA3*, a  $P_{1B}$ -type of ATPase  
18        affects root-to-shoot cadmium translocation in rice by mediating efflux into  
19        vacuoles. *New Phytol.* 189, 190–199.
- 20        Morel, M., Crouzet, J., Gravot, A., Auroy, P., Leonhardt, N., Vavasseur, A., Richaud,  
21        P., 2009. AtHMA3, a  $P_{1B}$ -ATPase allowing Cd/Zn/Co/Pb vacuolar storage in  
22        Arabidopsis. *Plant Physiol.* 149, 894–904.

- 1 Peralta-Videa, J.R., Lopez, M.L., Narayan, M., Saupe, G., Gardea-Torresdey, J., 2009.  
2 The biochemistry of environmental heavy metal uptake by plants: implications  
3 for the food chain. *Int. J. Biochem. Cell B.* 41, 1665–1677.
- 4 Satoh-Nagasawa, N., Mori, M., Nakazawa, N., Kawamoto, T., Nagato, Y., Sakurai, K.,  
5 Takahashi, H., Watanabe, A., Akagi, H., 2012. Mutations in rice (*Oryza sativa*)  
6 heavy metal ATPase 2 (*OsHMA2*) restrict the translocation of zinc and cadmium.  
7 *Plant Cell Physiol.* 53, 213–224.
- 8 Sasaki, A., Yamaji, N., Ma, J.F., 2014. Overexpression of *OsHMA3* enhances Cd  
9 tolerance and expression of Zn transporter genes in rice. *J. Exp. Bot.* 65,  
10 6013–6021.
- 11 Song, W.Y., Sohn, E.J., Martinoia, E., Yong, Y.Y., Jasinski, M., Forestier, C., Hwang,  
12 I., Lee, Y., 2003. Engineering tolerance and accumulation of lead and cadmium in  
13 transgenic plants. *Nat. Biotechnol.* 8, 914–919.
- 14 Takahashi, R., Bashir, K., Ishimaru, Y., Nishizawa, N.K., Nakanishi, H., 2012. The  
15 role of heavy-metal ATPases, HMAs, in zinc and cadmium transport in rice. *Plant*  
16 *Signal. Behav.* 7, 1605–1607.
- 17 Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011.  
18 MEGA5: molecular evolutionary genetics analysis using maximum likelihood,  
19 evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28,  
20 2731–2739.
- 21 Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. Higgins, D.G., 1997. The  
22 ClustalX windows interface: flexible strategies for multiple sequence alignment

- 1 aided by quality analysis tools. *Nucleic. Acids Res.* 24, 4876–4882.
- 2 Ueda, T., Yamaguchi, M., Uchimiya, H., Nakano, A., 2001. *Ara6*, a plant-unique  
3 novel type Rab GTPase, functions in the endocytic pathway of *Arabidopsis*  
4 *thaliana*. *EMBO J.* 20, 4730–4741.
- 5 Ueno, D., Yamaji, N., Kono, I., Huang, C.F., Ando, T., Yano, M., Ma, J.F., 2010. Gene  
6 limiting cadmium accumulation in rice. *Proc. Natl Acad. Sci. USA.* 107,  
7 16500–16505.
- 8 Ueno, D., Milner, M.J., Yamaji, N., Yokosho, K., Koyama, E., Clemencia Zambrano,  
9 M., Kaskie, M., Ebbs, S., Kochian, L.V., Ma, J.F., 2011. Elevated expression of  
10 *TcHMA3* plays a key role in the extreme Cd tolerance in a Cd-hyperaccumulating  
11 ecotype of *Thlaspi caerulescens*. *Plant J.* 66, 852–862.
- 12 Verbruggen, N., Hermans, C., Schat, H., 2009. Mechanisms to cope with arsenic or  
13 cadmium excess in plants. *Curr. Opin. Plant Biol.* 12, 364–372.
- 14 Williams, L.E., Mills, R.F., 2005. P<sub>1B</sub>-ATPases—an ancient family of transition metal  
15 pumps with diverse functions in plants. *Trends Plant Sci.* 10, 491–502.
- 16 Wojas, S., Hennig, J., Plaza, S., Geisler, M., Siemianowski, O., Sklodowska, A.,  
17 Ruszczyńska, A., Bulska, E., Antosiewicz, D.M., 2009. Ectopic expression of  
18 *Arabidopsis* ABC transporter MRP7 modifies cadmium root-to-shoot transport  
19 and accumulation. *Environ. Pollut.* 157, 2781–2789.
- 20 Yoo, S.D., Cho, Y. H., Sheen, J., 2007. *Arabidopsis* mesophyll protoplasts: a versatile  
21 cell system for transient gene expression analysis. *Nat. Protoc.* 2, 1565–1572.  
22

## 1 **Figure Legends**

2 **Fig. 1.** Sequence alignment of FIHMA3 with other HMA3s from higher plants.

3 Sources of P<sub>1B2</sub>-ATPases and their GenBank accession numbers are as follows:

4 AtHMA3 (*Arabidopsis thaliana*, NM\_119158), OsHMA3 (*Oryza sativa*,

5 XM\_015791882), and TaHMA3 (*Triticum aestivum*, KF683298). The sequences were

6 aligned using DNAMAN 8.0 software. Amino acid residues highlighted in black are

7 conserved in the three transporters. Identical and different amino acid residues are

8 indicated with white and blue, respectively. Eight putative transmembrane domains

9 (TM 1–TM 8) and several motifs are boxed.

10 **Fig. 2.** Subcellular localization of an FIHMA3-GFP fusion protein transiently

11 expressed in *Arabidopsis thaliana* mesophyll cells. (A) Images obtained using GFP

12 alone as the control. The red dye FM 4-64 was used to indicate the plasma membrane

13 location. (B) Images obtained when GFP was fused to the C terminus of FIHMA3. In

14 panels A and B from left to right, GFP signals, FM 4-64 signals, merged images of

15 GFP and FM 4-64 signals, and bright-field differential interference contrast (DIC)

16 images are shown. Bar = 5  $\mu$ m.

17 **Fig. 3.** *FIHMA3* expression in *F. loliaceum* exposed to (A) different concentrations of

18 CdCl<sub>2</sub> (0, 10, 25, 50, and 100 mg L<sup>-1</sup>) for 168 h or to 25 mg L<sup>-1</sup> CdCl<sub>2</sub> (B) or 100 mg

19 L<sup>-1</sup> CdCl<sub>2</sub> (C) over a 168-h period. The relative expression level of *FIHMA3* in shoots

20 and roots was analyzed by quantitative real-time RT-PCR. *Actin* was used as an

21 internal control. Experiments were repeated three times. Data are means  $\pm$  SD ( $n = 3$ )

22 and bars indicate SD. Different letters indicate significant differences at  $P < 0.05$

23 (Duncan's test).

1 **Fig. 4.** Cd content of tissues of *F. loliaceum* exposed to 10, 25, 50, and 100 mg L<sup>-1</sup>  
2 CdCl<sub>2</sub> for 168 h. (A) Cd content of shoots and roots. Five plants were pooled per  
3 replicate (*n* = 8). (B) Relationship between relative *FlHMA3* expression and Cd  
4 content of roots subjected to 10 (◆), 25 (▲), 50 (■), and 100 (●) mg L<sup>-1</sup> CdCl<sub>2</sub>  
5 treatment for 168 h (*n* = 3–8). Data are means ± SD and bars indicate SD. Different  
6 letters indicate significant differences at *P* < 0.05 (Duncan's test).

7 **Fig. 5.** Time course of Cd content of *F. loliaceum* exposed to 25 mg L<sup>-1</sup> CdCl<sub>2</sub> for 3 to  
8 168 h. (A) Cd content of shoots and roots. Five plants were pooled per replicate (*n* =  
9 8). (B) Relationship between relative *FlHMA3* expression and Cd content of roots  
10 subjected to 25 mg L<sup>-1</sup> CdCl<sub>2</sub> treatment for 3–168 h. Data are means ± SD (*n* = 3–8)  
11 and bars indicate SD.

12 **Fig. 6.** Time courses of Cd content of *F. loliaceum* exposed to 100 mg L<sup>-1</sup> CdCl<sub>2</sub> for 3  
13 to 168 h. (A) Cd content of shoots and roots. Five plants were pooled per replicate (*n*  
14 = 8). (B) Relationship between relative *FlHMA3* expression and Cd content of roots  
15 subjected to 100 mg L<sup>-1</sup> CdCl<sub>2</sub> treatment for 3–168 h. Data are means ± SD (*n* = 3–8)  
16 and bars indicate SD.

#### 17 **Supplementary Figure Legends**

18 **Supplementary Fig. 1.** Phylogenetic tree of *HMA*s. The tree was constructed by the  
19 neighbor-joining method. Genes and GenBank accession numbers are as follows:  
20 *AdHMA3* (*Arachis duranensis*, XM\_016078710), *AiHNA3* (*Arachis ipaensis*,  
21 XM\_016346129), *AhHMA3* (*Arabidopsis halleri*, AJ556182), *AhHMA4* (*Arabidopsis*  
22 *halleri*, AY960757), *AtHMA2* (*Arabidopsis thaliana*, NM\_119157), *AtHMA3*

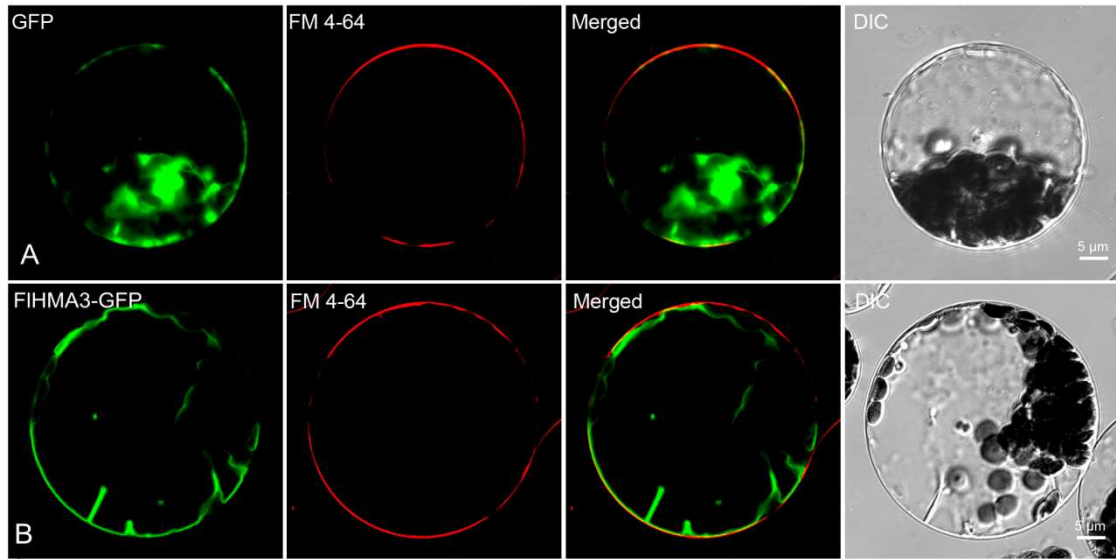
1 (*Arabidopsis thaliana*, NM\_119158), *AtHMA4* (*Arabidopsis thaliana*, AF412407),  
2 *BdHMA3* (*Brachypodium distachyon*, XM\_003561234), *BnHMA3* (*Brassica napus*,  
3 XM\_013849300), *BrHMA3* (*Brassica rapa*, XM\_009139644), *CaHMA3* (*Cicer*  
4 *arietinum*, XM\_012717947), *CsHMA3* (*Camelina sativa*, JX402100), *CmHMA3*  
5 (*Cucumis melo*, XM\_008455480), *EgHMA3* (*Elaeis guineensis*, XM\_010928912),  
6 *EugHMA3* (*Eucalyptus grandis*, XM\_010048654), *ErgHMA3* (*Erythranthe guttata*,  
7 XM\_012995791), •*FlHMA3* (*Festulolium loliaceum*), *FvHMA3* (*Fragaria vesca*,  
8 XM\_011464053), *GmHMA3* (*Glycine max*, XM\_006593460), *GrHMA3* (*Gossypium*  
9 *raimondii*, XM\_012589041), *HvHMA2* (*Hordeum vulgare*, GU177852), *HvHMA3*  
10 (*Hordeum vulgare*, KU212808), *JcHMA3* (*Jatropha curcas*, XM\_012211439),  
11 *MaHMA3* (*Musa acuminata*, XM\_009417251), *MnHM3* (*Morus notabilis*,  
12 XM\_010112413), *NbHMA3* (*Nicotiana tabacum*, XM\_016654239), *NcHMA4*  
13 (*Noccaea caerulescens*, JQ904704), *ObHMA3* (*Oryza brachyantha*, XM\_006658354),  
14 *OsHMA2* (*O. sativa*, HQ646362), *OsHMA3* (*O. sativa*, XM\_015791882), *PdHMA3*  
15 (*Phoenix dactylifera*, XM\_008803179), *PeHMA3* (*Populus euphratica*,  
16 XM\_011021683), *PmHMA3* (*Prunus mume*, XM\_008225567), *RcHMA3* (*Ricinus*  
17 *communis*, XM\_015727254), *SaHMA2* (*Sedum alfredii*, JQ012929), *SbHMA3*  
18 (*Sorghum bicolor*, XM\_002459533), *SiHMA3* (*Setaria italica*, XM\_012843843),  
19 *SlHMA3* (*Solanum lycopersicum*, XM\_004242795), *TaHMA2* (*Triticum aestivum*,  
20 HM021132), *TaHMA3* (*Triticum aestivum*, KF683298), *ThHMA3* (*Tarenaya*  
21 *hassleriana*, XM\_010550291), *TiHMA3* (*Triticum turgidum*, KF683295), *VvHMA3*  
22 (*Vitis vinifera*, XM\_010658478), and *ZmHMA3* (*Zea mays*, XM\_008671782).

- 1 **Supplementary Fig. 2.** Relative growth rate (RGR) of *F. loliaceum* exposed to 0, 10,
- 2 25, 50, or 100 mg L<sup>-1</sup> CdCl<sub>2</sub> for 168 h. Five plants were pooled per replicate ( $n = 8$ ).
- 3 Data are means  $\pm$  SD and bars indicate SD. Different letters indicate significant
- 4 differences at  $P < 0.05$  (Duncan's test).

ACCEPTED MANUSCRIPT





**Fig. 2**

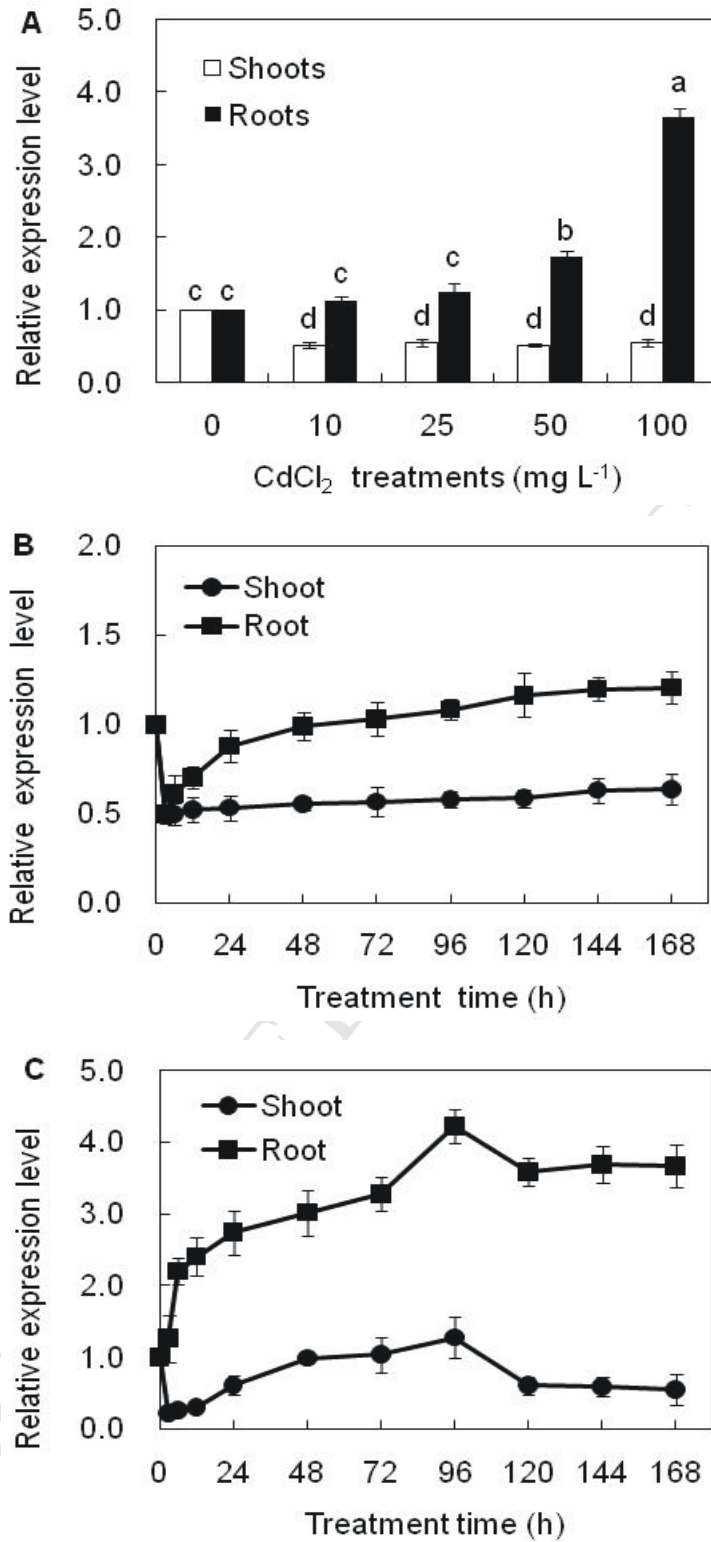


Fig. 3

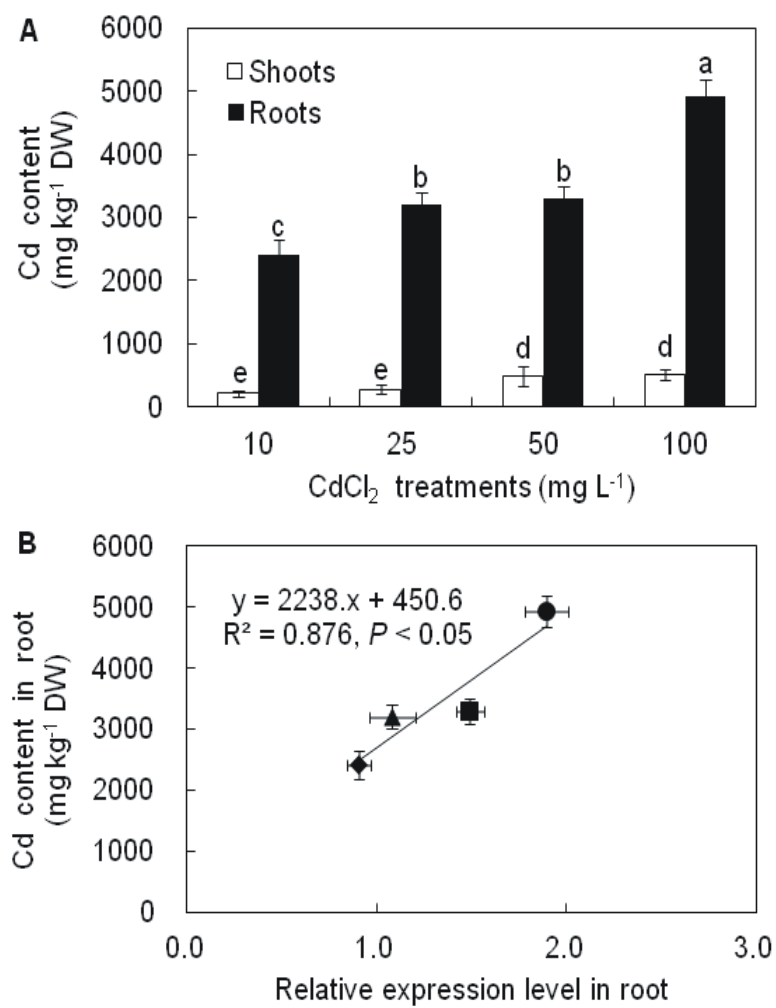


Fig. 4

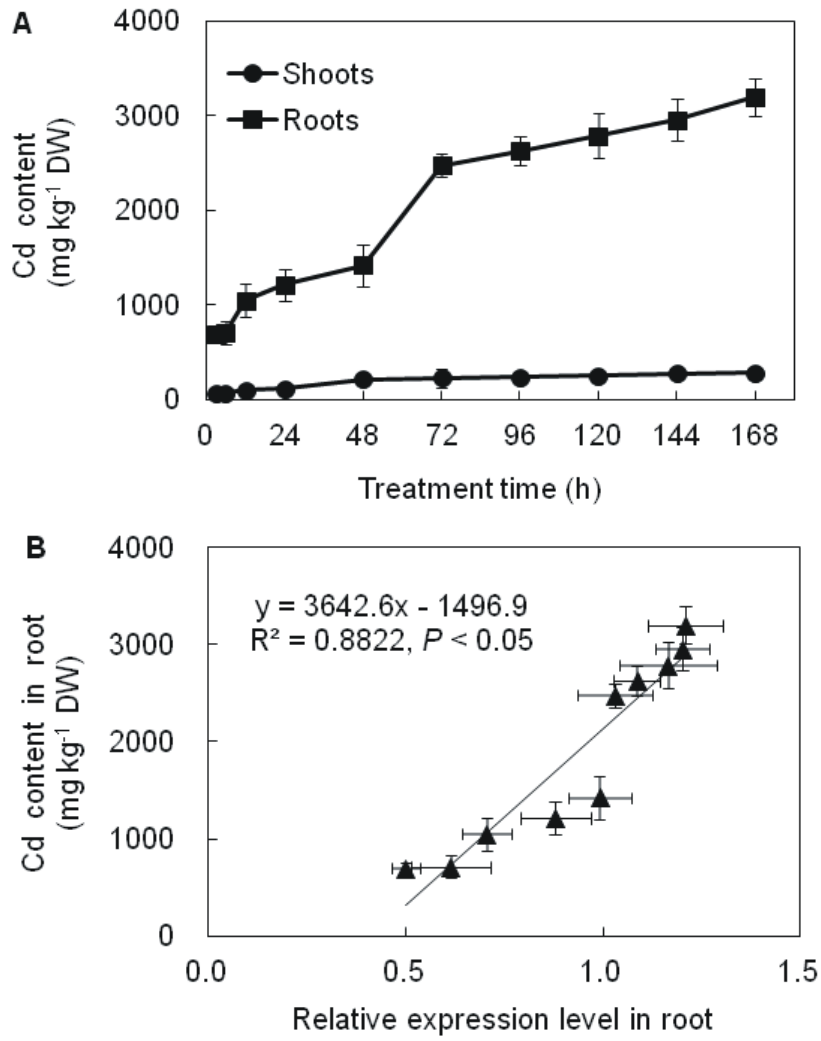


Fig. 5

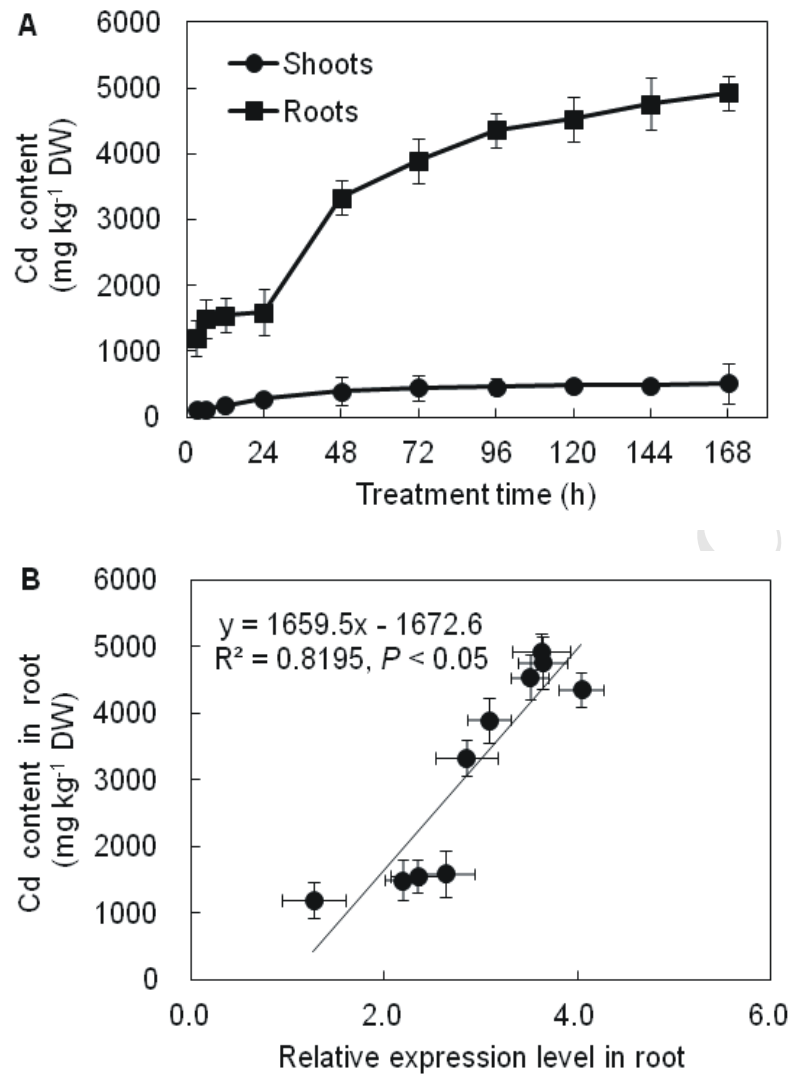
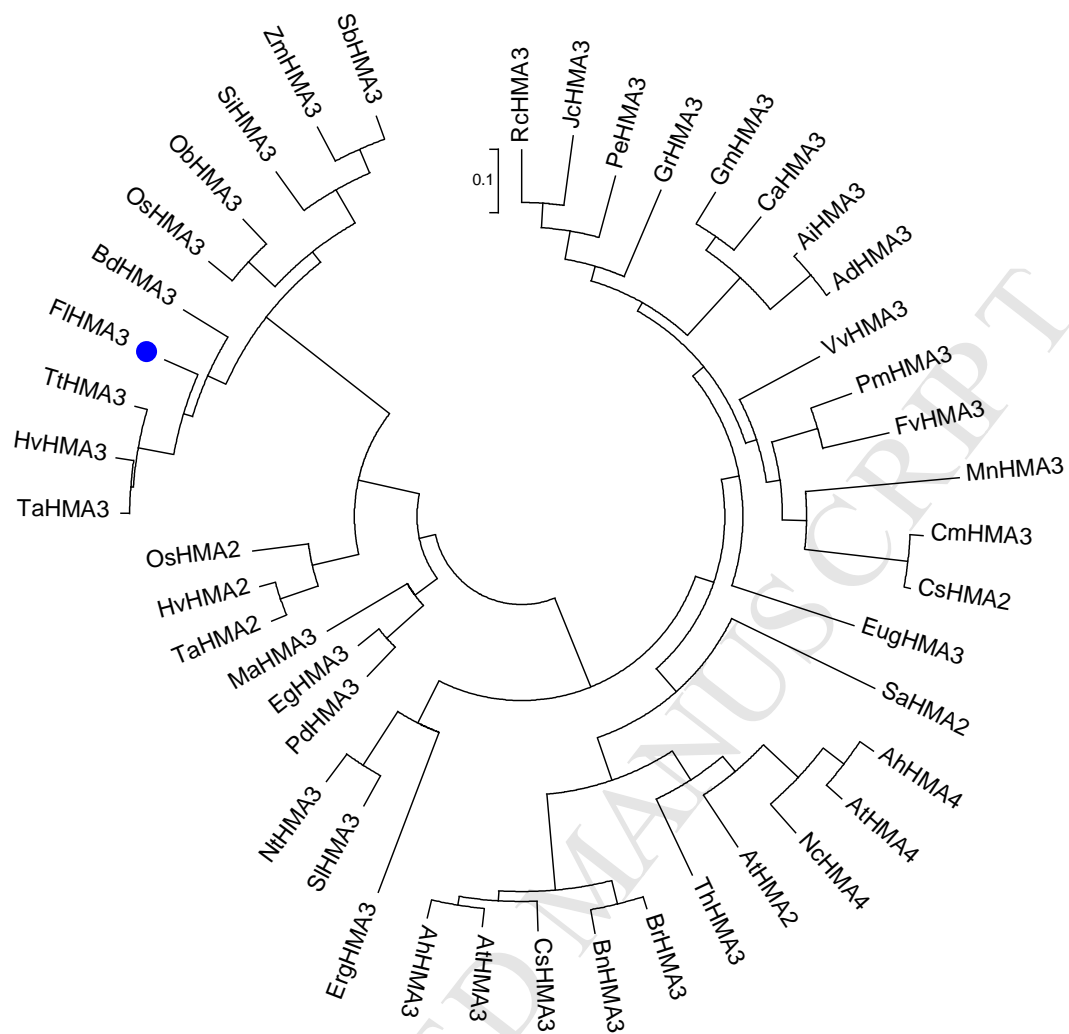
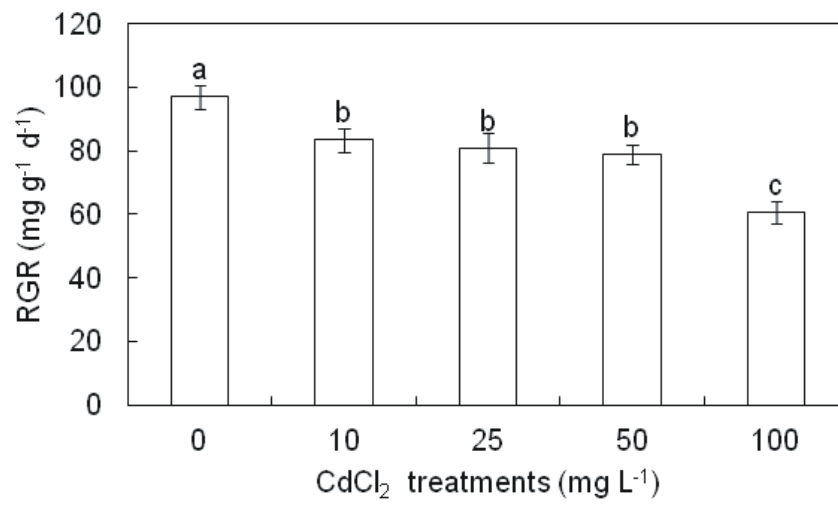


Fig. 6



Supplementary Fig. 1



**Supplementary Fig. 2**



**Supplementary Table 1**

Primer sequences used in the experiments

Primer	Sequence (5'-3')
P1	ATCAACRTYCTSATGCTYATCGC
P2	GGTGATSGTSCCGGTCTTGTCGA
P3	GGTTACATTGCCGTGAGGACGAC
P4	TCGACAAGACCGGCACCATCACC
P5	GGGGAATTCATGACGGACGGTGGCGAGAAC
P6	GGGGGTACCGTTTCACCAGAGCAGCATCCT
P7	GCTCAACCTGGACGGTTACA
P8	AACCATCGCTCCAGATCACC
A1	TCGAGACTGCGAAGAGTAGC
A2	TCCATGCCGATGATGGAAGG

## Highlights

- *FIHMA3* was mainly expressed in roots and up-regulated by excess Cd.
- *FIHMA3* was localized at the vacuolar membrane.
- A significant positive correlation was found between expression levels of *FIHMA3* and Cd accumulations in roots of *F. loliaceum* under Cd stress.
- $\text{Cd}^{2+}$  taken up by root cells may be sequestered into the vacuole via a pathway mediated by *FIHMA3* to reduce the concentration of this toxic metal in the cytoplasm.

**Author contributions**

Qiang Guo, Lin Meng and Mike W. Humphreys conceived and designed the experiments. Qiang Guo performed all the experiments and wrote the manuscript. John Scullion and Luis A.J. Mur review and polish the manuscript.