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Expression of *FIHMA3*, a vacuolar P_{1B2}-ATPase from *Festulolium Ioliaceum*, correlates with response to cadmium stress

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1	Title: Expression of <i>FlHMA3</i> , a vacuolar P_{1B2} -ATPase from <i>Festulolium loliaceum</i> ,
2	correlates with response to cadmium stress
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1 Abstract

2	Heavy metal ATPase 3 (HMA3), a P_{1B2} -ATPase, is a key tonoplast transporter
3	involved in mediating the vacuolar sequestration of cadmium (Cd) to detoxify the
4	intake of this element by plants. HMA3 expression in response to Cd stress has not
5	been previously examined in the grass hybrid species Festulolium loliaceum (Huds.) P.
6	Fourn. In this study, FlHMA3 isolated from F. loliaceum was found to comprise 833
7	amino acid residues with 77% homology to the rice OsHMA3. Transient expression of
8	FlHMA3 fused to enhanced green fluorescent protein in Arabidopsis protoplasts
9	suggested its localization to vacuolar membranes. Quantitative real-time RT-PCR
10	analysis of F. loliaceum revealed that FlHMA3 is expressed predominantly within
11	roots and up-regulated by excess Cd. Over the 168 h treatment, Cd content of F.
12	loliaceum roots was significantly higher than that of shoots, regardless of external
13	CdCl ₂ concentrations. A significant positive correlation was found between <i>FlHMA3</i>
14	expression and Cd accumulation in roots of F. loliaceum seedlings subjected to
15	10–100 mg L^{-1} CdCl ₂ for 168 h or, in a separate experiment, to 25 or 100 mg L^{-1}
16	$CdCl_2$ for the same duration. These findings provide evidence that <i>FlHMA3</i> encodes a
17	vacuolar P_{1B2} -ATPase that may play an important role in Cd^{2+} sequestration into root
18	cell vacuoles, thereby limiting the entry of Cd^{2+} into the cytoplasm and reducing Cd^{2+}
19	toxicity.

Keywords: cadmium, *Festulolium loliaceum*, *FlHMA3*, phytomediation, vacuole
sequestration

22

1 1. Introduction

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

16 Plants have evolved alternative adaptation strategies to cope with Cd stress. One such mechanism involves production of phytochelatins (PCs), which are 17 glutathione-derived peptides. PCs are synthesized in the cytosol, where they form 18 PC-Cd complexes that are subsequently sequestered into vacuoles to reduce the 19 deleterious effects of Cd accumulation in the cytosol (Mendoza-Cózatl et al., 2005; 20 Clemens, 2006). This process plays an important role in heavy metal homeostasis and 21 detoxification (Colangelo and Guerinot, 2006; Hanikenne and Nouet, 2011). 22 Generally, vacuolar sequestration of Cd²⁺ can be mediated by heavy metal 23

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_{1B} -ATPases (heavy metal
3	P _{1B} -ATPases, HMAs), a large group of ATP-driven pumps implicated in the transport
4	of monovalent Cu^+/Ag^+ (P _{1B1} -ATPases) and divalent $Zn^{2+}/Cd^{2+}/Co^{2+}/Pb^{2+}$
5	(P _{1B2} -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	$Zn^{2+}/Cd^{2+}/Co^{2+}/Pb^{2+}$ subgroup; localized in the tonoplast, it helps detoxify essential
12	biological (Zn^{2+}) and non-essential $(Cd^{2+}, Co^{2+}, and Pb^{2+})$ 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	$Zn^{2+}/Cd^{2+}/Co^{2+}/Pb^{2+}$ subgroup (Miyadate et al., 2011; Takahashi et al., 2012).

OsHMA3, a tonoplast-localized transporter of Cd within root cells, plays a role in the 1 sequestration of Cd²⁺ into root cell vacuoles (Ueno et al., 2010). OsHMA3 has been 2 3 identified as the locus responsible for regulation of Cd accumulation in shoots of rice cultivars Anjana Dhan and Cho-Ko-Koku. When the function of this protein is lost, 4 Cd^{2+} passage through the xylem is increased, thereby leading to Cd^{2+} accumulation in 5 the shoots (Ueno et al., 2010; Miyadate et al., 2011). Sasaki et al. (2014) recently 6 found that overexpression of OsHMA3 contributed to reduced Cd^{2+} accumulation in 7 the grain and to enhanced Cd tolerance in rice. Taken together, the available evidence 8 implies that HMA3-mediated vacuolar sequestration of heavy metals plays an 9 important role in metal detoxification. 10

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

flooding (Macleod et al., 2013). Festulolium loliaceum was developed following the 1 hybridisation of Lolium perenne (perennial ryegrass) with Festuca pratensis (meadow 2 3 fescue). Eventhough F. loliaceum has been broadly recognized for its resilience to stress conditions, studies in this regard have been primarily confined to climatic rather 4 than edaphic stresses; consequently, the potential use of this species for 5 bioremediation and its tolerance to Cd-contaminated soils have not been previously 6 explored. In this context, we isolated and characterized FlHMA3 from F. loliaceum 7 and verified its subcellular localization. We also analyzed FIHMA3 expression in 8 9 plants exposed to high Cd concentrations, which revealed patterns consistent with a role in the conferral of heavy metal tolerance. Our findings provide useful initial 10 information on the potential future agricultural application of this grass hybrid on 11 12 Cd-contaminated soils.

13

1 **2. Materials and Methods**

2 2.1 Plant growth conditions and treatments

Seeds of *Festulolium loliaceum* variety Prior (2n = 4x = 28) were provided by the 3 Institute of Biological, Environmental, and Rural Sciences (IBERS), Aberystwyth 4 University. Seeds were sterilized with 5% sodium hypochlorite solution for 5 min, 5 rinsed thoroughly with distilled water, and then germinated on moistened filter paper 6 for 168 h at 25°C in dark. After emergence of plumules, uniform seedlings were 7 selected and transferred into plastic containers filled with 0.6 L modified Hoagland's 8 solution containing 2 mM KNO₃, 1 mM NH₄H₂PO₄, 0.5 mM Ca(NO₃)₂·4H₂O, 0.5 9 mM MgSO₄·7H₂O, 60 µM Fe-citrate, 92 µM H₃BO₃, 18 µM MnCl₂·4H₂O, 1.6 µM 10 ZnSO₄·7H₂O, 0.6 μ M CuSO₄·5H₂O, and 0.7 μ M (NH₄)₆Mo₇O₂₄·4H₂O for 5 weeks. 11 The nutrient solution was renewed every 2 d. All seedlings were grown in a CE 12 growth chamber under a 16 h/8 h day/night cycle at 25°C/18°C, a relative humidity of 13 50% to 60%, and a light intensity of 200 μ mol m⁻² s⁻¹. Five-week-old plants were 14 used for all treatments, with each treatment replicated eight times and each replicate 15 16 comprising five individual plants. Containers for all treatments were arranged in a completely randomized block design. Two treatment approaches were used: (i) 17 Hoagland's nutrient solution supplemented with 0, 10, 25, 50, and 100 mg L^{-1} CdCl₂ 18 for 168 h; and (ii) Hoagland's nutrient solution containing 25 or 100 mg L^{-1} CdCl₂, 19 with plants removed following 0, 3, 6, 12, 24, 48, 72, 96, 120, 144, and 168 h 20 exposure. These exposure concentrations were not selected to simulate field 21 conditions. Rather they were determined to observe Cd uptake and stress of the plants 22 over a short period of exposure in order to better understand plant mitigation 23

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 = $(\ln Wj - \ln Wi)/\Delta t$, where Wi and Wj are dry weights before and after 168 h
5	treatment, respectively, and Δt is elapsed time between the two measurements
6	(Martínez et al., 2005).

7 2.3. Cloning of FlHMA3

Total RNA was extracted according to Guo et al. (2012) from roots of F. 8 *loliaceum* seedlings exposed to 100 mg L^{-1} CdCl₂ for 24 h. First-strand cDNA was 9 synthesized from 2 µg total RNA using an oligo(dT) primer and PrimeScriptRTase 10 (Takara). The partial cDNA fragment of *FlHMA3* was amplified by PCR using 11 degenerate primers P1 and P2 (Table S1) designed based on the gene sequences of 12 BdHMA3 (Brachypodium distachyon, XM_003561234), HvHMA3 (Hordeum vulgare, 13 KU212808), OsHMA3 (Oryza sativa, XM_015791882), TaHMA3 (Triticum aestivum, 14 KF683298), and ZmHMA3 (Zea mays, XM_008671782). PCR cycling conditions 15 were as follows: 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 16 s, and 72°C for 40 s, with a final extension at 72°C for 10 min. The PCR product was 17 purified from agarose gels, ligated into a pMD-19T vector (Takara), and sequenced by 18 Sangon Biotech (China). The 5'- and 3'-ends of FlHMA3 were obtained with 5'- and 19 3'- Rapid Amplification of cDNA Ends kits (SMARTer RACE, Clontech) according 20 to the manufacturer's instructions and specific primers P3 and P4, respectively (Table 21 S1). These fragments were assembled to obtain the full-sequence of *FlHMA3* cDNA. 22

1 2.4. Sequence analysis

performed 2 Α BLAST search using the NCBI platform was 3 (http://www.ncbi.nlm.nih.gov/BLAST). Sequence analysis of cDNA and multiple alignments were performed with DNAMAN 8.0 software. Molecular mass and 4 isoelectric point of the deduced protein encoded by FlHMA3 was predicted using the 5 ExPASy proteomics server (http://www.expasy.org). Hydrophobicity values were 6 calculated using the program TMPRED available at http://www.ch.embnet.org/. 7 Phylogenetic relationship of FlHMA3 with other plant HMAs multiple sequence 8 9 alignment was analyzed by multiple alignments using Clustal X software (Thompson et al., 1997). Then a phylogenetic tree was constructed by MEGA6.0 software using 10 the neighbor-joining method with 1,000 bootstrap replicates (Tamura et al., 2011). 11

12 2.5. Subcellular localization of FlHMA3

The open reading frame (ORF) of FlHMA3 excluding the stop codon was 13 amplified using PrimeSTAR HS DNA polymerase with primers P5 (Table S1, EcoRI 14 restriction site underlined) and P6 (Table S1, KpnI restriction site underlined), cloned 15 into a pMD-19T vector, and sequenced by Sangon Biotech. The amplified fragment 16 17 was cut from the pMD-19T plasmid using EcoRI and KpnI restriction enzymes and cloned into a pBSHES-NL vector to generate a fusion with enhanced green 18 fluorescent protein (EGFP) under the control of the CaMV35S promoter. The 19 FlHMA3-GFP fusion construct or a non-GFP-tagged vector construct was transiently 20 expressed in protoplasts isolated from A. thaliana Col-0 cell suspensions using the 21 polyethylene glycol-mediated method (Yoo et al., 2007). Protoplasts containing the 22

1	plasmids were incubated at 23°C for 2-3 d in darkness. For FM4-64 staining,
2	protoplasts were transferred into 50 μ M 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 <i>FlHMA3</i> 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	loliaceum (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 $2^{-\Delta\Delta Ct}$

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

3 2.7. Determination of Cd content

Harvested plants were thoroughly washed with deionized water, divided into
shoots and roots, and oven dried at 80°C to a constant weight. Dried shoots and roots
were ground and digested in a mixture of HNO₃/HClO₄ (5/1, v/v) for 24 h and then
heated at 150–200°C to near dryness. After cooling, the residue was dissolved in
distilled deionized water to a total volume of 20 mL. Cd content was determined
using an atomic absorption spectrophotometer (AA-6300C, Shimadza, Kyoto, Japan).
2.8. Statistical analysis

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

1 3. Results

2 3.1. Characterization of FlHMA3

The full-length cDNA of FlHMA3 isolated from roots was obtained by RT-PCR 3 and rapid amplification of cDNA ends. The sequence, which comprised 2,957 bp, 4 contained a 2,502 bp ORF encoding 833 amino acid residues with an estimated 5 molecular mass of 87.58 kDa and a theoretical isoelectric point of 5.33. As shown in 6 Fig. 1, multiple sequence alignment revealed that *FlHMA3* shares high homology 7 with amino acid sequences of orthologs TaHMA3 from wheat (83%) and OsHMA3 8 from rice (77%) and, to a lesser extent, AtHMA3 from A. thaliana (54%). This 9 similarity indicates that F1HMA3 is a P_{1B2}-ATPase. A previous study has shown that 10 OsHMA3 encodes a divalent metal ion (Cd^{2+}) transporter P_{1B2}-ATPase involved in 11 mediating the sequestration of Cd into vacuoles (Miyadate et al., 2011). Our 12 phylogenetic analysis placed FlHMA3 into a clade with other HMA3 genes from 13 closely related monocotyledonous species such as wheat (TaHMA3) and rice 14 (OsHMA3) (Fig. S1). These results suggest that FlHMA3 encodes a P_{IB2}-ATPase 15 transporter. 16

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

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

To determine the subcellular localization of FlHMA3, a GFP reporter construct 1 was developed to express a fusion protein consisting of GFP fused to the C terminus 2 3 of FlHMA3 (FlHMA3:GFP) under the control of the CaMV35S promoter. This construct was then transfected into mesophyll cells of A. thaliana protoplasts using 4 the polyethylene glycol method. Expression of the control (35S:GFP) resulted in 5 distribution of GFP signals throughout the cytoplasm and the nucleus of mesophyll 6 protoplasts (Fig. 2A). When FIHMA3:GFP was transfected into the protoplasts of 7 mesophyll cells, however, the GFP signals were clearly separated, with FM 4-64 8 signals in the area of the plasma membrane and tonoplast (Fig. 2B). This observation 9 suggests that FlHMA3, in line with its presumed function, is localized specifically on 10 the vacuolar membrane. 11

12 3.3. Expression patterns of FlHMA3 in F. loliaceum exposed to Cd

To investigate the effect of Cd on transcription of FlHMA3, plants were treated 13 with different concentrations of CdCl₂ for 168 h. As shown in Fig. 3A, mRNA levels 14 of FlHMA3 were 2.2–6.6 folds higher in roots than in shoots at external CdCl₂ 15 concentrations of 10–100 mg L^{-1} . Compared with the control, exposure to 10–100 mg 16 L^{-1} CdCl₂ led to significantly increased *FlHMA3* transcription in roots, with the 17 highest transcription levels observed with the most concentrated solution (100 mg L^{-1} 18 CdCl₂). The expression of *FlHMA3* was significantly lower in shoots and did not 19 change in response to the increase in CdCl₂ concentration. 20

To further determine the kinetics for Cd^{2+} induced activation of *FlHMA3* in tissues, *FlHMA3* transcript levels were examined in plants exposed to 25 and 100 mg

 L^{-1} CdCl₂ over a 168 h period. When exposed to 25 mg L^{-1} CdCl₂, *FlHMA3* transcript 1 levels increased gradually in both roots and shoots from 3 to 168 h, but the magnitude 2 was higher in roots than in shoots (Fig. 3B). In the case of 100 mg L^{-1} CdCl₂ 3 treatment, *FlHMA3* transcript levels increased rapidly in both shoots and roots from 3 4 to 72 h; after peaking at 96 h, levels declined and remained constant following 5 exposure for 120–168 h. Under this treatment, *FlHMA3* expression was always higher 6 in roots than in shoots (Fig. 3C). Taken together, these results provide strong evidence 7 to suggest that *FlHMA3* at least within the 168 h timeframe used in this study is 8 expressed primarily in roots, with the extent of its transcription determined by the 9 level of CdCl₂ exposure. 10

3.4. Cd accumulation in tissues and its relationship with the expression levels of
FlHMA3

As external CdCl₂ concentrations were increased, Cd contents of shoots and roots 13 increased progressively after 168 h of growth; however, Cd contents of roots were 14 significantly higher than those of shoots at concentrations of $10-100 \text{ mg } \text{L}^{-1} \text{CdCl}_2$ 15 (Fig. 4A). Moreover, an increase in $CdCl_2$ concentration from 50 to 100 mg L^{-1} had 16 no impact on Cd contents of shoots (Fig. 4A). We further observed a significant 17 positive correlation between Cd content and FlHMA3 expression level in roots of F. 18 *loliaceum* exposed to 25–100 mg L^{-1} CdCl₂ for 168 h (Fig. 4B). Despite the increased 19 FlHMA3 expression, however, the increase in Cd had a negative impact on plant 20 growth rate even though roots did not appear to be necrotic or changed in overall 21 length or branching and were therefore considered to be functional. A 14%-38% 22

reduction in relative growth rate compared with the control was recorded between the treatment extremes (10–100 mg L^{-1} CdCl₂) (Fig. S2). Despite this effect the *F*. *loliaceum* plants survived following the treatments without further evidence of toxicity symptoms such as chlorosis or necrosis, consistent with our presumption that root functionality was maintained.

To monitor the differences in Cd accumulation in root and shoot tissue over time, 6 we recorded changes in plants exposed to 25 or 100 mg L^{-1} CdCl₂ over a 168 h period. 7 The addition of either 25 or 100 mg L^{-1} CdCl₂ significantly increased Cd content in 8 both shoots and roots, but the content was always higher in roots than in shoots (Fig. 9 5A). In plants undergone 100 mg L^{-1} CdCl₂ treatment, the concentration of Cd in both 10 shoots and roots was significantly greater than in tissues exposed to 25 mg L^{-1} CdCl₂ 11 from 3 to 168 h (Fig. 6A). In addition, we observed no significant difference in Cd 12 accumulation by shoots in the presence of either 25 or 100 mg L^{-1} CdCl₂ between 48 13 and 168 h (Figs. 5A-6A). A significant positive correlation was found between Cd 14 content and expression levels of FlHMA3 in roots of F. loliaceum exposed to either 25 15 or 100 mg L^{-1} CdCl₂ concentrations within the 3–168 h time frame (Figs. 5B–6B). 16 This result provides supporting evidence to suggest an association between Cd 17 tolerance and FlHMA3 expression in F. loliaceum. 18

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1 **4. Discussion**

P_{1B}-ATPases are involved in heavy metal transport through biological membranes 2 3 via an ATP-dependent process. P_{1B2}-ATPases, which are unique to plants and have attracted much attention, play a critical role in controlling the translocation of Zn^{2+} or 4 Cd^{2+} from roots to shoots and in sequestration of Cd^{2+} from the cytoplasm into the 5 vacuole (Cobbett et al., 2003; Morel et al., 2009; Mendoza-Cózatl et al., 2011). 6 However, studies of similar proteins in non-model plants have been lacking. In this 7 paper, we have presented the first characterization of a homologous P_{1B2} -ATPase from 8 a synthetic hybrid of the forage grass species F. loliaceum, an amphiploid species 9 hybrid of the agricultural grasses Lolium perenne and Festuca pratensis. FIHMA3 has 10 amino acid homologies of 54% and 77%, respectively, with AtHMA3 of A. thaliana 11 and OsHMA3 of rice, both belonging to Zn²⁺/Cd²⁺/Co²⁺/Pb²⁺ transporting group of 12 P_{1B2}-ATPases (Gravot et al., 2004; Miyadate et al., 2011). The FIHMA3 polypeptide 13 sequence was found to possess the expected features of eight transmembrane domains 14 and a CPC motif. The motifs DKTGTLT, HP, and GDGxNDx shown in Fig. 1 are 15 considered to be the domains of ion transduction, phosphorylation, and translocation 16 of metal ions in the large cytoplasmic loop and for ATP binding. Similar motifs have 17 been reported for AtHMA3 from A. thaliana (Gravot et al., 2004), GmHMA8 from 18 soybean (Bernal et al., 2007), OsHMA3 from rice (Miyadate et al., 2011), TcHMA3 19 from T. caerulescens (Ueno et al., 2011), HvHMA2 from barley (Mills et al., 2012), 20 and CsHMA3/4 from cucumber (Migocka et al., 2015). The GxCCxxE motif, which 21 occurs in the N or C terminus of all plant P_{1B2}-ATPases, is generally thought to be 22

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 FlHMA3,
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 Cd^{2+} and the ability to transport Cd^{2+} into vacuoles, which indicates that <i>HMA3</i> is
6	responsible for sequestration of Cd^{2+} into vacuoles (Ueno et al., 2010; Miyadate et al.,
7	2011). The methodologies we employed to demonstrate that FlHMA3 similarly
8	encodes a P_{1B2} -ATPases transporter involved in sequestration of Cd^{2+} into the vacuole.
9	First, we fused GFP to FlHMA3 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 Cd^{2+} from the
16	cytoplasm into the vacuole across the vacuolar membrane. Furthermore, gene
17	expression analysis indicated that $FlHMA3$ was mainly expressed in roots of F .
18	<i>loliaceum</i> exposed to 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 FlHMA3 as a
20	determinant factor in the root in Cd tolerance. FlHMA3 expression patterns in F.
21	<i>loliaceum</i> do reflect a possible adaptation response to CdCl ₂ stress. Ueno et al. (2010)
22	reported that OsHMA3 from a low Cd ²⁺ accumulating cultivar (Nipponbare) functions

as a firewall by sequestrating Cd^{2+} into the vacuoles of roots, thereby separating Cd^{2+} 1 from areal parts. A subsequent study confirmed that OsHMA3 overexpression 2 enhances tolerance to Cd^{2+} toxicity by increasing sequestration of Cd^{2+} into the 3 vacuoles of root cells and then decreasing the translocation of toxic Cd²⁺ into shoots 4 (Sasaki et al., 2014). One likely explanation is that only a limited amount of Cd^{2+} is 5 loaded into the xylem from the root cells and subsequently translocated to the shoots 6 (Miyadate et al., 2011). We note that a significant positive correlation was found 7 between FlHMA3 expression levels and Cd accumulation in roots of F. loliaceum 8 exposed to different concentrations of Cd (10–100 mg L^{-1}) for 168 h or to 25 or 100 9 mg L^{-1} over the same time period (Figs. 4B–6B). This speculates that *FlHMA3* was 10 detoxification of Cd^{2+} in F. loliaceum cells by enhancing the sequestration of Cd^{2+} 11 into the vacuole. 12

Both shoots and roots of F. loliaceum plants reached their highest Cd contents at 13 the highest applied $CdCl_2$ concentration (100 mg L⁻¹). The effect of this high 14 concentration was morphologically reflected by a significant reduction in plant 15 growth compared with the other Cd treatments (Figs. S2 and 4A). Most 16 heavy-metal-accumulating plants generally exhibit slow growth rates, low biomass 17 accumulation, and a tendency for altered root morphologies and necrosis (Clemens, 18 2006). Although the use of F. loliaceum for bioremediation of polluted soils requires 19 further scrutiny, the pilot study described herein suggests that this grass hybrid has 20 potential in such an application. One limitation of the current study is that our Cd 21 treatments, although extremely concentrated and highly toxic to plant growth, were 22

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 ²⁺ 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 <i>FlHMA3</i> encoding a P_{1B2} -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.
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1 Acknowledgements

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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_{1B} -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., Vavasseu, A., Richaud, P., 2004.
7	AtHMA3, a plant P1B-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 <i>PtSOS1</i> 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., Tezuka1, 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

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, NK., 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 _{1B} -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., Antosiewic, 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.
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1 Figure Legends

Fig. 1. Sequence alignment of FIHMA3 with other HMA3s from higher plants. 2 Sources of P_{1B2}-ATPases and their GenBank accession numbers are as follows: 3 AtHMA3 (Arabidopsis thaliana, NM_119158), OsHMA3 (Oryza 4 sativa, XM 015791882), and TaHMA3 (Triticum aestivum, KF683298). The sequences were 5 aligned using DNAMAN 8.0 software. Amino acid residues highlighted in black are 6 conserved in the three transporters. Identical and different amino acid residues are 7 indicated with white and blue, respectively. Eight putative transmembrane domains 8 (TM 1–TM 8) and several motifs are boxed. 9

Fig. 2. Subcellular localization of an FIHMA3-GFP fusion protein transiently expressed in *Arabidopsis thaliana* mesophyll cells. (A) Images obtained using GFP alone as the control. The red dye FM 4-64 was used to indicate the plasma membrane location. (B) Images obtained when GFP was fused to the C terminus of FIHMA3. In panels A and B from left to right, GFP signals, FM 4-64 signals, merged images of GFP and FM 4-64 signals, and bright-field differential interference contrast (DIC) images are shown. Bar = 5 μ m.

Fig. 3. *FlHMA3* expression in *F. loliaceum* exposed to (A) different concentrations of CdCl₂ (0, 10, 25, 50, and 100 mg L⁻¹) for 168 h or to 25 mg L⁻¹ CdCl₂ (B) or 100 mg L⁻¹ CdCl₂ (C) over a 168-h period. The relative expression level of *FlHMA3* in shoots and roots was analyzed by quantitative real-time RT-PCR. *Actin* was used as an internal control. Experiments were repeated three times. Data are means \pm SD (n = 3) and bars indicate SD. Different letters indicate significant differences at P < 0.05(Duncan's test).

1	Fig. 4. Cd content of tissues of <i>F. loliaceum</i> exposed to 10, 25, 50, and 100 mg L^{-1}
2	$CdCl_2$ for 168 h. (A) Cd content of shoots and roots. Five plants were pooled per
3	replicate $(n = 8)$. (B) Relationship between relative <i>FlHMA3</i> expression and Cd
4	content of roots subjected to 10 (\blacklozenge), 25 (\blacktriangle), 50 (\blacksquare), and 100 (\bullet) mg L ⁻¹ CdCl ₂
5	treatment for 168 h ($n = 3-8$). Data are means \pm 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 <i>F. loliaceum</i> exposed to 25 mg L^{-1} CdCl ₂ 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 ⁻¹ CdCl ₂ treatment for 3–168 h. Data are means \pm SD ($n = 3-8$)
11	and bars indicate SD.
12	Fig. 6. Time courses of Cd content of <i>F. loliaceum</i> exposed to 100 mg L^{-1} CdCl ₂ 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 ⁻¹ CdCl ₂ treatment for 3–168 h. Data are means \pm SD ($n = 3-8$)
16	and bars indicate SD.

17 Supplementary Figure Legends

Supplementary Fig. 1. Phylogenetic tree of *HMAs*. The tree was constructed by the
neighbor-joining method. Genes and GenBank accession numbers are as follows: *AdHMA3* (*Arachis duranensis*, XM_016078710), *AiHNA3* (*Arachis ipaensis*,
XM_016346129), *AhHMA3* (*Arabidopsis halleri*, AJ556182), *AhHMA4* (*Arabidopsis 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), TtHMA3 (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⁻¹ CdCl₂ 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).

Chillip Marker

F1HMA3 TaHMA3 OsHMA3 AtHMA3	NTOGONRRGATSDTALEESLLPKEAAAASTRRKYEKTYLDVLGVCCSAE GALVERULAPLDGVRAMSVVMESRTVLVBHDPEATSGSRIVEWINGGAGLEASVRAYGSSGIJSRWFSEYIVAGGVLLLASSSGLLPURALALGAGAGAEEMULRGPAAASRLTLDIN UWLLAVAGAVALKDY MMGGGSYPALEASLLAEEAAARROWEKTYLDVLGVCCSAEGALVERULAPLDGVRAVAVWESRTVVVBHDPAAVSGSRIVEWINGGAGLEASVRAYGSSGIJSRWFSEYIVAGALLLASSRRVLLPHOMLALGAGAGAEEMVLRGUPAASRLALDIN UWLLAVAGAVALKDY MAGKDDAEGLEARLLLLIPPEAAAEEPTRCGGGDGGGGGRRKKKTUDVLGVCCSAEGALVERUVEWINGGRUVSVVVASRTVVVBHDPAAPSGIVKAUGSSGVVSRWFSEYIVAGAULFEFEQOLAVVAVVAGAEEMVRRGPAASRLSLDIN UWLLAVAGAVALKDY MAGKDDAEGLEARLLLLIPPEAAAEEPTRCGGGDGGGGRRKKKTUDVLGVCCSAEGALVERUVEWINGSRTVVVBHDFALSGVRAKGSSGVVSRWFSEYIVAGEVLASSGVULFFFQOLAVVAVVAGAEEMVRRGPAASRLSLDIN UWLLAVAGAUCUGDY MAGKDDAEGLEARLLLLIPPEAAAEEPTRCGGGDGGGGRRKKKTUDVLGVCCSAEGALVERUVEWINGSRTVVVBHDFALSGVVRAKGSSGVVSRWFSEYIVAGAE	186 177 192 163
F1HMA3 TaHMA3 OsHMA3 AtHMA3	TM1 TM2 TM3 TEAGRIVELETTARNIDTIRCTRASAGUSSINGNIERKAVIAPTERVAVARDIEVEAVIAURAGEVUEVDEVDEVDEVDEVDEVDEVDEVDEVDEVDEVDEVDEVDE	386 377 392 363
F1HMA3 TaHMA3 OsHMA3 AtHMA3	TM 4 TM 5 An EC2LLTAR RMGIIVEGED TAVAE DETGTID AGESTID AGESTID AGENVED. EVQITE LIVE ISSID RESERVADAN VEYACEN SET OF REEVANDERI INCOMENTAL RESERVED IN SALAR RSCHTDTVPEKIDDLEGVSIG VICED DIVEVESSIGUES SET OF RESERVED AND VEYACEN SET OF REEVANDERI INCOMENTAL RESERVED ISSUES AGEN RAVAE DETGTID AGENT DEVELOPERINGLES VER DIVER SET OF REEVANDERI INCOMENTAL RESERVED AGENT AD AGE	585 575 591 514
F1HMA3 TaHMA3 OsHMA3 AtHMA3	QU GGVLEELHSATHEEDKVRLVGNIKARAGFTMMVGDGINDA GALAMADVGVSMGISGSAAAMETSHATIMSSTILKVEAAVTIGRFVRRTILVNVVASVAAKAAVIAUGTCLLVVINSMULLGEGRTTGRGKEDACRATARSUDMRRSQLAAIASSTAAPTTASGEKDCHCCCKQSESD RUGGALEELHSETHEEDKVRLVGALKARAGFTMMVGDGNDA GALATADVGVSMGISGSAAAMETSHATIMSSTILKVEAAVTIGRFARRTIAVNVSSVAAKAAVIAUAVAAVLADVGTCLLVVINSMULLGEGRRTGRGKEDACRATARSUDMRRSQLAAIASSTAAPTTASGEKDCHCCCKQSESD GU GGVMEELHSETHEEDKVRLVGGIKARFGFTMMVGDGNDA GALATADVGVSMGISGSAAAMETSHATIMSSTILKVEAAVIGRFARRTIAVNVSSVAAKAAVIAUAVAAVLADVGTCLLVVINSMULLGEGRRTGRGKEDACRATARSUDMRRSQLAAIASSTAAPTTASGEKDCHCCCKKSRSP QU GGVMEELHSETHEEDKVRLVGGIKARFGFTMMVGDGNDA GALATASUGKSSAAAMETSHATIMSSTILKVEAAVIGRFARRTIAVNVAGSVAAKAAVIAUAVAAVLADVGTCLLVVINSMULLGEGRRF.RKKED QU GGVMEELHSETHEEDKVRLVGGIKARFGFTMMVGDGNDA GALATASUGKSGSAAAMETSHATIMSSTILKVEGARGAGREQTNGCRCCFKFGMSP QU ENALDINHSETHEEDKVRLVGGIKARFGFTMMVGDGINDA GALAKADIGISGSAAAMETSHATIMSTILKEEKKGGAKEDGACRATARSUMAAVAADAGAAGREQTNGCRCCFKFGMSP	781 774 790 709
F1HMA3 TaHMA3 OsHMA3 AtHMA3	TM 6 TM 7 TM 8 EHTVAIDIPADEHRQEELMCAFMAGHVTAGSDCPSVMPISSSCASQCCSGE EHSVAIDVRVDECRE	833 816 990 711
F1HMA3 TaHMA3 OsHMA3 AtHMA3	RCCARTMNSGEVK	833 816 1003 711
	Fig. 1	
	Fig. 1	





Fig. 2



Fig. 3











Supplementary Table 1

Primer	Sequence (5'-3')
P1	ATCAACRTYCTSATGCTYATCGC
P2	GGTGATSGTSCCGGTCTTGTCGA
P3	GGTTACATTGCCGTGAGGACGAC
P4	TCGACAAGACCGGCACCATCACC
P5	GGG <u>GAATTC</u> ATGACGGACGGTGGCGAGAAC
P6	GGG <u>GGTACC</u> GTTTCACCAGAGCAGCATCCT
P7	GCTCAACCTGGACGGTTACA
P8	AACCATCGCTCCAGATCACC
A1	TCGAGACTGCGAAGAGTAGC
A2	TCCATGCCGATGATGGAAGG

Primer sequences used in the experiments

Highlights

- FlHMA3 was mainly expressed in roots and up-regulated by excess Cd.
- FlHMA3 was localized at the vacuolar membrane.
- A significant positive correlation was found between expression levels of FlHMA3

and Cd accumulations in roots of F. loliaceum under Cd stress.

 \bullet Cd²⁺ 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.

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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.