

Genome-wide characterization of the HIPP gene family in *Panicum virgatum* reveals the novel role of PvHIPP23 in response to cadmium stress

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Abstract

Background

The heavy metal-associated isoprenylated plant protein (*HIPP*) gene family contributes to metal homeostasis regulation and stress adaptation; however, its biological significance in the bioenergy crop switchgrass (*Panicum virgatum* L.) remains largely unexplored.

Results

In this investigation, 79 *PvHIPP* genes were systematically identified at the genome-wide level and phylogenetically assigned into five groups. Among these genes, *PvHIPP23* exhibited substantial induction following cadmium (Cd) exposure in both roots and shoots. Suppression of *PvHIPP23* through RNA interference improved Cd tolerance in switchgrass, accompanied by diminished Cd accumulation in roots and enhanced Cd movement from roots toward shoots. In contrast, ectopic expression of *PvHIPP23* in *Arabidopsis thaliana* reduced Cd tolerance, as indicated by impaired root elongation, decreased chlorophyll levels, and reduced biomass production. Furthermore, *PvHIPP23* was demonstrated to interact with the heavy-metal transporter *PvHMA2.1* at the plasma membrane and negatively regulate *PvHMA2.1* expression. Independent overexpression of *PvHMA2.1* enhanced Cd tolerance in switchgrass, producing phenotypic outcomes comparable to those observed following *PvHIPP23* silencing.

Conclusions

These findings identify *PvHIPP23* as a negative regulator of Cd tolerance that attenuates *PvHMA2.1* transcription through protein–protein interaction. The characterized *PvHIPP23*–*PvHMA2.1* regulatory module expands current knowledge of the molecular mechanisms controlling Cd responses in switchgrass and provides a potential genetic target for developing switchgrass lines with enhanced Cd phytoextraction potential.

Introduction

The rapid expansion of industrial activities has resulted in severe environmental challenges [1], among which heavy metal Cd contamination has emerged as a critical concern in contemporary agriculture and environmental protection. As a non-essential element, Cd disrupts chlorophyll biosynthesis, thereby impairing photosynthetic efficiency, restricting plant growth, and inducing necrosis [2–4]. Cd is absorbed through plant root cells and subsequently transported to stems and leaves, where it promotes chlorophyll degradation and leaf chlorosis, ultimately restricting plant development and potentially causing plant mortality. Cd accumulation in plant tissues may eventually facilitate its entry into the food chain, posing substantial risks to human health and highlighting the urgent need for effective

remediation strategies [5]. Phytoremediation, an approach that employs plants to extract pollutants from contaminated soils [6], offers a promising solution owing to its low cost, environmental compatibility, and limited secondary pollution [7, 8].

Panicum virgatum L, colloquially known as switchgrass, is an important perennial C4 bioenergy grass characterized by high biomass production, strong stress resistance, and broad environmental adaptability [9]. It is recognized as a globally cultivated perennial forage crop with substantial economic and ecological importance. Switchgrass exhibits considerable efficiency in Cd removal from contaminated soils [10]. Numerous studies have investigated its potential application as a phytoremediation resource. Chen et al. [11] showed that the lowland ecotype Alamo switchgrass removed significantly more Cd from Cd-amended soils than the highland ecotype Blackwell and demonstrated greater suitability for Cd phytoextraction from contaminated environments. Overexpression of *PvBiP2* in switchgrass has been shown to markedly enhance vegetative growth and Cd tolerance, resulting in a 90%–140% increase in Cd accumulation within plants [12]. Lin et al. [13, 14] established an m6A-mediated regulatory network involved in Cd transport between switchgrass roots and shoots and demonstrated that *PvFIP37* enhances Cd tolerance by activating *PvYSL7*, *PvYSL17*, *PvABCC4*, and *PvABCC9*, thereby facilitating Cd sequestration into vacuoles. These findings provide potential strategies for candidate gene manipulation in phytoremediation applications involving heavy metal contamination. Furthermore, previous research has demonstrated that *PvHMA2.1* enhances Cd tolerance in *Arabidopsis thaliana* and may promote root-to-shoot Cd translocation, suggesting its potential utility in the remediation of Cd-contaminated soils [15]. Switchgrass cultivation in Cd-contaminated soils contributes to soil restoration and ecological improvement, and generates economic value through bioenergy production.

Heavy metal-associated isoprenylated plant proteins (HIPPs) are characteristic of vascular plants that contain a heavy metal-associated (HMA) domain and a C-terminal isoprenylation site (CaaX motif); they are regarded as metallochaperone proteins associated with metal transport and isoprenylation processes [16, 17]. HIPPs are important regulators of stress responses and heavy metal homeostasis, particularly during Cd exposure [18, 19]. Cd treatment induces *AtHIPP44* expression in *Arabidopsis* leaves and roots, and plants overexpressing *AtHIPP44* exhibit elevated Cd accumulation [20]. Overexpression of the tomato *SITCMP-1* gene in *Arabidopsis* increases endogenous *AtHIPP26* expression and decreases Cd accumulation in aerial tissues [21]. Overexpression of *OsHIPP42* in the Cd-sensitive yeast mutant $\Delta ycf1$ enhances Cd tolerance, while transient expression in tobacco leaves alleviates Cd-induced damage, demonstrating that *OsHIPP42* positively regulates Cd tolerance in plants [22]. Under Cd stress, *OsHIPP29*-overexpressing plants accumulate less Cd in roots and shoots and exhibit improved growth performance, whereas *OsHIPP29* mutants and interference lines display increased Cd accumulation and enhanced Cd sensitivity, indicating that *OsHIPP29* mitigates Cd toxicity by reducing Cd accumulation [23]. Heterologous expression of *OsHIPP9* modifies Cd tolerance in yeast, whereas *OsHIPP9* chelates Cd and regulates Cd accumulation in plants [24]. Collectively, these studies indicate that Cd stress can stimulate *HIPP* gene expression and that manipulation of *HIPP* genes through

overexpression or suppression influences Cd tolerance by altering Cd distribution and accumulation in plants.

As metallochaperone proteins, HIPPs are involved in plant responses to Cd stress. Currently, investigations into the identification and functional characterization of the HIPP family in plants have progressively expanded, including studies in *Arabidopsis* [19], *Oryza sativa* [25], *Triticum aestivum* [26], *Zea mays* [27], *Sorghum bicolor* [28], *Medicago sativa* [29], *Nicotiana tabacum* [30], and *Nelumbo nucifera* [31]. However, the HIPP family in switchgrass has not yet been systematically identified and functionally investigated. Therefore, in this study, the *HIPP* gene family in *P. virgatum* was systematically identified and characterized using an integrated bioinformatics approach. Furthermore, transgenic switchgrass and *Arabidopsis* plants expressing or silencing *PvHIPP23* were generated to investigate and validate the function of *PvHIPP23* under Cd stress. The results preliminarily demonstrate that *PvHIPP23* negatively regulates Cd tolerance by interacting with the previously identified *PvHMA2.1* [15] and repressing its expression in switchgrass. These findings elucidate the regulatory mechanism of the *PvHIPP* family during Cd stress responses and provide valuable genetic resources and potential molecular targets for improving Cd tolerance and phytoremediation efficiency in Cd-contaminated soils.

Materials and methods

Plant materials

The seeds of wild-type (WT) lowland switchgrass cultivar Alamo and WT *Arabidopsis thaliana* Columbia used in this study were provided by the Forage Breeding Laboratory of China Agricultural University. All switchgrass plants were cultivated in a greenhouse under natural light conditions, with the temperature maintained between 25°C and 35°C during both daytime and nighttime periods. Leaves, internodes, roots, and tiller buds at the elongation stage (E3) [32], as well as inflorescences at the reproductive stages R1 and R3, were collected from switchgrass plants for RNA extraction and tissue-specific expression analysis of *PvHIPP23*. Transgenic switchgrass plants subjected to Cd treatment were grown in a vermiculite-based culture system under greenhouse conditions. The expression levels of target genes in transgenic switchgrass were evaluated at the E2 developmental stage.

Sterilized WT switchgrass seeds were vernalized in darkness at 4°C for 3 days and subsequently germinated on moistened filter paper for 7 days. Uniformly growing switchgrass seedlings were transferred to 1/4 Hoagland's nutrient solution for 7 days and subsequently cultivated in 1/4 Hoagland's nutrient solution supplemented with 50 µM CdCl₂ or without Cd supplementation (control). Seedlings were maintained in a growth chamber under a 16 h/8 h light/dark photoperiod for 7 days. Shoots and roots were collected at different time points during the 7-day Cd treatment period (0, 0.25, 0.5, 1, 3, 5, and 7 days), rapidly rinsed with deionized water three to five times, gently dried using filter paper, transferred into centrifuge tubes, immediately frozen in liquid nitrogen, and stored at – 80°C until RNA extraction.

Identification and phylogenetic analysis of HIPPs in *P. virgatum*

The protein sequences of *Arabidopsis thaliana* HIPPs were obtained from the Arabidopsis Information Resource (TAIR; <https://www.arabidopsis.org/>), while *Oryza sativa* HIPP sequences were retrieved from the Rice Genome Annotation Project (<http://rice.plantbiology.msu.edu/annotation.shtml>). The genome sequence of *P. virgatum* was downloaded from the *Panicum virgatum* var. AP13 HAP1 v6.1 database in Phytozome 14 (<https://phytozome-next.jgi.doe.gov/>). A local BLASTP search was performed to identify candidate *PvHIPP* proteins using *AtHIPP* sequences as queries. Additionally, the Hidden Markov Model profile of the HMA (PF00403) domain was obtained from the Pfam database (<http://pfam.xfam.org/>) and applied for the identification of candidate *PvHIPP* proteins. The resulting homologous proteins were further screened by verifying the presence of a C-terminal prenylation motif, leading to the final determination of candidate *PvHIPP* members.

Multiple sequence alignments of HIPPs from *A. thaliana*, *O. sativa*, and *P. virgatum* were performed using MAFFT (<https://www.ebi.ac.uk/Tools/msa/mafft/>). A phylogenetic tree was generated using MEGA 11 based on the maximum likelihood (ML) method with 1000 bootstrap replicates and subsequently visualized using iTOL (<https://itol.embl.de/>). The sequence identifiers of HIPPs from *A. thaliana*, *O. sativa*, and *P. virgatum* are provided in Table S1.

Chromosomal localization and synteny analysis of *PvHIPP* genes

Chromosomal distribution information of *PvHIPP* genes was obtained from the *P. virgatum* genome GFF annotation file and visualized using the Gene Location Visualize function in the GTF/GFF module in TBtools [33]. Gene duplication events within the *PvHIPP* gene family were investigated using MCScanX [34, 35] with default parameters. The resulting collinear *HIPP* gene pairs were visualized using TBtools. To characterize interspecies syntenic relationships among *P. virgatum*, *A. thaliana*, and *O. sativa*, comparative synteny maps were constructed using the dual synteny plotter function in TBtools.

Conservation and structural analysis of HIPPs in *P. virgatum*

Conserved motifs and domains of *PvHIPPs* were identified using MEME (<https://meme-suite.org/meme/tools/meme>) with ten motif parameters and the Conserved Domain Search tool of NCBI (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) with default parameters, respectively. The gene structures of *PvHIPP* members were obtained from the *P. virgatum* genome GFF annotation file. TBtools was subsequently utilized to generate graphical representations of gene structures, conserved motifs, and conserved domains.

Cis-regulatory element analysis of *PvHIPP* genes

The promoter sequences extending 2000 bp upstream of the translation initiation site (ATG) of *PvHIPP* genes were analyzed to identify cis-regulatory elements using PlantCARE (<https://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) [36]. Repeated cis-regulatory elements with associated functional annotations were selected and visualized using the Simple BioSequence Viewer and HeatMap modules implemented in TBtools.

Structural and physicochemical property analysis of PvHIPP23

Amino acid sequence alignment and tertiary structure prediction of Pavir.2NG597900.2 (*PvHIPP23*) and its homologous proteins were conducted using DNAMAN 6.0 and SWISS-MODEL (<http://swissmodel.expasy.org/interactive>) with default parameters, respectively. The physicochemical properties of the *PvHIPP23* protein, including molecular weight, isoelectric point, instability index, and hydrophilicity, were predicted using the Multiple Protein Profiler (MPP) tool (https://mproteinprofiler.microbiologyandimmunology.dal.ca/protein_profiler/prot_char/). Predictions of the signal peptide, transmembrane domain, and subcellular localization of *PvHIPP23* were performed using SignalP 6.0 (<https://services.healthtech.dtu.dk/services/SignalP-6.0/>), DeepTMHMM-1.0 (<https://services.healthtech.dtu.dk/services/DeepTMHMM-1.0/>), and Plant-mPLOC (<http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/>), respectively, using the initial parameters.

Subcellular localization

The seeds of *Nicotiana benthamiana* were provided by the Forage Breeding Laboratory of China Agricultural University. The coding sequence of *PvHIPP23* was amplified by PCR and inserted into the expression vector pMDC83–GFP under the regulation of the 35S promoter. The constructs 35S:*PvHIPP23*–GFP, control 35S:GFP, p19, and the nuclear marker AHL22–mRFP were independently introduced into *Agrobacterium tumefaciens* strain GV2260. The infiltration mixtures containing 35S:*PvHIPP23*–GFP, p19, and AHL22–mRFP were transiently expressed in leaves of 3-week-old *N. benthamiana*, whereas the combination of 35S:GFP, p19, and AHL22–mRFP served as the control. Subcellular localization of *PvHIPP23* was examined using Nikon A1 confocal laser scanning microscopy at 2–3 days after infiltration. All primers used in this study are listed in Table S2.

RNA extraction and transcript analysis by RT-qPCR

Total RNA was extracted using the Quick General Plant RNA Extraction Kit (Huayueyang, China) and subsequently reverse-transcribed into cDNA using the PrimeScript™ RT reagent Kit (Perfect Real Time; TaKaRa, China). Real-time quantitative PCR (RT-qPCR) was conducted using the TB Green™ Premix Ex Taq™ II Kit (TaKaRa, China). The switchgrass reference gene for ubiquitin (GenBank: FL899020) was employed as the internal control for the switchgrass samples, whereas *Arabidopsis* Actin2 was used as

the reference gene for the *Arabidopsis* samples. Three technical replicates were performed for each analysis. Relative transcript abundance was calculated using the $2^{-\Delta\Delta CT}$ method [37].

Vector construction and generation of transgenic switchgrass and *Arabidopsis*

The coding sequence of *PvHIPP23* was amplified from the switchgrass ecotype Alamo. The verified sequence was inserted into the binary vector pUbi1301 [38] using the restriction enzymes BamHI/KpnI (New England BioLabs, Ipswich, MA, USA). A specific 200-bp fragment of *PvHIPP23*, designated *Pvhipp23*, was selected for RNA interference (RNAi) construction by generating a hairpin structure in pKannibal and subsequently recombined into the destination vector pCambia1305.2 through LR recombination using Gateway LR Clonase according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The constructed vectors and the previously generated pUbi1301-*PvHMA2.1* vector [15] were separately introduced into *A. tumefaciens* strain EHA105 through freeze–thaw transformation. These constructs were subsequently introduced into switchgrass calli using *Agrobacterium*-mediated transformation [39]. Generation and selection of transgenic switchgrass lines were conducted through antibiotic resistance screening of calli and further verified by PCR and RT-qPCR analyses. Non-transformed switchgrass plants regenerated from the same Alamo callus were used as the WT control.

For transgenic *Arabidopsis*, the constructed pUbi1301-*PvHIPP23* vector was introduced into *A. tumefaciens* strain GV3101 through freeze–thaw transformation and subsequently transferred into *Arabidopsis* Columbia WT plants using the floral-dip method [40, 41]. Putative transgenic *Arabidopsis* plants were selected on 1/2 MS medium supplemented with 50 mg/L hygromycin and 100 mg/L ampicillin and further confirmed by PCR and RT-qPCR analyses. Three independent T4 transgenic lines with elevated *PvHIPP23* expression levels were selected for investigation of *PvHIPP23*-mediated responses to Cd stress.

Cd treatment assay in *PvHIPP23* transgenic switchgrass and *Arabidopsis*

PvHIPP23 transgenic switchgrass plants cultivated in the vermiculite-based culture system described in Section 2.1 were utilized for Cd stress experiments involving detached leaves and whole plants, with WT switchgrass plants serving as controls. Detached leaves collected from switchgrass plants at the E5 stage were treated with different concentrations of CdCl₂ solutions (0, 100, 150, 200, 300, and 500 µM) at 25°C in darkness for 14 or 20 days. Whole switchgrass plants grown to the E2 stage were treated with 1/4 Hoagland solution containing 0 or 200 µM CdCl₂ for 50 days. Subsequently, growth-related parameters of each genotype were determined under Cd stress, including plant height, leaf length and width of the second fully expanded leaf from the top, length and diameter of the second internode from the bottom, tiller number, and shoot and root biomass.

Three independent T4 *Arabidopsis* transgenic lines (OP2-10, OP6-3, and OP8-4) heterologously expressing *PvHIPP23*, together with WT seeds, were surface sterilized with 6% NaClO for 10–15 min, vernalized in darkness at 4°C for 3 days, and germinated on 1/2 MS medium for 3 days. Uniformly growing *Arabidopsis* seedlings were transferred to 1/2 MS medium supplemented with 0, 25 μ M, or 50 μ M CdCl₂ for 8 days, after which phenotypic parameters were evaluated. Primary root lengths of individual seedlings were measured using ImageJ 1.52 following photographic documentation. Chlorophyll content was determined from batches of ten *Arabidopsis* seedlings according to the previously described method [42]. Seedling shoots and roots were separated, surface moisture was removed using filter paper, and the fresh weights of shoots and roots from each group of ten plants were recorded. Subsequently, all samples were dried at 80°C to a constant weight and measured to four decimal places using a precision analytical balance.

Determination of heavy metal content

Following Cd stress treatment, switchgrass shoots and roots were separated, rinsed with deionized water, and dried using filter paper to remove surface moisture. All samples were dried at 85°C until a constant weight was achieved, accurately weighed, and subsequently digested with HNO₃ [43]. The concentrations of Cd, Cu, Zn, Fe, and Al in the digested samples were determined by inductively coupled plasma mass spectrometry (ICP-MS) after filtration through a 0.22- μ m membrane filter. The Cd translocation factor (Tf) was calculated as the ratio of Cd concentration in shoots to that in roots [44].

Phenotypic analysis and Cd treatment of *PvHMA2.1*-overexpressing switchgrass

Three *PvHMA2.1*-overexpressing switchgrass lines (OE2, OE4, and OE5) cultivated as described in Section 2.1 were selected for phenotypic evaluation. Growth-related parameters of each genotype were measured at the R2 stage, including plant height, leaf length and width of the second fully expanded leaf from the top, internode length and diameter of the second internode from the bottom, spike length, and the numbers of leaves, nodes, and spikelets. Detached leaves collected from *PvHMA2.1*-overexpressing switchgrass plants grown to the E5 stage were treated with different concentrations of CdCl₂ solutions (0, 100, 150, and 200 μ M) at 25°C in darkness for 15 days, with WT switchgrass leaves used as the control. For whole-plant Cd treatment, *PvHMA2.1*-overexpressing switchgrass plants grown to the R0 stage in a vermiculite-based culture system as described in Section 2.1 were treated with 1/4 Hoagland solution containing 0 or 500 μ M CdCl₂ for 40 days. Subsequently, growth-related parameters were measured under Cd stress conditions.

BiFC assays

Bimolecular fluorescence complementation (BiFC) assays were performed using a split-YFP system. Briefly, the coding sequence of *PvHIPP23* was inserted into the *pGTQL1211-YN* vector, whereas the coding sequence of *PvHMA2.1* was inserted into the *pGTQL1221-YC* vector through LR recombination. The combinations of *PvHMA2.1*-cYFP, *PvHIPP23*-nYFP, p19, and the plasma membrane marker *AtCBL1*-

RFP were co-infiltrated into leaves of 3-week-old *N. benthamiana*. After 2–3 days of co-cultivation, fluorescence signals were detected using Nikon A1 confocal laser scanning microscopy.

Statistical analysis

Statistical analyses were conducted using Excel and SPSS 23.0. Data are presented as the mean $\pm$ standard deviation (SD) from three biological replicates. Statistical significance was evaluated using Student's *t*-test, as indicated in the figures (*, **, and *** represent $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively).

Results

Phylogenetic analysis of the HIPP family

To identify HIPP family members in switchgrass, 45 *Arabidopsis* HIPP protein sequences were retrieved from TAIR. A local BLASTP search identified 79 putative HIPP members in switchgrass, all of which contained the HMA domain and the C-terminal CaaX isoprenylation motif. To elucidate the evolutionary relationships of HIPPs among switchgrass, *Arabidopsis*, and rice, an additional 59 rice HIPP sequences were obtained from the Rice Genome Annotation Project. A phylogenetic tree was constructed using MEGA 11 based on 183 aligned protein sequences generated by the online MAFFT platform. All HIPPs were classified into five groups (Groups I–V) (Fig. 1). The corresponding accession numbers are provided in Table S1.

Group I consisted of 11 PvHIPPs, 13 AtHIPPs, and 17 OsHIPPs, including Pavir.2NG330103.1, Pavir.5KG159300.1, Pavir.5NG156100.1, Pavir.7KG431400.1, Pavir.7NG430300.1, Pavir.7KG385500.3, Pavir.7NG430400.1, Pavir.9KG547900.1, Pavir.9NG771600.3, Pavir.9KG307900.2, Pavir.9NG498514.1, AtHIPP2, AtHIPP10–AtHIPP16, AtHIPP39, AtHIPP41, AtHIPP43, AtHIPP46, AtHIPP47, OsHIPP12–OsHIPP27, and OsHIPP49. Group II contained 23 PvHIPPs, 7 AtHIPPs, and 21 OsHIPPs. Group III comprised 16 PvHIPPs, 8 AtHIPPs, and 10 OsHIPPs. Group IV represented the smallest group and included only Pavir.7NG258700.1, Pavir.7KG230200.5, AtHIPP17, AtHIPP18, and AtHIPP19. Group V was the largest group, containing 27 PvHIPPs, 14 AtHIPPs, and 11 OsHIPPs. Within this group, Pavir.2NG597900.2 was closely associated with Pavir.2KG543800.1, OsHIPP45, and AtHIPP23, and was designated *PvHIPP23* for further functional investigation in this study. The close evolutionary relationships among members within each group suggest potential functional conservation among HIPPs.

Chromosomal location and collinearity relationship analysis of PvHIPPs

The 79 switchgrass *HIPP* genes were distributed across 18 chromosomes, with 1–9 *HIPP* genes unevenly located on each chromosome (Fig. S1). The K and N genomes represent two homologous subgenomes of switchgrass ($2n = 4x = 36$). Specifically, Pavir.2NG597900.2 (*PvHIPP23*) is located on

Chr02N, whereas Pavir.2KG543800.1 is positioned at the corresponding region of Chr02K. Only one *HIPP* gene was identified on chromosome Chr04 (Pavir.4KG247300.1 located on Chr04K and Pavir.4NG164300.2 located on Chr04N) and chromosome Chr06 (Pavir.6KG238200.5 located on Chr06K and Pavir.6NG124500.1 located on Chr06N), whereas the remaining chromosomes contained two or more *HIPP* genes.

Gene duplication analysis within the *PvHIPP* family demonstrated that 83.54% of duplication events were attributed to segmental duplication, whereas dispersed, tandem, and proximal duplications accounted for 7.60%, 5.06%, and 3.80%, respectively (Fig. 2a). Comparative syntenic analysis involving *A. thaliana* and *O. sativa* revealed evolutionary conservation patterns within the *HIPP* gene family. Specifically, only six collinear *HIPP* gene pairs were detected between *P. virgatum* and *A. thaliana*, whereas 87 orthologous gene pairs were identified between *P. virgatum* and *O. sativa* (Fig. 2b). These results indicate that switchgrass shares a greater number of conserved syntenic *HIPP* genes with monocot species than with dicot species, suggesting closer evolutionary relationships of *PvHIPP* genes within monocots.

Conservation and structural analysis of *PvHIPP* genes

A total of 10 conserved motifs and three conserved domains were identified within the *PvHIPP* gene family (Fig. 3). Motif analysis demonstrated that *PvHIPP* gene family members contained three to eight conserved motifs. All members of Group I possessed motifs 3, 6, and 7, whereas all members of Group V contained motifs 1 and 2. Among Group V members, Pavir.2NG597900.2 (*PvHIPP23*) contained motifs 1, 2, 3, 4, 5, and 10. All *PvHIPP*s harbored at least one conserved HMA domain, while 12 of the 16 members in Group III contained two HMA domains, with the exceptions of Pavir.2NG291300.2, Pavir.5NG635700.1, Pavir.7NG343600.1, and Pavir.9KG376405.1. Furthermore, gene structure analysis indicated that all *PvHIPP* genes consisted of two to five exons. Members belonging to Groups II and III generally exhibited more complex gene structures, with most members containing three to four exons (Fig. 3). Collectively, *PvHIPP* members within the same phylogenetic group exhibited similar motif compositions and gene structures, whereas distinct structural characteristics were observed among members from different groups. These findings suggest that *PvHIPP*s possess both conserved and diversified structural features during evolutionary differentiation.

Analysis of cis-regulatory elements in *PvHIPP* gene promoters

The 2000-bp promoter regions upstream of the translation initiation sites of *PvHIPP* genes were analyzed using the PlantCARE database. A total of 3212 cis-regulatory elements were identified, comprising 40 distinct types of cis-elements that were classified into four functional categories (Fig. S2). Among these elements, 1260 (39.23%) were associated with stress responses, 892 (27.77%) were related to plant hormone responses, 590 (18.37%) were involved in light responsiveness, and 470 (14.63%) were associated with plant growth and development. Notably, 74 of the 79 *PvHIPP* genes contained one to nine MYC recognition sites, with the exceptions of Pavir.6KG238200.5,

Pavir.8KG363300.1, *Pavir.5KG233200.1*, *Pavir.4KG247300.1*, and *Pavir.3KG361400.1*. In addition, stress-responsive elements (STREs) and abscisic acid-responsive elements (ABREs) represented the most abundant abiotic and biotic stress-related elements identified in *PvHIPP* promoters. Regarding hormone-responsive elements, ABREs, methyl jasmonate-responsive elements (TGACG-motif and CGTCA-motif), salicylic acid-responsive elements (TCA-element), and ethylene-responsive elements (EREs) were detected in 11 *PvHIPP* genes. Furthermore, multiple light-responsive elements were identified in *PvHIPP* promoters, including G-Box, Box 4, GATA-motif, GT1-motif, and TCT-motif. Several cis-elements associated with plant growth and development were additionally detected, including CAT-box, MSA-like AAGAA-motif, A-box, CCGTCC-motif, and O2-site. The promoter region of *PvHIPP23* contained 24 types of cis-regulatory elements, among which MYC recognition sites, STRE, A-box, CCGTCC-motif, CCGTCC-box, and GATA-motif showed relatively high frequencies. Overall, prediction of cis-regulatory elements within *PvHIPP* promoter regions provides valuable information for investigating the transcriptional regulatory mechanisms of *HIPPs* in switchgrass.

Protein structure and physicochemical property analysis of *PvHIPP23*

To further characterize the structural features of *Pavir.2NG597900.2* (*PvHIPP23*), the protein structures of *PvHIPP23* and its three closest homologous proteins were predicted and comparatively analyzed. Multiple sequence alignment revealed that *PvHIPP23* shared 77.71% sequence identity with the three homologous proteins. All four proteins contained one conserved HMA domain located at amino acid positions 29–92 and one conserved C-terminal CaaX motif (Fig. S3a). Tertiary structure prediction demonstrated that *PvHIPP23* exhibited a highly similar three-dimensional structure to its homologous proteins (Fig. S3b). Physicochemical property analysis indicated that the *PvHIPP23* protein consists of 158 amino acids, with a predicted molecular weight of 17.31 kDa, an isoelectric point of 9.1, and an instability index of 41.58, suggesting that *PvHIPP23* is an unstable and hydrophilic protein (Table S3). Furthermore, prediction analyses revealed that *PvHIPP23* lacks both a signal peptide and transmembrane domain, while its predicted subcellular localization was within the nucleus. These findings provide structural information and potential functional insights for further investigation of *PvHIPP23*.

Subcellular localization and gene expression patterns of *PvHIPP23*

To determine the subcellular localization of *PvHIPP23*, the *PvHIPP23*–GFP fusion protein was expressed under the control of the 35S promoter, and *Agrobacterium* strains carrying 35S:*PvHIPP23*–GFP or 35S:GFP constructs were infiltrated into *N. benthamiana* leaves for fluorescence detection using confocal microscopy. The green fluorescence signals from 35S:GFP or 35S:*PvHIPP23*–GFP were examined together with the red fluorescence signal from the nuclear marker AHL22–mRFP. Similar to the 35S:GFP control, 35S:*PvHIPP23*–GFP fluorescence was detected not only in the nucleus but also along the plasma membrane (Fig. 4a). These observations indicate that *PvHIPP23* is localized in both

the nucleus and plasma membrane. To characterize the expression profile of *PvHIPP23*, various tissues were collected for transcript analysis. RT-qPCR results demonstrated that *PvHIPP23* was expressed in all examined tissues, with the lowest transcript abundance detected in leaves (Fig. 4b). Compared with leaf tissues, *PvHIPP23* expression levels were significantly higher in all other analyzed tissues ($P < 0.05$). Among these tissues, R1-stage inflorescences exhibited the highest *PvHIPP23* expression level, reaching 47.08-fold of the expression level detected in leaves, followed by R3-stage inflorescences, roots, internodes, and tiller buds (Fig. 4b).

To determine whether *PvHIPP23* responds to Cd stress, three-leaf-stage switchgrass seedlings were exposed to Cd treatment over a 7-day time course. Shoots and roots were separately collected at 0, 0.25, 0.5, 1, 3, 5, and 7 days for transcript abundance analysis. RT-qPCR results showed that *PvHIPP23* transcripts in shoots were induced after 1 day of Cd exposure and reached the maximum level at 3 days, exhibiting a 5.48-fold increase compared with the untreated control (Fig. 4c). Similarly, *PvHIPP23* expression in roots was stimulated by Cd treatment, showing significant elevation after 0.25 days and reaching a peak at 0.5 days, with approximately 5.34-fold higher expression than that in the control (Fig. 4c). After reaching the maximum expression level, *PvHIPP23* transcript abundance gradually declined in both shoots and roots and returned to undetectable levels after 3 days of Cd treatment (Fig. 4c). Collectively, these results demonstrate that *PvHIPP23* exhibits a rapid response in both shoots and roots under Cd stress, indicating its involvement in Cd-responsive regulatory processes in switchgrass.

Construction of *PvHIPP23* transgenic switchgrass plants

To investigate the biological function of *PvHIPP23*, the *PvHIPP23* gene fragment with the expected size was successfully amplified by PCR using specific primers (Fig. S4a). Following sequence confirmation of the amplified *PvHIPP23* fragment, the fragment was inserted into the *pUbi1301* dual-expression vector, and the recombinant plasmid was introduced into competent *Agrobacterium* EHA105 cells. PCR validation confirmed the successful generation of *PvHIPP23*-overexpressing *Agrobacterium* strains (Fig. S4b). Based on the coding sequence of *PvHIPP23*, an interference fragment (*Pvhipp23*) was designed for RNAi-mediated suppression. Using *PvHIPP23* as the template, the correctly sized interference fragment was amplified by PCR with specific primers (Fig. S4c). The amplified interference fragment was subsequently inserted into the intermediate vector through ligation, and after sequence verification, the recombinant construct was subjected to an LR recombination reaction with the *pCAMBIA1305.2* dual-expression vector to generate the *PvHIPP23*-RNAi interference vector. The recombinant vector was then transformed into competent *Agrobacterium* EHA105 cells. Colony PCR verification confirmed the successful acquisition of transformed *PvHIPP23*-RNAi *Agrobacterium* strains (Fig. S4d, e). Mature and fully developed switchgrass seeds were selected for callus induction (Fig. S5a). The constructed *PvHIPP23* overexpression and *PvHIPP23*-RNAi vectors were introduced into switchgrass calli through *Agrobacterium*-mediated transformation. Following antibiotic resistance screening of calli (Fig. S5b), differentiation and rooting procedures (Fig. S5c, d), *PvHIPP23* transgenic switchgrass plants were successfully generated (Fig. S5e).

The obtained *PvHIPP23*-overexpressing and *PvHIPP23*-RNAi switchgrass plants were cultivated to the E2 stage, and genomic DNA was extracted from leaf tissues for transgenic line identification. Three positive *PvHIPP23*-overexpressing lines, OE1–OE3 were identified (Fig. S6a, b). A total of 27 *PvHIPP23*-RNAi lines were obtained, among which RNAi-1, RNAi-8, and RNAi-33 contained only the forward interference fragment without the corresponding reverse interference fragment. Therefore, 24 positive *PvHIPP23*-RNAi switchgrass lines were ultimately selected (Fig. S6c–f). To evaluate *PvHIPP23* expression levels in the transgenic switchgrass lines, RNA was extracted from *PvHIPP23*-overexpressing and *PvHIPP23*-RNAi plants, and transcript abundance was analyzed by RT-qPCR. Expression levels of *PvHIPP23* in the three overexpression lines OE1–OE3 were significantly higher than those in WT plants ($P < 0.001$), exhibiting 55.02-, 14.80-, and 27.62-fold increases compared with WT, respectively (Fig. S7a). Among the 24 *PvHIPP23*-RNAi lines, 21 exhibited significantly reduced *PvHIPP23* expression relative to WT ($P < 0.05$). The RNAi-27 line displayed the strongest suppression, with *PvHIPP23* expression reduced to only 0.14-fold of the WT level (Fig. S7b).

RNAi of the *PvHIPP23* gene enhances Cd tolerance in switchgrass

Analysis of detached leaves from *PvHIPP23* transgenic switchgrass plants under Cd stress demonstrated that the stress symptoms of *PvHIPP23*-overexpressing plants progressively increased with increasing Cd concentrations after 14 days of treatment (Fig. 5a). Under 300 and 500 μM Cd treatments, detached leaves from the three *PvHIPP23*-overexpressing lines exhibited reduced greenness compared with WT plants (Fig. 5a), indicating that overexpression of *PvHIPP23* negatively affects Cd tolerance in detached switchgrass leaves. In contrast, detached leaves from three *PvHIPP23*-RNAi lines (RNAi-13, RNAi-21, and RNAi-25) treated with different Cd concentrations (0, 100, 150, and 200 μM) for 20 days retained greater greenness than WT leaves, whereas WT leaves displayed more severe chlorosis (Fig. 5b). These results indicate that suppression of *PvHIPP23* enhances Cd tolerance in detached switchgrass leaves.

To further evaluate the Cd tolerance of *PvHIPP23* transgenic switchgrass, *PvHIPP23*-RNAi plants were subjected to Cd stress treatment. Under Cd exposure, the growth performance of *PvHIPP23*-RNAi lines (RNAi-7, RNAi-12, RNAi-13, RNAi-14, RNAi-15, and RNAi-17) was superior to that of WT plants, with improved tolerance observed in both aerial tissues (Fig. 5c) and roots (Fig. 5d) after 50 days of 200 μM Cd treatment. Analysis of growth-related traits revealed that, compared with WT plants, *PvHIPP23*-RNAi plants exhibited increasing trends in plant height, leaf length, internode length, leaf width, stem diameter, and tiller number. Among these parameters, internode length and stem diameter were significantly greater than those of WT plants ($P < 0.001$) (Fig. 5e, f). Furthermore, biomass analysis under Cd stress showed that both fresh and dry weights of shoots and roots in *PvHIPP23*-RNAi plants were significantly higher than those in WT plants ($P < 0.05$) (Fig. 5g, h). These results demonstrate that *PvHIPP23* suppression improves Cd tolerance in switchgrass.

Determination of heavy metal contents in switchgrass shoots and roots under Cd stress revealed that, compared with WT plants, *PvHIPP23*-RNAi plants exhibited significantly reduced Cd accumulation in roots ($P < 0.05$), whereas Cd accumulation in shoots showed an increasing trend without statistical significance (Fig. 6a). The Cd translocation factor from roots to shoots was significantly increased in *PvHIPP23*-RNAi plants compared with WT plants ($P < 0.01$) (Fig. 6b), indicating that *PvHIPP23* suppression decreases root Cd accumulation while promoting Cd transport from roots to shoots under Cd stress. Compared with WT plants, Cu accumulation in the roots of *PvHIPP23*-RNAi plants was significantly decreased ($P < 0.05$), whereas Cu levels in shoots exhibited a declining trend without significant differences (Fig. 6c). Under Cd stress, Zn concentrations in both roots and shoots of *PvHIPP23*-RNAi plants showed decreasing trends compared with WT plants, with shoot Zn content exhibiting a significant reduction ($P < 0.05$) (Fig. 6d). No significant differences were detected in Fe or Al concentrations between *PvHIPP23*-RNAi and WT plants under Cd stress (Fig. 6e, f). These results indicate that *PvHIPP23* interference alters Cu and Zn homeostasis in switchgrass during Cd stress, while exerting limited effects on Fe and Al homeostasis. Collectively, these findings demonstrate that *PvHIPP23*-RNAi plants display enhanced Cd tolerance, reduced root Cd uptake, and increased Cd translocation from roots to shoots under Cd stress. Furthermore, Cd stress influences Cu and Zn ion homeostasis in *PvHIPP23*-RNAi switchgrass plants.

Heterologous expression of *PvHIPP23* reduces Cd tolerance in *Arabidopsis*

Because only a limited number of independent *PvHIPP23*-overexpressing switchgrass lines were obtained previously and these plants exhibited slow growth, Cd stress analysis of *PvHIPP23*-overexpressing switchgrass was not performed at this stage. To rapidly evaluate the effect of *PvHIPP23* overexpression on Cd tolerance, *PvHIPP23* was additionally introduced into *Arabidopsis*. The previously constructed *pUbi1301-PvHIPP23* recombinant plasmid was transformed into competent *Agrobacterium* GV3101 cells. After PCR confirmation of the recombinant construct (Fig. S8a), the validated vector was introduced into *Arabidopsis* Col-0 plants. The resulting T1 transgenic *Arabidopsis* plants were screened and verified, generating nine independent T1 *PvHIPP23* transgenic lines (Fig. S8b). Following genetic segregation screening of T2 progeny, seven T3 *Arabidopsis* lines heterologously expressing *PvHIPP23* with significantly elevated *PvHIPP23* transcript levels were obtained ($P < 0.05$) (Fig. S8c).

Three independent *PvHIPP23*-expressing *Arabidopsis* lines (OP2-10, OP6-3, and OP8-4) were randomly selected for Cd stress analysis under 25 and 50 μM Cd treatments. After 8 days of Cd exposure, primary root length gradually decreased in all *Arabidopsis* seedlings with increasing Cd concentrations (Fig. 7a). Compared with WT plants, the root lengths of *PvHIPP23*-expressing lines OP2-10 and OP8-4 were significantly reduced under both 25 and 50 μM Cd treatments ($P < 0.05$) (Fig. 7b), indicating that heterologous expression of *PvHIPP23* increases Cd sensitivity in *Arabidopsis*. With increasing Cd concentrations, chlorophyll contents gradually declined in all *Arabidopsis* seedlings. Compared with WT plants, *PvHIPP23*-expressing seedlings exhibited a decreasing trend in chlorophyll content under Cd

stress, and the OP6-3 line showed significantly lower chlorophyll content than WT under 25 μM Cd treatment ($P < 0.05$) (Fig. 7c). Compared with the control condition, shoot and root fresh weights of all *Arabidopsis* seedlings decreased after exposure to 50 μM Cd. Furthermore, *PvHIPP23*-expressing seedlings exhibited reduced shoot and root fresh weights compared with WT plants under Cd treatment, with shoot fresh weight of OP2-10 and root fresh weight of OP8-4 being significantly lower than those of WT ($P < 0.05$) (Fig. 7d, e). Collectively, these results demonstrate that Cd stress suppresses shoot and root growth of *Arabidopsis* seedlings and that heterologous expression of *PvHIPP23* reduces Cd tolerance compared with WT plants.

PvHMA2.1 enhances Cd tolerance in switchgrass

As a metallochaperone protein, *PvHIPP23* may interact with heavy metal transporters, including heavy-metal ATPase family proteins, to coordinately regulate heavy metal transport processes. To investigate the potential association between *PvHIPP23* and heavy-metal ATPase family proteins, a systematic analysis of the heavy-metal ATPase family in switchgrass was previously conducted. The heterologous expression of *PvHMA2.1* was shown to reduce Cd uptake in *Arabidopsis* roots while simultaneously promoting Cd translocation from roots to shoots, thereby enhancing Cd tolerance in *Arabidopsis* [15]. These effects are consistent with the Cd tolerance phenotype observed in *PvHIPP23*-RNAi switchgrass plants in the present study. To further characterize the function of *PvHMA2.1* in switchgrass responses to Cd stress, the previously constructed *pUbi1301-PvHMA2.1* overexpression vector was introduced into competent *Agrobacterium* EHA105 cells. PCR amplification was subsequently performed to identify positive *Agrobacterium* colonies (Fig. S9a). Through *Agrobacterium*-mediated transformation, the recombinant *pUbi1301-PvHMA2.1* vector was successfully introduced into switchgrass calli. Following molecular identification, five independent *PvHMA2.1*-overexpressing switchgrass lines (OE1–OE5) were obtained (Fig. S9b). *PvHMA2.1* expression levels were significantly elevated in these lines ($P < 0.05$) (Fig. S9c). Three *PvHMA2.1*-overexpressing lines (OE2, OE4, and OE5) were selected for phenotypic analysis. At the R2 developmental stage, no significant differences were observed between the three *PvHMA2.1*-overexpressing lines and WT plants in plant height (Fig. S9d), internode length (Fig. S9e), internode width (Fig. S9f), or the numbers of leaves and nodes (Fig. S9g). However, several individual traits exhibited differences compared with WT plants. The spike length and leaf width of OE2 were significantly greater than those of WT ($P < 0.05$) (Fig. S9e, f), whereas the leaf length of OE5 (Fig. S9e) and spikelet number of OE4 (Fig. S9g) were significantly lower than those of WT ($P < 0.05$). Collectively, these results indicate that *PvHMA2.1* overexpression does not substantially affect the overall growth phenotype of switchgrass plants at the R2 stage under normal growth conditions.

To investigate the role of *PvHMA2.1* in Cd stress responses in switchgrass, detached leaves from three *PvHMA2.1*-overexpressing lines (OE2, OE4, and OE5) were treated with different concentrations of Cd. After 15 days of Cd exposure, stress symptoms in *PvHMA2.1*-overexpressing detached leaves progressively increased with increasing Cd concentrations (Fig. 8a). However, under 100 μM , 150 μM , and 200 μM Cd treatments, detached leaves from the three *PvHMA2.1*-overexpressing lines maintained greater greenness than WT leaves (Fig. 8a), indicating improved Cd tolerance in *PvHMA2.1*-

overexpressing plants. Furthermore, under 500 μM Cd treatment for 40 days, *PvHMA2.1*-overexpressing switchgrass lines OE2, OE4, and OE5 exhibited stronger growth performance than WT plants, particularly in aboveground tissues (Fig. 8b). Measurement of growth-related traits under Cd stress demonstrated that *PvHMA2.1*-overexpressing plants showed significantly increased plant height, spike length, leaf length, internode length, leaf width, internode width, and spikelet number compared with WT plants ($P < 0.05$) (Fig. 8c–f). These results indicate that *PvHMA2.1* positively regulates Cd tolerance in switchgrass. This finding is consistent with previous observations of the Cd tolerance function of *PvHMA2.1* in *Arabidopsis* [15] and resembles the enhanced Cd tolerance phenotype observed following *PvHIPP23* RNAi in switchgrass.

PvHIPP23 negatively regulates PvHMA2.1 expression through interaction with PvHMA2.1 in switchgrass

Based on the aforementioned findings, *PvHIPP23* and *PvHMA2.1* were hypothesized to participate in a common regulatory pathway during Cd stress responses. To elucidate the functional relationship between *PvHIPP23* and *PvHMA2.1*, the expression levels of *PvHMA2.1* were examined in *PvHIPP23* transgenic switchgrass plants. Compared with WT plants, *PvHMA2.1* expression levels were significantly decreased in the *PvHIPP23*-overexpressing lines OE1–OE3 ($P < 0.01$) (Fig. 9a). In contrast, *PvHMA2.1* transcript levels were increased in *PvHIPP23*-RNAi switchgrass plants compared with WT plants, with significant upregulation observed in RNAi-15 and RNAi-21 lines ($P < 0.05$) (Fig. 9b). These results indicate that *PvHIPP23* negatively regulates *PvHMA2.1* expression in switchgrass. To further validate the interaction between *PvHIPP23* and *PvHMA2.1*, a BiFC assay was conducted in tobacco cells. Fluorescence signals were detected at the plasma membrane when *PvHIPP23* and *PvHMA2.1* were co-expressed, whereas no fluorescence signal was observed in the control combination (Fig. 9c). These results demonstrate that *PvHIPP23* physically interacts with *PvHMA2.1* in vivo.

Collectively, these results demonstrate that *PvHIPP23* suppresses *PvHMA2.1* expression and directly interacts with *PvHMA2.1*. The reduced Cd tolerance observed in plants overexpressing *PvHIPP23* may therefore result from negative regulation of *PvHMA2.1* expression by *PvHIPP23*. Conversely, enhanced Cd tolerance in *PvHIPP23*-RNAi switchgrass and *PvHMA2.1*-overexpressing plants in both *Arabidopsis* and switchgrass may be associated with increased *PvHMA2.1* expression following *PvHIPP23* suppression, thereby promoting Cd tolerance in switchgrass.

Discussion

Identification of the HIPP family in switchgrass for the first time

The HIPP family represents a class of proteins specific to vascular plants that play important roles in responses to heavy metal stress. These proteins are structurally characterized by the presence of one or

more HMA domains and a C-terminal isoprenylation CaaX motif. Previous studies have identified 45, 59, 114, 33, 66, 45, 23, 68, 33, 56, and 26 *HIPP* genes in *Arabidopsis* [19], rice [25], wheat, *Hordeum vulgare* [26], maize [27], sorghum [28], alfalfa [29], tobacco [30], lotus [31], *Camellia sinensis* [45], and *Citrus sinensis* [46], respectively. In this study, 79 *HIPP* genes were identified in switchgrass, all containing HMA domains and C-terminal CaaX motifs, representing a larger *HIPP* family size than that reported in most investigated plant species. Based on conserved motifs and domains, the 79 PvHIPPs were classified into five groups, consistent with previous classifications in *Arabidopsis* [19], rice, and wheat [26]. Distinct protein structural characteristics were observed among different groups. Most PvHIPPs in Group III contained two HMA domains, which may contribute to metal ion uptake and maintenance of metal homeostasis, similar to AtHIPP07 (originally AFTP3) [47, 48], OsHIPP29 [26], and OsHIPP33 [49]. In contrast, Group V contained the largest number of members, including 27 PvHIPPs, and exhibited abundant motif compositions, with motif 5 being uniquely present in this group.

PvHIPP23 overexpression reduces Cd tolerance

The *HIPP* family participates in plant responses to various abiotic stresses. In this study, the target protein PvHIPP23 (Pavir.2NG597900.2) was classified into Group V and exhibited the closest evolutionary relationships with AtHIPP23 and OsHIPP45, which have been reported to respond to cold stress [25, 50]. Meanwhile, another homologous protein of PvHIPP23 within Group V, OsHIPP44, showed increased expression in the roots of Cd-treated plants, suggesting its potential involvement in Cd homeostasis [25]. Similarly, *PvHIPP23* expression was significantly induced in both shoots and roots under Cd stress, indicating that PvHIPP23 may participate in Cd-responsive processes in switchgrass. To investigate the function of PvHIPP23 during Cd stress, transgenic switchgrass plants were generated and their Cd tolerance was evaluated. Detached leaves can partially reflect Cd toxicity and Cd tolerance in plants [51]. In this study, detached leaf analysis of *PvHIPP23* transgenic switchgrass demonstrated that leaves from *PvHIPP23*-overexpressing plants exhibited reduced greenness compared with WT plants, whereas *PvHIPP23*-RNAi plants showed improved maintenance of leaf greenness. The responses of HIPPs to heavy metal stress are highly diverse and species-specific. Most HIPPs enhance heavy metal tolerance in plants, such as ApHIPP26, which improves Cd tolerance in *Arabidopsis* [52], whereas several HIPPs exhibit opposite regulatory functions. *BcHIPP16* was significantly induced by Cd exposure, and its expression in yeast and *Arabidopsis* increased Cd sensitivity in yeast while promoting Cd accumulation in yeast cells and *Arabidopsis* seedlings [53]. Similarly, *OsHIPP17* overexpression increased Cd accumulation in yeast cells and impaired growth of *Arabidopsis* under Cd stress [54].

In this study, because only three independent *PvHIPP23*-overexpressing switchgrass lines were obtained and these plants exhibited slow growth, heterologous expression of *PvHIPP23* in *Arabidopsis* was performed to further evaluate its function in Cd tolerance. Consistently, *PvHIPP23*-expressing *Arabidopsis* plants exhibited reduced Cd tolerance compared with WT plants, as indicated by inhibited shoot and root growth, shorter primary roots, decreased chlorophyll content, and reduced fresh and dry biomass under Cd stress. Conversely, *PvHIPP23*-RNAi switchgrass plants displayed enhanced Cd tolerance, improved growth performance, reduced Cd uptake, and increased Cd translocation from roots

to shoots. These results are consistent with previous observations that heterologous expression of *PvHMA2.1* in *Arabidopsis* enhances Cd tolerance [15]. Similar effects were reported for *IbHIPP7*, whose overexpression significantly improved Cd tolerance and reduced Cd accumulation in roots and shoots [55]. Additionally, *PvHIPP23* suppression reduced Cu and Zn uptake and altered metal homeostasis under Cd stress. The influence of HIPPs on metal homeostasis may be mediated through interactions with other transporter proteins [21, 56, 57].

Cd toxicity negatively affects plants by disrupting overall physiological processes. Excessive Cd accumulation can induce chloroplast membrane damage [58]. In this study, chlorophyll content in *PvHIPP23*-expressing *Arabidopsis* seedlings was lower than that in WT plants under Cd stress, suggesting that Cd toxicity damages chloroplast function and subsequently affects photosynthesis. Furthermore, heterologous expression of *PvHIPP23* may aggravate Cd-induced chloroplast injury, thereby reducing Cd tolerance in transgenic plants. Malondialdehyde content serves as an indicator of lipid peroxidation and reflects responses to various environmental stresses [59]. In addition to antioxidant enzymes, free proline contributes to cell membrane stabilization due to its antioxidant properties [60]. However, physiological parameters including malondialdehyde content, proline content, and antioxidant enzyme activities were not measured in this study, limiting further clarification of the physiological mechanisms underlying *PvHIPP23*-mediated Cd responses. Future studies should incorporate additional physiological indicators to comprehensively elucidate the molecular regulatory network involved in switchgrass responses to Cd stress from multiple perspectives.

PvHMA2.1 overexpression enhances Cd tolerance

Heavy-metal ATPase family proteins (indicated by HMA appearing in their name) belong to the P1B-type ATPase subfamily and function as metal transporters by hydrolyzing ATP to drive metal ion transport [61]. Based on substrate specificity, heavy-metal ATPases are classified into the Zn/Cd/Co/Pb subgroup and the Cu/Ag subgroup [62], both of which contribute to heavy metal detoxification and sequestration processes [63]. Rice OsHMA2, a member of the Zn/Cd/Co/Pb subgroup [62], mediates Cd transport from roots to shoots through xylem vessels and enhances Cd tolerance in rice [64, 65]. Furthermore, OsHMA3, AtHMA3, and AtHMA4, which are homologous proteins of OsHMA2, have been demonstrated to improve Cd tolerance in plants [66–69]. In previous research, the switchgrass membrane transporter *PvHMA2.1*, an ortholog of OsHMA2, was shown to reduce Cd uptake in roots, promote Cd transport from roots to shoots, and enhance Cd tolerance in *Arabidopsis* [15]. In the present study, detached leaves of *PvHMA2.1*-overexpressing switchgrass exhibited improved Cd tolerance compared with WT plants. Moreover, overexpression of *PvHMA2.1* enhanced Cd tolerance in switchgrass, accompanied by improved growth performance and growth-related traits under Cd stress, which is consistent with previous findings. The functions of *PvHMA2.1* in switchgrass and *Arabidopsis* are comparable to those of OsHMA2 in promoting Cd translocation from roots to shoots and enhancing Cd tolerance, suggesting that *PvHMA2.1* plays an important role in Cd tolerance and redistribution in plants.

PvHIPP23 interacts with PvHMA2.1 to downregulate PvHMA2.1 gene expression

Metallochaperone proteins can facilitate heavy metal transport through interactions with heavy metal transporter proteins [70]. The *Arabidopsis* metallochaperone protein antioxidant protein 1 (ATX1) regulates Cu transport by interacting with the heavy metal transporters AtHMA5 and AtHMA7 [71–73]. Similarly, the metallochaperone protein OsATX1 mediates Cu ion transfer through interactions with OsHMA4, OsHMA5, OsHMA6, and OsHMA9, thereby maintaining Cu homeostasis in plants [74]. HIPPs are ATX1-like metallochaperone proteins in plants [25, 75], suggesting that they may interact with heavy metal transporters, such as heavy-metal ATPase family proteins, to coordinately regulate heavy metal transport. In this study, the enhanced Cd tolerance caused by *PvHIPP23* interference in switchgrass was consistent with the Cd tolerance effects of *PvHMA2.1* in switchgrass and *Arabidopsis* [15]. Therefore, a potential functional association between *PvHIPP23* and *PvHMA2.1* was proposed. The present results demonstrated that *PvHIPP23* suppressed *PvHMA2.1* expression in switchgrass and interacted with *PvHMA2.1* at the plasma membrane in vivo. These findings suggest that *PvHIPP23* negatively regulates *PvHMA2.1* expression through interaction with *PvHMA2.1*, thereby reducing Cd tolerance in switchgrass. Conversely, increased *PvHMA2.1* expression resulting from *PvHIPP23* suppression or direct *PvHMA2.1* overexpression enhances Cd tolerance and promotes Cd translocation in plants. However, the interaction between *PvHIPP23* and *PvHMA2.1* was verified only through BiFC analysis in this study. Additional evidence, including yeast two-hybrid assays and identification of specific interaction sites, is required to further elucidate the regulatory mechanism of the *PvHIPP23*–*PvHMA2.1* module during Cd stress responses.

As metallochaperone proteins, HIPPs primarily bind metal ions through their HMA domains [28] and utilize C-terminal isoprenylation modifications to anchor themselves to cellular membranes [29]. Subsequently, they may transfer bound metal ions to specific target proteins, such as the membrane-localized heavy metal transporter AtHMA2 [76], ultimately facilitating metal ion export from cells [74]. OsHMA6 and OsHMA9 are additional plasma membrane-localized HMA proteins involved in Cu efflux and enhancement of Cu tolerance in rice [77, 78]. The heavy metal efflux transporters belonging to the heavy-metal ATPase family in switchgrass have not yet been identified, and whether *PvHIPP23* promotes the expression of *PvHMA* efflux transporters and facilitates Cd efflux remains to be investigated. In summary, this study identifies *PvHIPP23* as a novel negative regulator involved in Cd stress responses and preliminarily reveals its potential regulatory mechanism through interaction with *PvHMA2.1*. The contrasting positive and negative regulatory functions observed within the HIPP family may result from species-specific characteristics, functional diversification of the *HIPP* gene family, and suppression of positive regulators such as *PvHMA2.1*. These findings provide additional evidence for understanding the functional diversity and regulatory complexity of the HIPP family.

Conclusion

This study provides the first comprehensive characterization of the PvHIPP family and identifies 79 *HIPP* genes in switchgrass. Functional analysis of PvHIPP23 demonstrates that it negatively regulates Cd tolerance by interacting with PvHMA2.1 and suppressing *PvHMA2.1* expression in switchgrass. In contrast, RNAi-mediated suppression of *PvHIPP23* enhances Cd tolerance by reducing Cd uptake in roots and promoting Cd translocation from roots to shoots, highlighting its potential application in phytoremediation. These findings advance the understanding of the molecular mechanisms underlying Cd stress responses in switchgrass and provide a potential target, PvHIPP23, for improving the remediation efficiency of Cd-contaminated soils.

Abbreviations

HIPP

heavy metal-associated isoprenylated plant protein

HMA domain

heavy metal-associated domain

WT

wild-type

RNAi

RNA interference

ICP-MS

inductively coupled plasma mass spectrometry

Tf

translocation factor

BiFC

bimolecular fluorescence complementation

UTR

untranslated region

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The data underlying the findings of this study are provided in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Hui Zang: Writing – review & editing, Writing – original draft, Supervision, Conceptualization, Funding acquisition. Qingrui Yan: Methodology, Data curation. Yajie Xue: Writing – review & editing, Data curation. Sisi Zhang: Methodology, Formal analysis. Dongliang Shang: Writing – review & editing, Writing – original draft, Project administration.

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Figures

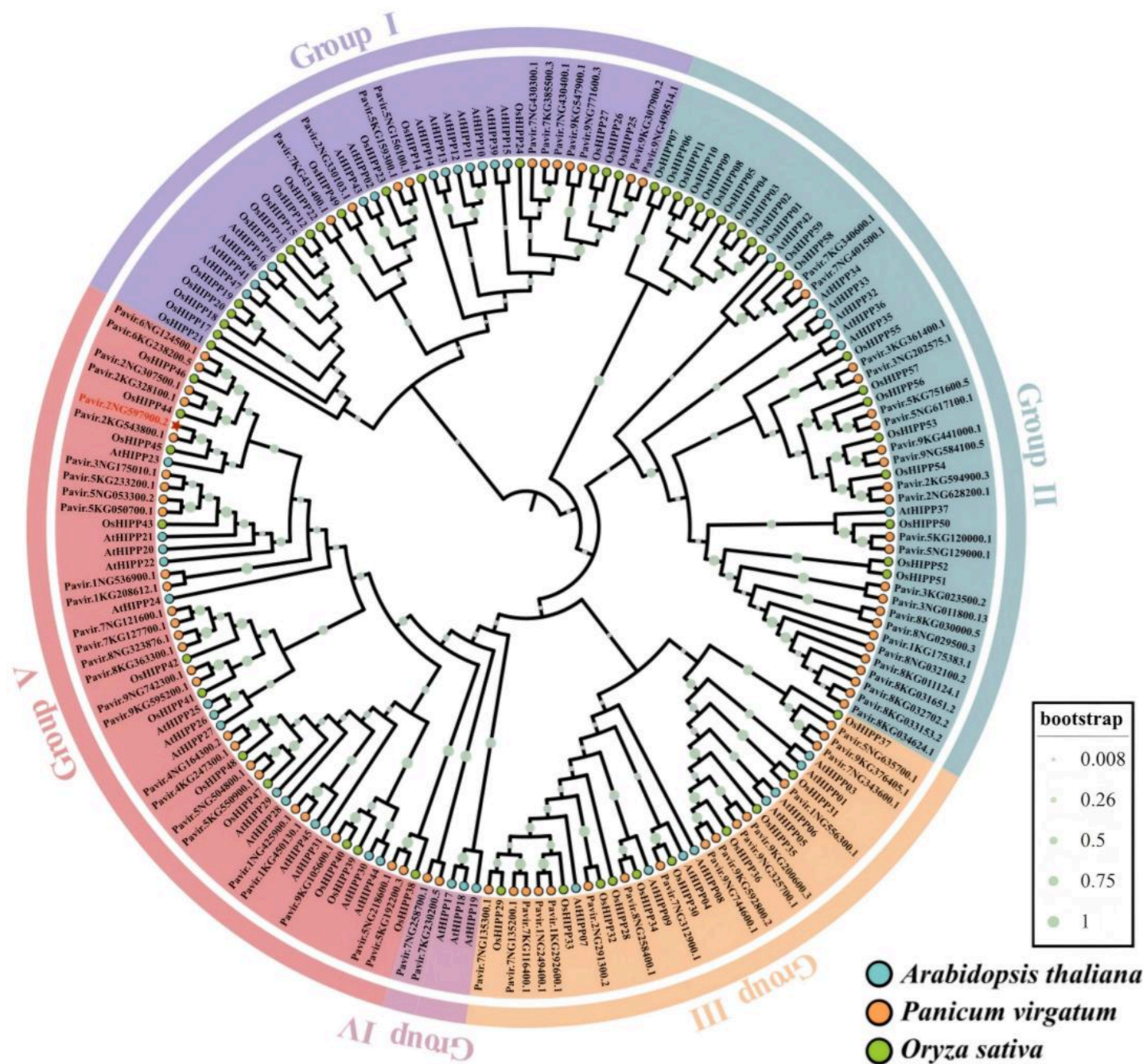


Figure 1

The unrooted phylogenetic tree of HIPPs from *Arabidopsis thaliana*, *Oryza sativa*, and *Panicum virgatum*. Multiple HIPP sequence alignment was initially performed using MAFFT, followed by phylogenetic tree construction using MEGA 11 based on the maximum likelihood method with 1000 bootstrap replicates. Green points adjacent to branch nodes indicate the percentage of bootstrap replicates supporting each branch. All HIPPs were classified into five groups, which are indicated by different colors. The sequence identifiers of HIPPs from *A. thaliana*, *O. sativa*, and *P. virgatum* are listed in Table S1

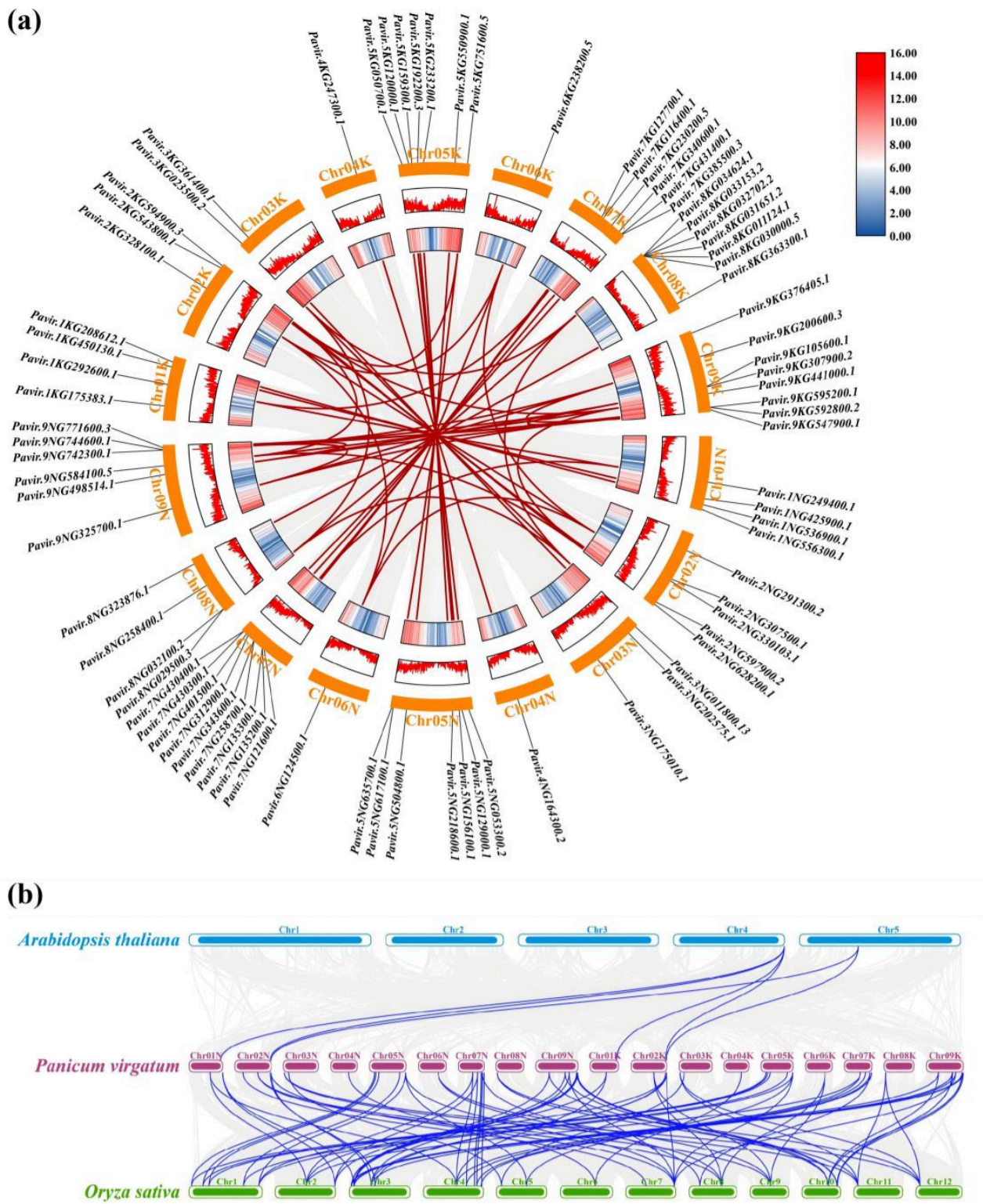


Figure 2

Synteny analysis of *HIPP* genes. **(a)** Intraspecific synteny analysis of *HIPP* genes in *P. virgatum*. Gray lines in the background represent all collinear blocks within the *P. virgatum* genome, whereas red lines indicate duplicated *PvHIPP* gene pairs. **(b)** Synteny analysis of the *HIPP* gene family among *P. virgatum*, *A. thaliana*, and *O. sativa*. Gray background lines represent all syntenic regions within the genomes, while

blue lines indicate collinear *HIPP* gene pairs among different species. Each horizontal bar, labeled with the corresponding chromosome number above, represents an individual chromosome

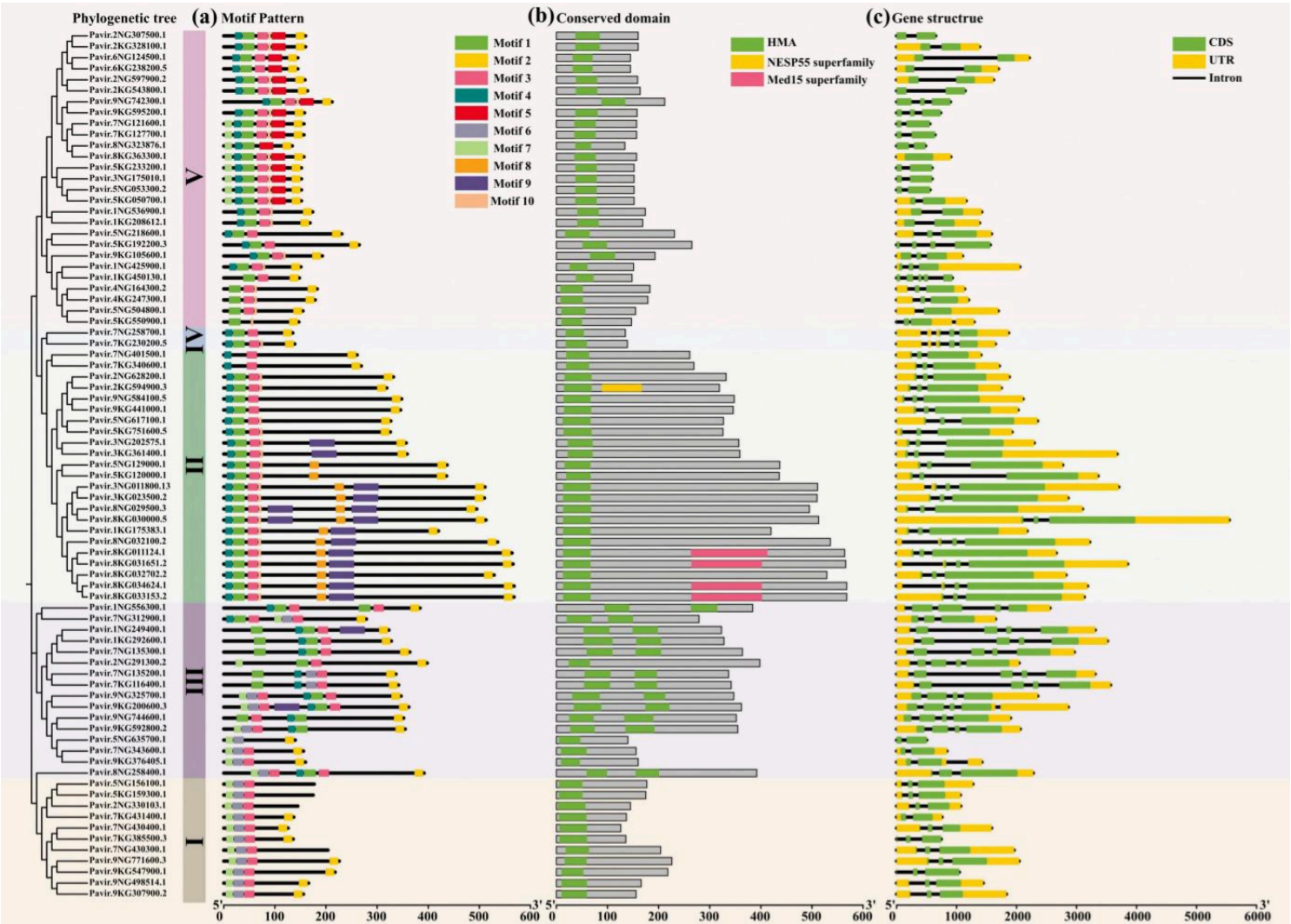


Figure 3

Conservation and structural analysis of *HIPPs* in *P. virgatum*. The phylogenetic tree of *HIPPs* was generated based on multiple sequence alignment using MAFFT and constructed in MEGA 11 using the maximum likelihood method with 1000 bootstrap replicates. **(a)** Conserved motif analysis of *PvHIPP* genes. Conserved motifs were identified using the online MEME program with 10 motif parameters and are represented by boxes with different colors. **(b)** Conserved domain analysis of *PvHIPP* genes. Boxes with distinct colors represent different conserved domains. **(c)** Gene structure analysis of *PvHIPP* genes. Green boxes and black lines represent exons and introns, respectively, whereas yellow boxes indicate untranslated regions (UTRs). The horizontal axis represents the sequence length of *PvHIPP* genes at the corresponding positions

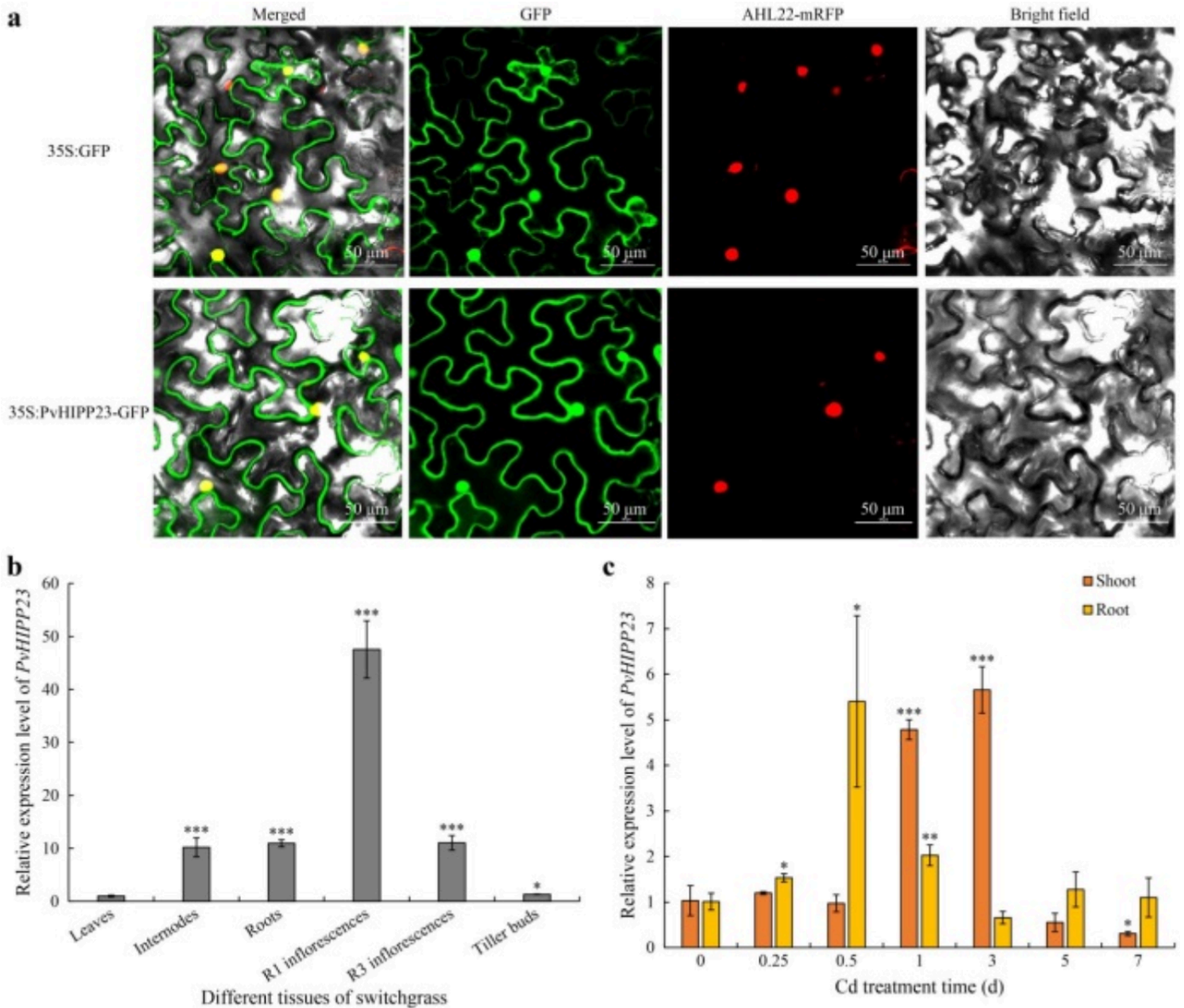


Figure 4

Subcellular localization and expression patterns of *PvHIPP23*. **a** Subcellular localization of *PvHIPP23* in tobacco leaf cells. GFP represents green fluorescent protein fluorescence, and AHL22-mRFP represents fluorescence from the nuclear marker protein. Scale bars = 50 μ m. **b** Relative expression levels of *PvHIPP23* in different switchgrass tissues. The transcript level of *PvHIPP23* in leaves was normalized to 1. **c** Relative expression levels of *PvHIPP23* in switchgrass shoots and roots during time-course Cd treatment. Transcript levels of *PvHIPP23* in shoots and roots under control conditions were independently normalized to 1. Data represent mean $\pm$ SD ($n = 3$). Asterisks indicate significant differences: * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$.

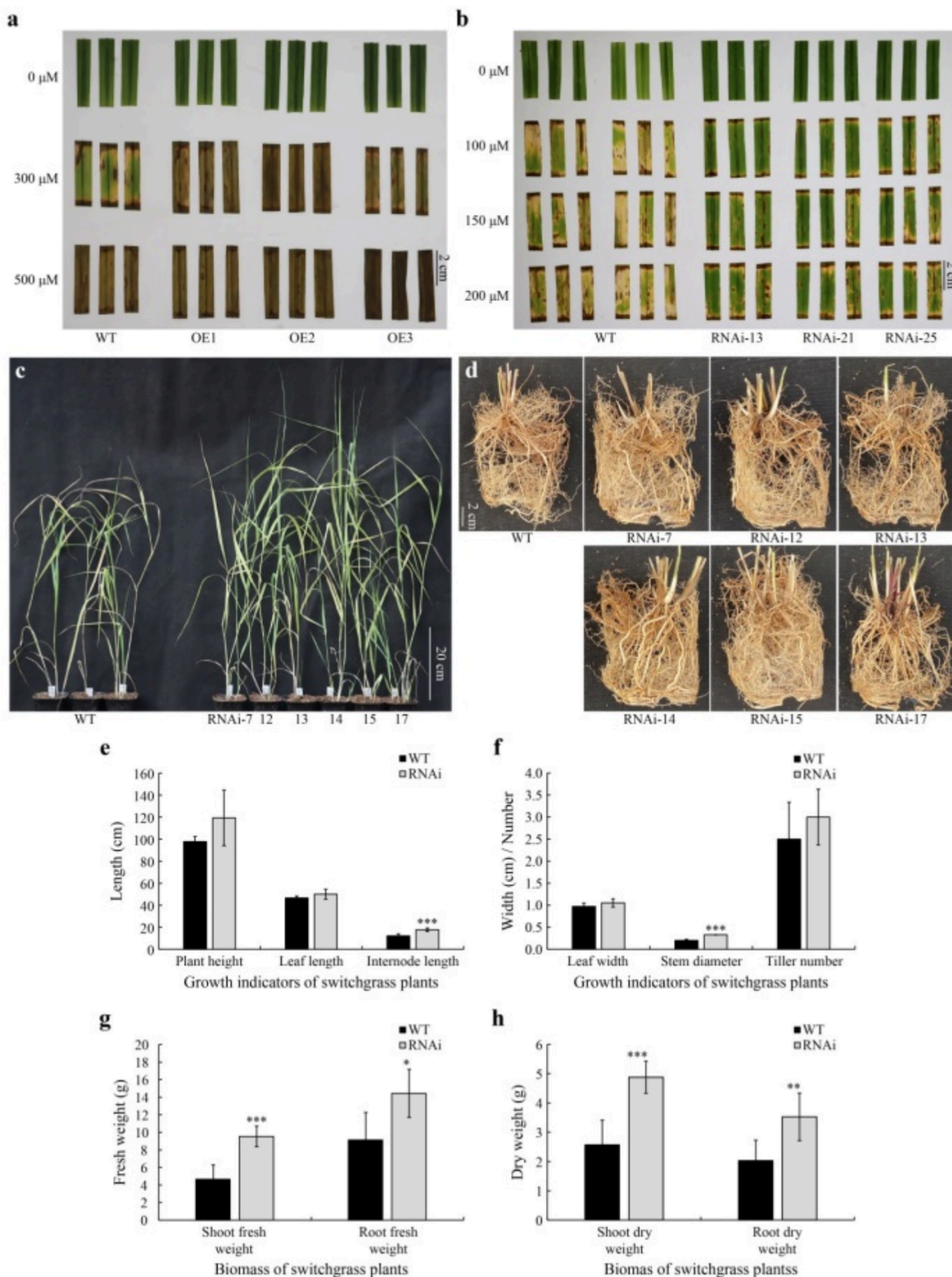


Figure 5

Analysis of *PvHIPP23* transgenic switchgrass under Cd stress. **a, b** Phenotypic analysis of detached leaves from *PvHIPP23*-overexpressing switchgrass (**a**) and *PvHIPP23*-RNAi switchgrass (**b**) under Cd stress. 0, 100, 150, 200, 300, and 500 μ M represent different concentrations of CdCl₂ solutions. WT: wild-type switchgrass; OE1, OE2, and OE3: different *PvHIPP23*-overexpressing switchgrass lines; RNAi-13, RNAi-21, and RNAi-25: different *PvHIPP23*-RNAi switchgrass lines. Scale bars = 2 cm. **c–h** Analysis of

PvHIPP23-RNAi switchgrass plants under Cd stress. **c, d** Phenotypic analysis of shoots (**c**) and roots (**d**) of *PvHIPP23*-RNAi switchgrass plants subjected to 200 μ M CdCl₂ treatment for 50 days. WT: wild-type switchgrass; RNAi-7, RNAi-12, RNAi-13, RNAi-14, RNAi-15, and RNAi-17: different *PvHIPP23*-RNAi switchgrass lines. Scale bars = 20 cm for shoots and 2 cm for roots. **e–h** Analysis of plant height, leaf length, and internode length (**e**); leaf width, stem diameter, and tiller number (**f**); shoot and root fresh weight (**g**); and shoot and root dry weight (**h**) of *PvHIPP23*-RNAi switchgrass plants under 200 μ M CdCl₂ stress. Data represent mean $\pm$ SD (n = 6), where n represents different switchgrass lines. Asterisks indicate significant differences compared with WT: * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$

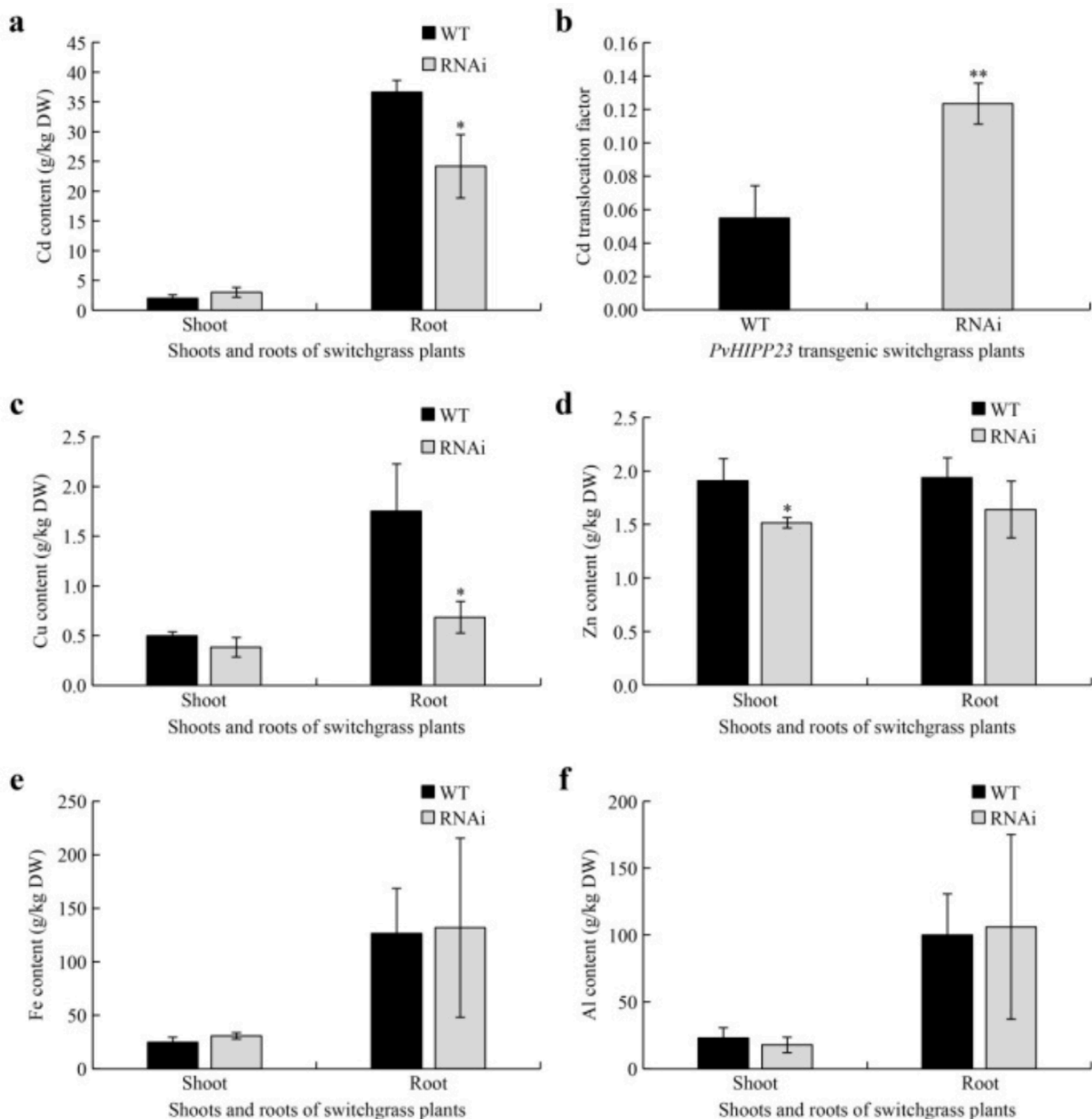


Figure 6

Metal contents in *PvHIPP23*-RNAi switchgrass plants under Cd stress. **a** Cd contents in shoots and roots of *PvHIPP23*-RNAi switchgrass plants under Cd stress. **b** Cd translocation factor from roots to shoots in *PvHIPP23*-RNAi switchgrass plants under Cd stress. **c–f** Cu (**c**), Zn (**d**), Fe (**e**), and Al (**f**) contents in shoots and roots of *PvHIPP23*-RNAi switchgrass plants under Cd stress. Data represent mean $\pm$ SD ($n = 3$), where n represents different switchgrass lines. Asterisks indicate significant differences compared with WT: * indicates $P < 0.05$ and ** indicates $P < 0.01$

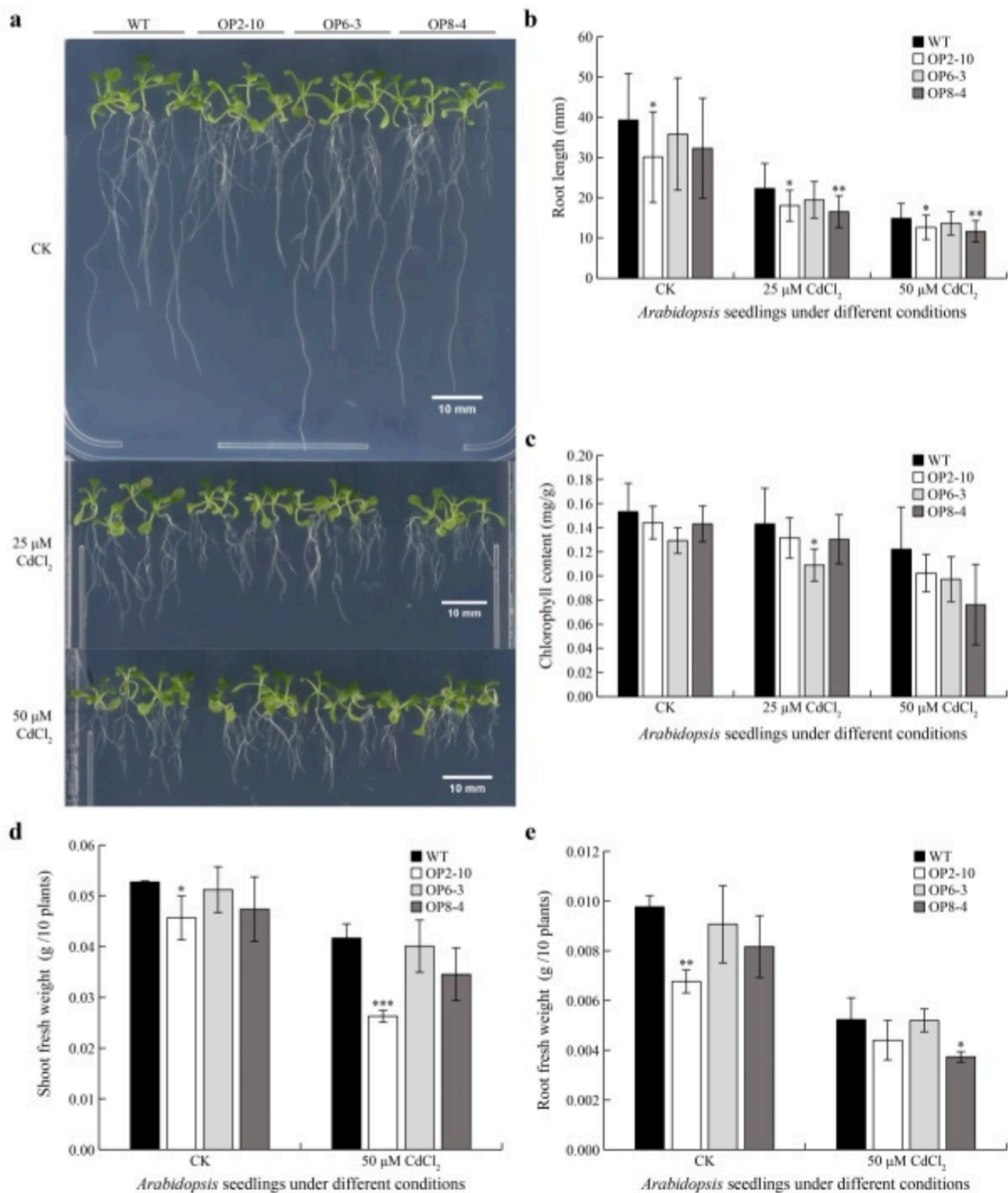


Figure 7

Analysis of *PvHIPP23*-expressing *Arabidopsis* in response to Cd stress. **a** Phenotypic analysis of *Arabidopsis* seedlings under different Cd concentrations. Scale bars = 10 mm. **b** Analysis of primary root lengths of *Arabidopsis* seedlings under different Cd treatments. Data represent mean $\pm$ SD ($n = 20$). **c** Analysis of chlorophyll contents in shoots of *Arabidopsis* seedlings under different Cd treatments. Data represent mean $\pm$ SD ($n = 5$). **d, e** Shoot fresh weight (**d**) and root fresh weight (**e**) of

Arabidopsis seedlings under Cd stress. Data represent mean $\pm$ SD ($n = 3$). WT: *Arabidopsis* Col-0. OP2-10, OP6-3, and OP8-4: T4 *PvHIPP23*-expressing *Arabidopsis* lines. Asterisks indicate significant differences compared with WT: * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$

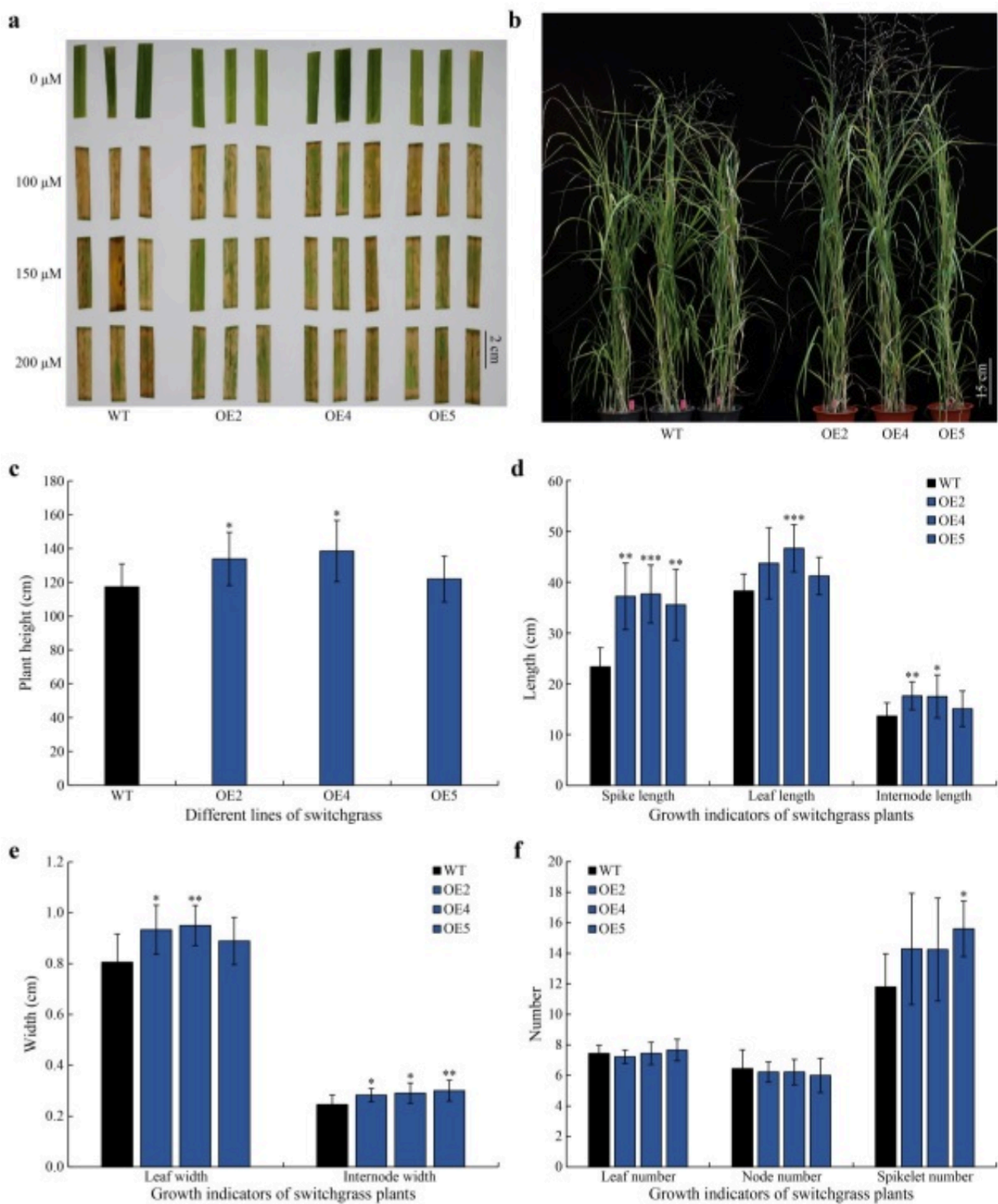


Figure 8

Analysis of *PvHMA2.1*-overexpressing switchgrass plants under Cd stress. **a** Phenotypic analysis of detached leaves from *PvHMA2.1*-overexpressing switchgrass under Cd stress for 15 days. 0 μM , 100 μM , 150 μM , and 200 μM represent different concentrations of CdCl_2 solutions. Scale bar = 2 cm. **b** Phenotypic analysis of *PvHMA2.1*-overexpressing switchgrass plants under 500 μM CdCl_2 stress for 40 days. Scale bar = 15 cm. **c–f** Growth trait analysis of *PvHMA2.1*-overexpressing switchgrass under Cd stress. Analysis of plant height (**c**); spike, leaf, and internode length (**d**); leaf and internode width (**e**); and numbers of leaves, nodes, and spikelets (**f**) under 500 μM CdCl_2 treatment. WT: wild-type switchgrass. OE2, OE4, and OE5: different *PvHMA2.1*-overexpressing switchgrass lines. Data represent mean $\pm$ SD ($n \geq 5$). Asterisks indicate significant differences compared with WT: * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$

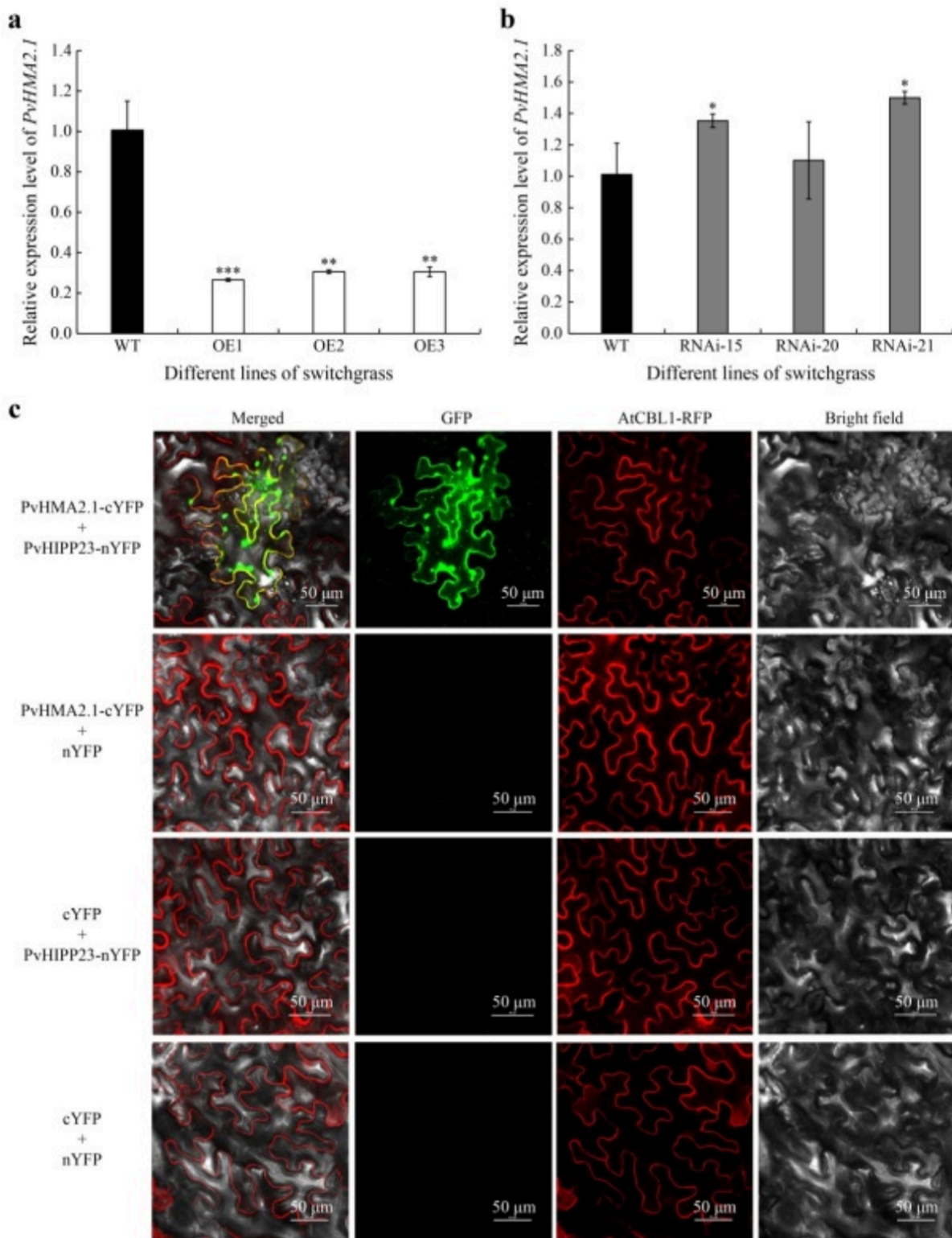


Figure 9

Identification of the relationship between *PvHIPP23* and *PvHMA2.1*. **a, b** Analysis of *PvHMA2.1* expression levels in *PvHIPP23* transgenic switchgrass. *PvHMA2.1* expression levels in *PvHIPP23*-overexpressing switchgrass (**a**) and *PvHIPP23*-RNAi switchgrass (**b**). WT: wild-type switchgrass. OE1–OE3: different *PvHIPP23*-overexpressing switchgrass lines. RNAi-15, RNAi-20, and RNAi-21: different *PvHIPP23*-RNAi switchgrass lines. Asterisks indicate significant differences compared

with WT: * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$. **c** BiFC analysis of PvHIP23 and PvHMA2.1 interactions in tobacco leaf cells. GFP: green fluorescent protein fluorescence. AtCBL1-RFP: fluorescence of the plasma membrane marker protein. Scale bars = 50 μm

Supplementary Files

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