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Genome-Wide Identification of the HMA Gene Family and Expression Analysis under Cd Stress in Barley

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Abstract: In recent years, cadmium (Cd) pollution in soil has increased with increasing industrial activities, which has restricted crop growth and agricultural development. The heavy metal ATPase (HMA) gene family contributes to heavy metal stress resistance in plants. In this study, 21 HMA genes (HvHMAs) were identified in barley (Hordeum vulgare L., Hv) using bioinformatics methods. Based on phylogenetic analysis and domain distribution, barley HMA genes were divided into five groups (A–E), and complete analyses were performed in terms of physicochemical properties, structural characteristics, conserved domains, and chromosome localization. The expression pattern analysis showed that most HvHMA genes were expressed in barley and exhibited tissue specificity. According to the fragments per kilobase of exon per million fragments values in shoots from seedlings at the 10 cm shoot stage (LEA) and phylogenetic analysis, five HvHMA genes were selected for expression analysis under Cd stress. Among the five HvHMA genes, three (HvHMA1, HvHMA3, and HvHMA4) were upregulated and two (HvHMA2 and HvHMA6) were downregulated following Cd treatments. This study serves as a foundation for clarifying the functions of HvHMA proteins in the heavy metal stress resistance of barley.

Keywords: barley; HMA gene family; bioinformatics analysis; Cd stress

1. Introduction

Cadmium (Cd) pollution is one of the negative consequences of industrialization. Cd is highly toxic to plants and is easily absorbed by the roots and accumulates in the tissues [1], which influences various processes including water and mineral uptake, respiration, and photosynthesis, and leads to the inhibition of growth and even death [2]. Cd ions with a lack of specificity enter the plant through other transporters (Fe²⁺/Fe³⁺, Zn²⁺, and Mn²⁺) and compete with other nutrients for plant uptake, resulting in deficient nutrition [3,4]. In response to Cd poisoning, various defense mechanisms have evolved in plants, such as extrusion across plasma membrane, chelation in the cytosol, and vacuolar sequestration [5]. Previous studies have identified multiple proteins related to Cd transport, including heavy metal ATPase (HMA) [6], yellow stripe-like proteins (YSL) [7], and natural resistance-associated macrophage proteins (NRAMP) [8], to name a few.

HMA, also known as P₁β-ATPase, is a type of protein combining ATP hydrolysis with metal ion transport across membranes [9,10] participating in absorbing and transporting heavy metal ions (Cu²⁺, Zn²⁺, Co²⁺, Cd²⁺, and Pb²⁺) [6]. Typical HMA proteins contain the E1–E2 ATPase domain and haloacid dehalogenase-like hydrolase (Hydrolase) domain [11]. Additionally, both sides of the N-terminal and C-terminal metal-binding sites may possess one or more soluble metal-binding domains (MBDs) that interact with or bind to specific metal ions [6]. At present, a number of HMA genes have been identified in plants, including 8 in Arabidopsis thaliana [12], 9 in rice (Oryza sativa L.) [12] 11 in maize (Zea mays L.) [13], 11 in sorghum (Sorghum bicolor L.) [13], 17 in Populus trichocarpa [11], and
Studies have demonstrated that AtHMA2 and AtHMA4 are two essential genes mediating Cd translocation in A. thaliana [15]. The translocation of Cd from the roots to shoots was near-completely abolished in the hma2 hma4 double mutant. TcHMA3, a tonoplast-localized transporter highly specific for Cd, is responsible for sequestering Cd into the leaf vacuoles so as to detoxify Cd in Thlaspi caerulescens [16]. OsHMA3, which localizes to vacuolar membranes, was identified as the gene that controls root-to-shoot Cd translocation rates in rice [17]. These results indicate that the HMA gene family plays diverse roles in plant resistance to Cd stress.

2. Materials and Methods

2.1. Plant Materials and Treatment

The ‘ZJU3’ barley variety was used in this study. Seeds uniform in size and with a full shape were selected and sterilized in 2.5% NaClO for 10 min, rinsed with distilled water four times, and then germinated at 28 °C under dark conditions. After 48 h, seedlings with a root length of approximately 0.5 cm were moved to hydroponic culture boxes (day/night temperatures of 26 °C/24 °C, light/dark photoperiod of 14 h/10 h, and light intensity of 18000 Lx). At the one-leaf stage, the seedlings were treated with 1/4 Hoagland’s nutrient solution. At the two-leaf stage, Cd stress experiments were performed. The CdCl₂ solutions (50 µmol/L and 100 µmol/L) prepared with Hoagland’s nutrient solution were used to simulate Cd stress, and Hoagland’s nutrient solution without CdCl₂ was used as the control. After 120 h of treatment, more than 10 barley seedlings were selected for each sample, and quickly stored at −80 °C until analysis. The experiment was performed in triplicate. The Hoagland’s nutrient solution formula was as described by Zhang et al. [26].

2.2. RNA Isolation and cDNA Synthesis

The total RNA was isolated from barley leaves using an RNA extraction kit (Tiangen, Beijing, China) and reverse transcribed to generate cDNA using a reverse transcription kit (Yeasen, Shanghai, China). The cDNA obtained was stored at −20 °C for qRT-PCR analysis.

2.3. Bioinformatics Analysis of the Barley HMA Gene Family

2.3.1. Identification and Structural Analysis of Barley HMA Genes

The total RNA was isolated from barley leaves using an RNA extraction kit (Tiangen, Beijing, China) and reverse transcribed to generate cDNA using a reverse transcription kit (Yeasen, Shanghai, China). The cDNA obtained was stored at −20 °C for qRT-PCR analysis.
accessed on 17 July 2020) [29] to remove sequences without E1-E2 ATPase and Hydrolase domains. Afterward, the non-redundant barley HMA proteins were obtained by manually removing the redundant sequences. The batch sequence search function in the Pfam database was used to obtain gene annotation files, and TBtools v1.087 (Chen, C.C., South China Agricultural University (SCAU), Guangdong, China) was then used to draw the domain map. The molecular characteristics of the HvHMA proteins were analyzed in ExPASy (Compute pI/Mwtool) (https://web.expasy.org/protparam/; accessed on 26 July 2020) including the number of amino acid (aa) residues, molecular weight (MW), theoretical isoelectric point (pI), and grand average of hydropathicity (GRAVY). WoLF PSORT (https://wolfpsort.hgc.jp/; accessed on 31 July 2020) [30] was used to predict the subcellular localization of the HvHMA proteins. The conserved motifs of the HvHMA proteins were mapped using the MEME online tool (http://meme-suite.org/tools/meme; accessed on 4 August 2020) [31]. The intron-exon organizations of the HvHMA genes were generated using Gene Structure Display Server v2.0 (GSDS v2.0, http://gsds.cbi.pku.edu.cn/; accessed on 6 August 2020) (Center for Bioinformatics (CBI), Beijing, China) [32] by comparing the cDNAs to their corresponding genomic DNA sequences. The HvHMA genes were mapped to barley chromosomes based on physical location information from the EnsemblPlants database (http://plants.ensembl.org/Hordeum_vulgare/Info/Index; accessed on 6 August 2020) using Tbtools v1.087.

2.3.2. Phylogenetic Analysis of the Barley HMA Family

The HMA protein sequences of A. thaliana, rice, and barley were imported into the MEGA v7.0 program (Sudhir Kumar, Temple University, Philadelphia, PA, USA) and multiple sequence alignments were performed using ClustalW. The alignment file was then used to construct a neighbor-joining (NJ) phylogenetic tree, with the following parameters: p-distance model, 1000 bootstrap replications, and other default parameters [33]. The tree was displayed and modified using the iTOL website (https://itol.embl.de/; accessed on 12 August 2020) [34].

2.3.3. Expression Profiling of the Barley HMA Family and Candidate Gene Selection

The RNA-Seq data of 15 developmental stages were downloaded from the IPK website (https://apex.ipk-gatersleben.de/apex/?p=284:10; accessed on 20 August 2020) for mapping HvHMA expression profiles. The 15 developmental stages were as follows: 4-day embryos (EMB); roots from seedlings (10 cm shoot stage) (ROO1); shoots from seedlings (10 cm shoot stage) (LEA); developing inflorescences (INF2); developing tillers, 3rd internode (5 DAP) (NOD); developing grain (5 DAP) (CAR5); developing grain (15 DAP) (CAR15); etiolated seeding, dark conditions (10 DAP) (ETI); inflorescences, lemma (42 DAP) (LEM); inflorescences, lodicule (42 DAP) (LOD); dissected inflorescences (42 DAP) (PAL); epidermal strips (28 DAP) (EPI); inflorescences, rachis (35 DAP) (RAC); roots (28 DAP) (ROO2); and senescing leaves (56 DAP) (SEN). The expression of HvHMA genes was normalized and represented in fragments per kilobase of exon per million fragments mapped (FPKM). The HvHMA expression profile based on the FPKM values was then drawn using the Multiple Experiment Viewer (MeV) (J. Craig Venter Institute, La Jolla, CA, USA) [35]. Based on the FPKM values at the 10 cm shoot stage and phylogenetic analysis, the candidate genes were selected for qRT-PCR experiments.

2.4. Quantitative RT-PCR Analysis of Barley HMA Genes

Five pairs of primers related to specific genes were designed using Primer Premier v5.0 (PREMIER Biosoft, San Francisco, CA, USA) for qRT-PCR (Table 1). The barley actin gene HvActin (HORVU1Hr1G002840) was used as an internal control. The qRT-PCR analysis was performed on the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), and the data were analyzed using the 2−ΔΔCt method with three biological replicates [36]. IBM SPSS Statistics v20 (IBM, Armonk, NY, USA) statistical software was then used to
analyze significance (*, **, and *** indicates $p < 0.05$, $p < 0.01$, and $p < 0.001$ respectively). Histograms were drawn with SigmaPlot v10.0 (SYSTAT, San Jose, CA, USA).

Table 1. Primer sequences designed for qRT-PCR analysis.

| Gene Name  | Gene ID                      | Forward Primer Sequence (5′-3′) | Reverse Primer Sequence (5′-3′) |
|------------|------------------------------|--------------------------------|--------------------------------|
| HvHMA1     | HORVU7Hr1G097240.1           | TGGCGAAGAAATGCTGTGCT           | AACCAGCTGTGGATACATCCT          |
| HvHMA2     | HORVU6Hr1G033380.2           | TGGAGGTGTCATTTCAGAACTG         | CAACCACATAACTGGAACCTT          |
| HvHMA3     | HORVU5Hr1G094430.8           | ACATCGCCGTAGGAGGAAAGT          | GCGTCTGGCTGACTGCTGG            |
| HvHMA4     | HORVU7Hr1G108890.1           | TCAGCCTAAGTCAAGAAGACCATTG      | CTCGAGGATTCATCTGGTCC           |
| HvHMA5     | HORVU7Hr1G100160.2           | GCTAAGGCATCTATCGGTTC           | ATGCAGAACACTTATCTGGC          |
| HvActin    | HORVU1Hr1G002840             | TGGATCGGAGGGTCATCCT           | GCACCTTCTGTGGACGATGCTG         |

3. Results

3.1. Identification and Molecular Characteristics of Barley HMA Proteins

Through multiple bioinformatics analyses, a total of 21 barley HMA proteins were screened by removing redundant sequences and validating domains, which were named HvHMA1-21. The basic information on HvHMA1-21, including the number of amino acid residues, molecular weight, theoretical isoelectric point, grand average of hydropathicity, and subcellular localization, which indicated molecular characteristics of barley HMA proteins, is listed in Table 2. The results showed that the 21 HvHMA proteins contained 672 (HvHMA5) to 1083 (HvHMA21) amino acid residues with molecular weights ranging from 73112.29 (HvHMA5) to 118116.93 (HvHMA21) Da. The theoretical isoelectric points of the 21 HvHMA proteins ranged from 5.00 (HvHMA8) to 7.82 (HvHMA17), with the majority constituting acidic proteins. The GRAVY numeric values of the 21 HvHMA proteins varied from $-0.090$ (HvHMA1) to $0.422$ (HvHMA3), indicating that these proteins were likely amphoteric proteins. Additionally, the subcellular localization results showed that the 18 HvHMA proteins were localized in the plasma membrane, whereas three proteins (HvHMA9, HvHMA14, and HvHMA19) were localized in the endoplasmic reticulum.

Table 2. Physicochemical properties and subcellular localization of HMA proteins in barley.

| Gene Name  | Protein Number | ORF (aa) | MW (Da) | PI     | Subcellular Localization | Hydrophilicity Index |
|------------|----------------|----------|---------|--------|--------------------------|---------------------|
| HvHMA1     | A0A287XH51     | 1009     | 108464.81 | 6.68   | Plasma membrane           | −0.090              |
| HvHMA2     | A0A287TV87     | 987      | 106112.96 | 5.58   | Plasma membrane           | 0.171               |
| HvHMA3     | A0A287SMB8     | 765      | 80511.64  | 5.80   | Plasma membrane           | 0.422               |
| HvHMA4     | A0A287XS00     | 1023     | 102819.64 | 5.27   | Plasma membrane           | 0.268               |
| HvHMA5     | A0A287J245     | 672      | 73112.29  | 6.19   | Plasma membrane           | 0.277               |
| HvHMA6     | A0A287KH5      | 871      | 92584.78  | 7.59   | Plasma membrane           | 0.106               |
| HvHMA7     | M0X9Y2         | 761      | 80246.05  | 5.36   | Plasma membrane           | 0.223               |
| HvHMA8     | A0A287NAD1     | 1050     | 113188.47 | 5.00   | Plasma membrane           | 0.193               |
| HvHMA9     | A0A287N6A1     | 1000     | 109664.92 | 5.72   | Endoplasmic reticulum     | 0.171               |
| HvHMA10    | M0WLW4         | 946      | 100370.69 | 7.04   | Plasma membrane           | −0.016              |
| HvHMA11    | A0A287FY78     | 981      | 107543.64 | 6.42   | Plasma membrane           | 0.083               |
| HvHMA12    | A0A287E4C3     | 1039     | 114071.38 | 6.95   | Plasma membrane           | −0.042              |
| HvHMA13    | A0A287Q47      | 1033     | 111820.68 | 6.07   | Plasma membrane           | 0.183               |
| HvHMA14    | A0A287EJS4     | 1049     | 113618.24 | 5.49   | Endoplasmic reticulum     | 0.140               |
| HvHMA15    | M0WME5         | 962      | 105942.79 | 6.15   | Plasma membrane           | 0.094               |
| HvHMA16    | A0A287DYP4     | 983      | 107457.12 | 6.10   | Plasma membrane           | 0.063               |
| HvHMA17    | A0A287JG07     | 1010     | 106141.11 | 7.82   | Plasma membrane           | 0.074               |
| HvHMA18    | A0A287KKR1     | 690      | 73747.50  | 6.73   | Plasma membrane           | 0.219               |
| HvHMA19    | M0WLS2         | 1020     | 110187.74 | 6.21   | Endoplasmic reticulum     | 0.220               |
| HvHMA20    | A0A287Q5Y4     | 994      | 109774.70 | 7.45   | Plasma membrane           | 0.142               |
| HvHMA21    | A0A287P785     | 1083     | 118116.93 | 5.49   | Plasma membrane           | 0.034               |
3.2. Phylogenetic Analysis and Classification of Barley HMA Genes

To explore the evolutionary characteristics of HvHMA genes and the evolutionary relationships between the AtHMA, OsHMA, and HvHMA genes, HMA sequences from A. thaliana, rice, and barley, including 8 AtHMA proteins, 8 OsHMA proteins, and 21 HvHMA proteins, were subjected to phylogenetic analysis. As shown in Figure 1, the HvHMA genes were divided into five groups (A–E). Among the 21 HvHMA genes, 2 belong to group A, 6 to group B, 6 to group C, 3 to group D, and 4 to group E. The phylogenetic tree indicated that members of groups A, D, and E were homologous to the AtHMA and OsHMA proteins. Moreover, compared to dicotyledonous A. thaliana, monocotyledonous barley and rice were more closely related. With the exception of the E1-E2 ATPase and Hydrolase domains, some HvHMA proteins contained other domains including an HMA domain, Cation_ATPase_N domain, Cation_ATPase_C domain, Hydrolase_3 domain, and CaATP_NAI domain, which revealed that the HvHMA proteins contained more abundant domains than the AtHMA and OsHMA proteins. Thus, it is inferred that members of the barley HMA gene family are more functionally diverse and therefore worth exploring. Additionally, there were some differences among groups in the domain distribution of the HvHMA proteins. The HMA domains were concentrated in groups D and E, the Cation_ATPase_N domains and Cation_ATPase domains were distributed in groups B and C, and Cation_ATPase_C domains were all distributed in group B. In relation to other groups, there were more types of domains in group B, indicating that members of group B might be complex in terms of function.

![Figure 1. The phylogenetic tree was constructed using the HMA protein sequences of Hordeum vulgare (Hv), Oryza sativa (Os), and Arabidopsis thaliana (At) in MEGA v7.0. All HMA members were classified into five groups (A–E). The HMA proteins of different species are differentiated by different colors. The black letters represent HvHMAs, the green letters represent OsHMAs, and the red letters represent AtHMAs. Different-colored rectangles represent different structural domains. The three green rectangles from light to dark represent Cation_ATPase, E1–E2 ATPase, and Hydrolase_3, respectively; the yellow rectangle represents Hydrolase; the pink rectangle represents HMA; the red rectangle represents Cation_ATPase_C; and the two purple rectangles from light to dark represent Cation_ATPase_N and CaATP_NAI, respectively.](image-url)
3.3. Chromosomal Location of Barley HMA Genes

According to the genome annotations, 18 of the 21 HvHMA genes were distributed on the six barley chromosomes (Figure 2), with the largest number of genes located on Chr4 (5), followed by Chr5 (4), Chr7 (4), Chr1 (2), Chr2 (2), and Chr6 (1). However, HvHMA7, HvHMA12, and HvHMA16, without clear localization information, were not positioned onto barley chromosomes. In addition, most of the HvHMA genes were concentrated on or near the end of the barley chromosomes. These results suggested that the distribution of HvHMA genes was uneven.

3.4. Motif Composition of the Barley HMA Proteins

Conserved motif analysis of the HvHMA proteins helped elucidate the conservation as well as the diversification of this family, and a total of 10 distinct conserved motifs were revealed. As exhibited in Figure 3, all HvHMA proteins contained common motifs including motif 1 and motif 10, which suggested that the two motifs might be the characteristic motifs of HvHMA proteins. With the exception of HvHMA21, all HvHMA proteins contained motif 3. Additionally, HvHMA proteins within the same group were generally found to show a similar motif composition. For example, motif 2 was distributed in all groups except group A, whereas motif 4, motif 5, motif 6, motif 8, and motif 9 were unique to group C. Moreover, the differences in motif composition among groups combined with the phylogenetic analysis results supported the reliability of the group classifications and indicated that HvHMA genes in distinct groups might be functionally divergent.

3.5. Intron-Exon Structure of Barley HMA Genes

The introns disrupted the coding sequences of most HvHMA genes. As shown in Figure 4, there were some differences among the HvHMA genes in terms of the number and size of the introns, which might be caused by intron deletion and insertion events. With the exception of HvHMA16 without introns, all HvHMA genes contained 2–33 introns (13 with 2–8 introns, 6 with 12–20 introns, and 1 with 33 introns). Additionally, several non-coding regions were distributed in the 18 HvHMA genes, with the exception of HvHMA16, HvHMA18, and HvHMA20.
3.6. Expression Pattern Analysis of Barley HMA Genes and Target Gene Screening

The analysis of gene expression patterns contributed to gene function prediction. The expression profiles of the HvHMA genes (Figure 5) revealed that the expression of some genes was tissue-specific. For example, the expression levels of HvHMA5, HvHMA9, and HvHMA15 were high during grain development; the expression levels of HvHMA3, HvHMA6, HvHMA7, HvHMA8, HvHMA13, and HvHMA18 were high in the leaves; HvHMA2, HvHMA12, and HvHMA19 were specifically expressed in the inflorescences; and HvHMA4, HvHMA10, and HvHMA11 were specifically expressed in the tillers, roots, and epidermal strips, respectively. These results indicated that these genes might play specific roles in the relevant tissues. Moreover, the clustering results of the expression data unclearly correspond to the groupings based on phylogenetic analysis, implying that the expression pattern similarity incompletely depended on the sequence similarity. Based on a comprehensive consideration of the FPKM values at the 10 cm shoot stage and phylogenetic analysis, a total of five HvHMA genes were screened for expression analysis under Cd stress.
3.6. Expression Pattern Analysis of Barley HMA Genes and Target Gene Screening

The analysis of gene expression patterns contributed to gene function prediction. The expression of HvHMA genes was tissue-specific. For example, the expression levels of HvHMA12, HvHMA13, HvHMA18, and HvHMA19 were specifically expressed in the inflorescences; HvHMA4 and HvHMA6 were high during grain development; the expression levels of HvHMA5, HvHMA7, and HvHMA8 were high in the leaves; HvHMA10, HvHMA11, and HvHMA12 were upregulated and two genes (HvHMA4, HvHMA9) were downregulated. Compared with the control, the expression levels of all five genes were significantly different under the 50 μmol/L CdCl2 treatment. With the 100 μmol/L CdCl2 treatment, whereas four genes, except HvHMA4, were significantly different under the 50 μmol/L CdCl2 treatment.

3.7. Expression Analysis of Barley HMA Genes in Response to Cd Treatment

Intron-exon structures of HvHMA genes in barley. The exons and introns are indicated by yellow boxes and black lines, respectively (color figure online).

Figure 4. Intron-exon structures of HvHMA genes in barley. The exons and introns are indicated by yellow boxes and black lines, respectively (color figure online).

Figure 5. Expression profiles of HvHMA genes in different tissues and development stages. Data were obtained from a publicly available database. Columns represent HvHMA members, while rows show different developmental stages and tissues. The expression level of HvHMA genes is shown by the intensity of the color, where red represents high expression and green represents low expression. EMB, 4-day embryos; ROO1, roots from seedlings (10 cm shoot stage); LEA, shoots from seedlings (10 cm shoot stage); INF2, developing inflorescences; NOD, developing tillers, 3rd internode (5 DAP); CAR5, developing grain (5 DAP); CAR15, developing grain (15 DAP); ETI, etiolated seeding, dark conditions (10 DAP); LEM, inflorescences, lemma (42 DAP); LOD, inflorescences, lodicule (42 DAP); PAL, dissected inflorescences (42 DAP); EPI, epidermal strips (28 DAP); RAC, inflorescences, rachis (35 DAP); ROO2, roots (28 DAP); and SEN, senescing leaves (56 DAP) (color figure online).
3.7. Expression Analysis of Barley HMA Genes in Response to Cd Treatment

HMA proteins participate in the distribution of non-essential heavy metal ions including Cd\(^{2+}\), which greatly affect the plant response to heavy metal stress. To analyze the expression of HvHMA genes under Cd stress, five members (Table 1) were carefully selected from 21 HvHMA genes, and qRT-PCR experiments were further performed at the seedling stage. The results (Figure 6) revealed that three genes (HvHMA1, HvHMA3, and HvHMA4) were upregulated and two genes (HvHMA2 and HvHMA6) were downregulated. Compared with the control, the expression levels of all five genes were significantly different under the 100 µmol/L CdCl\(_2\) treatment, whereas four genes, except for HvHMA4, were significantly different under the 50 µmol/L CdCl\(_2\) treatment. With the increase of Cd concentration, the expression of HvHMA1 and HvHMA4 was significantly under the high-concentration stress than under the low-concentration stress. Moreover, the expression changes of HvHMA2, HvHMA3, and HvHMA6 were similar after the two Cd treatments: the expression of HvHMA2, HvHMA3, and HvHMA6 was slightly higher under the high-concentration stress than under the low-concentration stress.

![Figure 6](image)

**Figure 6.** Relative expression analysis of five HvHMA genes under Cd stress in barley seedling leaves. Different treatments are represented by three different colors, and columns in black represent CK, columns in red represent 50 µmol/L CdCl\(_2\), and columns in green represent 100 µmol/L CdCl\(_2\). ANOVA was used to test significance. * indicates \(p < 0.05\), ** indicates \(p < 0.01\), and *** indicates \(p < 0.001\). Error bars represent the standard deviation.

4. Discussion

The HMA family, which plays a significant role in heavy metal transport, exists widely in plants. In this study, 21 HMA genes were identified in barley. According to phylogenetic analysis, the 21 HvHMA genes could be divided into five groups (A–E). Compared to groups B and C, members belonging to groups A, D, and E possessed higher homology to the proteins of *A. thaliana* and rice. Except for the E1-E2 ATPase and Hydrolase domains, members of groups B and C contained the Cation_ATPase_N domains. In addition, members of group B contained the Cation_ATPase_C domains. The Cation_ATPase_N domains and the Cation_ATPase_C domains, which are metal-binding domains, participate in metal ion transport in plants. As indicated by the conserved motif analysis, motifs 4–6 and motifs 8–9 were only distributed in group C. Therefore, it is speculated that the domains as well as motifs unique to groups B and C resulted in the
separation of groups B and C from the other groups in the phylogenetic tree. Furthermore, the characteristics of groups B and C illustrated the differences in evolution between barley and other species.

The subcellular localization results revealed that most of the HvHMA members were predicted as plasma membrane proteins, with the exception of HvHMA9, HvHMA14, and HvHMA19, which were all located in the endoplasmic reticulum and were placed in the same group (group B). These results suggested that there were certain corresponding relationships between the phylogenetic groupings based on sequence similarity and subcellular localization. Therefore, homologous genes may be similar in gene function and signal transduction process.

According to the presence or absence of introns, eukaryotic genes can be divided into intron-containing and intronless genes. Most eukaryotic genes belong to the former, but some belong to the latter. Previous studies identified that there are 5846 (21.7%) intronless genes in A. thaliana and 11,109 (19.9%) in rice [37]. Among the 21 HvHMA genes, 20 members with 2–33 introns are intron-containing genes, whereas HvHMA16 is an intronless gene. Some plausible explanations may account for the origin of intronless genes. It has been suggested that intronless genes evolved owing to a loss of introns [38]. Another probability is that intronless genes formed as a result of reverse transcription [39]. During the process of retroposition, mRNAs are reverse-transcribed into cDNAs and inserted into new genomic positions that lack introns [40]. Therefore, it can be inferred that intron loss or retrotransposition events impacted on the intron-exon structures of HvHMA genes, leading to the presence of an intronless gene (HvHMA16) in the barley HMA gene family. By comprehensively analyzing the results of the evolutionary tree and the expression values at the 10 cm shoot stage, five genes were screened that might be related to stress responses. Among them, the expressions of HvHMA1, HvHMA3, and HvHMA4 were significantly upregulated under Cd stress. HvHMA1 was highly homologous to OsHMA2, which participates in the root-to-shoot translocation of Cd [41–43]. Compared to the wild-type (WT), the Cd concentration in the grains of OsHMA2-overexpressing rice was decreased by approximately half [44]. HvHMA3 was homologous to OsHMA3, which sequesters Cd into the vacuoles of root cells in rice, thereby controlling the rate of Cd translocation from the roots to shoots [45]. Additionally, HvHMA4 was homologous to OsHMA9, whose expression was induced by a high concentration of Cd [46]. The above results indicated that the changes in expression after Cd treatment were in line with theoretical expectations and that the phylogenetic analysis results were credible.

Cd stress negatively effects plant growth and development. The qRT-PCR results suggested that Cd stress can promote or inhibit the expression of HvHMA genes, which indicated that different HvHMAs exhibit diverse mechanisms to protect barley from stress damage. The expression of HvHMA1 gradually increased with the growth of Cd concentration. Mills [47] found that the corresponding HvHMA gene conferred Cd sensitivity to wild-type yeast due to transport activity. It was speculated that HvHMA1 changed the transport activity of Cd in response to Cd stress in barley. Lei [48] found that HvHMA3 played a crucial role in grain Cd accumulation. In this study, HvHMA3 were significantly upregulated after Cd treatment. Therefore, it was speculated that HvHMA3 might be involved in Cd distribution in stress condition. Following Cd stress, the expression levels of HvHMA2 and HvHMA6 decreased significantly, indicating that Cd stress negatively regulated the expression of the two genes. According to this, it is inferred that the expression regulation pathways related to HvHMA2 and HvHMA6 may be similar. Among experimental groups, HvHMA1 and HvHMA4 were significantly upregulated with increasing Cd concentration, which indicated that the two genes were sensitive to the change of Cd stress. However, the molecular mechanisms of these HvHMA genes in response to Cd stress need further exploration.
5. Conclusions

The barley HMA gene family was explored herein using bioinformatics analysis. The results revealed the characteristics of the barley HMA gene family in terms of physicochemical properties, phylogenetic relationships, domain distribution, chromosomal location, motif composition, intron-exon structure, as well as tissue expression. Moreover, five HvHMA genes were selected for expression analysis which indicated that the five genes responded differently to Cd stress. HvHMA1, HvHMA3, and HvHMA4 were strongly activated by Cd stress, whereas HvHMA2 and HvHMA6 were significantly restrained. This study preliminarily confirms that HvHMA1, HvHMA3, and HvHMA4 play vital roles in Cd tolerance, providing a theoretical basis for further research on the functions of related genes and the improvement of barley varieties.

Author Contributions: D.X., X.Z. (Xiaolin Zhang), and Y.F. manage this project; C.Z., Q.Y., T.Y., X.Z. (Xian Zhang) and Y.W. performed the experiments; C.Z., D.X. and Y.F. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the New Talent College Student Plan in Zhejiang Province (2020R427033; 2020R427034), the National Undergraduate Training Programs for Innovation and Entrepreneurship (202010346043).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We thank LetPub (www.letpub.com; accessed on 11 August 2021) for its linguistic assistance during the preparation of this manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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