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Response of *MaHMA2* gene expression and stress tolerance to zinc stress in mulberry (*Morus alba* L.)

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Abstract: *HMA2* (heavy metal ATPase 2) plays a crucial role in extracellular and intracellular Zn^{2+} transport across biomembranes, maintaining ion homeostasis, and playing an important role in the normal physiological metabolism, growth, and development of plants. In our study, a novel *HMA2* gene, named *MaHMA2*, was isolated and cloned from white mulberry (*Morus alba* L.). The gene sequence obtained was 1,342 bp long, with an open reading frame of 1,194 bp, encoding a protein of 397 amino acids, with a predicted molecular mass of 42.852 kD and an isoelectric point of 7.53. This protein belonged to the PIB-type ATPase transport protein family. We analyzed the expression of the *MaHMA2* gene by quantitative real-time PCR. The results showed that the level of *MaHMA2* gene expression decreased to a Zn concentration of 800 mg/kg. Malondialdehyde and proline levels increased and responded to increasing Zn when the *MaHMA2* gene was silenced, whereas the activities of peroxidase and superoxide dismutase tended to increase in response to increasing Zn^{2+} ion stress concentrations but were lower in the gene-silenced plants. These findings suggested that the *MaHMA2* gene played an active role in the tolerance response of mulberry to Zn stress.

Introduction

Zinc (Zn) plays an important role in many biological processes, including respiratory electron transport chain, photosynthesis, oxidative stress protection, hormone signaling, cell wall metabolism, and pollen formation (Rizwan *et al.*, 2019). Zn Deficiencies or excess can have adverse effects on plants. Soil organic matter has a strong adsorption capacity for Zn, which significantly reduces Zn effectiveness. Zn bioavailability is reduced at a high soil pH and under other soil conditions, leading to Zn deficiency in the plant (Wu *et al.*, 2020). Plants exhibit a range of injurious effects, such as inhibition of photosynthesis, reduced lignification, the inhibition of protein synthesis, reduced pollen viability, and decreased disease resistance in response to Zn-deficiency. Due to this host of injuries, plant leaves suffer chlorosis, susceptibility to deformation, slow growth and development, reduced yield, and poor tolerance to abiotic stresses. As a consequence of the rapid development of industrial, agricultural, and mining activities, heavy metal elements such as Zn are deposited in increasing quantities in soils, leading to heavy metal pollution; thus, endangering the growth and development of plants and animals and posing a threat to human health, and planting Zn-tolerant mulberry species is a very effective bioremediation measure (Qin *et al.*, 2018). The Zn content of Chinese soils ranges from 3 to 790 mg/kg, with an average of 100 mg/kg, which is twice the world average, and the Zn content of soils near zinc mining areas is as high as 4025 mg/kg (Liu *et al.*, 2020). Excessive Zn concentrations cause cycling between the reduced and oxidized Zn^{+} states of Zn ions to generate highly toxic hydroxyl radicals, which, in turn, damage macromolecules (Wu *et al.*, 2016). Therefore, it is imperative that plants maintain Zn homeostasis for normal growth and development.

To maintain Zn homeostasis, plants have evolved a tightly regulated network to control the uptake and transport of Zn. Although excess Zn is generally highly toxic to plants, some plants can still grow normally in environments with considerable Zn^{2+} contamination through hyper-accumulation

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of Zn^{2+} in their tissues to levels toxic to most other plant spp. This suggests the existence/deployment of Zn-tolerance mechanisms in Zn-hyperaccumulating plants that allows them to survive in Zn^{2+}-contaminated soils (Guzmán-Rangel *et al.*, 2017). Studying Zn^{2+}-tolerance mechanisms in plants is important for agricultural production and the phytoremediation of Zn^{2+}-contaminated soils (Versieren *et al.*, 2016).

The top priority in phytoremediation is to improve plant tolerance to heavy metals (Wu *et al.*, 2021). This can be achieved through various routes, such as increasing the activity of stress-related antioxidant enzymes, promoting compartmentalization, and increasing the quantities of metal-chelating molecules synthesized. The heavy metal-transporting ATPases (*HMAs*) are transport proteins that actively transport heavy metal cations across membranes in a process driven by ATP hydrolysis. The *HMA* gene is widely distributed in plants, and *HMA1*, *HMA3*, and *HMA4* have been identified and isolated from plants and shown to play important roles in plant responses to environmental stresses (Takahashi *et al.*, 2012). The different *HMA* proteins are selective for specific heavy metal ion transport. For instance, *HMA2-4* are Zn/Cd transporters, whereas *HMA5-8* are Cu^{+}/Ag^{+} transporters. The metal specificity of the *HMA1* transporter is still not completely understood. The *HMAs* are predicted to be localized at the plasma membrane, chloroplast, Golgi membrane (*HMA7*), or tonoplast (*HMA3*) (Blindauer and Leszczyszyn, 2010; Romero-Isart and Vasák, 2002; Leszczyszyn *et al.*, 2013). The gene sequences, protein structural domains, and conserved motif patterns among members of the different subgroups are closely related to the phylogenetic diversity, reflecting the fact that they all have different metal ion transport-related functions and differences in the specific heavy metal ions transported (Lee *et al.*, 2007; Cun *et al.*, 2014).

Virus-induced gene silencing (VIGS) is an RNA interference-based technique widely used to down-regulate various plant-specific transcripts and knocks-down the expression of target genes using modified plant viral genomes (Zhang *et al.*, 2015). VIGS has been developed as a new reverse genetics technique for the rapid functional characterization of plant genes. So far, VIGS systems have been established with RNA viruses, DNA viruses, satellite viruses, and DNA satellite molecules as vectors (Geuten *et al.*, 2013). Compared with transgenic plant techniques such as knockout and antisense, VIGS does not require the construction of transgenic plants. It has a short cycle time, is simple to perform, and obtains novel phenotypes quickly and at a low cost.

The mulberry plant (*Morus alba* L.) is native to central and northern China, and is now cultivated from the northeast to southwest provinces and regions of China (Zhao *et al.*, 2007). The soil environment conditions in these areas are harsh and usually heavily overloaded with heavy metals, thus, providing a poor soil environment for the large-scale cultivation of mulberry. Although research on *HMA* gene expression in response to heavy metal stress has been reported in other plant spp., little is known about how mulberry plant *HMA* genes help in response to metal stress such as Zn (Imran *et al.*, 2016). In this study, the response of *MaHMA2* gene expression and stress tolerance to Zn stress in mulberry was investigated through a bioinformatics analysis and molecular approaches. This work serves as a foundation for understanding the *MaHMA2* gene regulatory effect in mulberry under Zn stress and help breed mulberry variety to withstand heavy metal contaminated soils (Fan *et al.*, 2018).

Materials and Methods

Plant materials and growth conditions

Morus alba seedlings were obtained from the National Mulberry Germplasm Nursery at Jiangsu University of Science and Technology, Zhenjiang, China. The seedlings were transplanted in pots containing vermiculite and loamy soil (pH 7.0). When the seedlings reached about 20 cm above soil, they were randomly divided into five groups. The groups were treated with $ZnSO_4{\cdot}5H_2O$ at concentrations of 0 (control), 100, 200, 400 and 800 mg/kg to represent different Zn stress treatments. Three biological replicates of each treatment group were set up and sampled simultaneously after five days of Zn treatment. For the VIGS suppression of the *MaHMA2* gene, *M. alba* seeds were sown in seedling trays, and when the two cotyledons had emerged, they were injected with either recombinant virus pTRV2-*MaHMA2*, empty pTRV2 vector, or buffer. Thirty days after the inoculation, seedlings with uniform growth and condition were selected and transplanted into pots containing vermiculite and loamy soil (pH 7.0) with the $ZnSO_4{\cdot}5H_2O$ concentrations described above. The seedlings injected with buffer were set as the blank control, pTRV2 empty vector as the negative control and the recombinant virus pTRV2-*MaHMA2* as the experimental group. A volume (100 mL) of Murashige & Skoog (MS) nutrient solution was added to each pot at a fixed time every day for five days and sampled at the same time point, with three biological replicates (three seedlings per replicate) of each group.

Bioinformatic analysis

Primers for PCR, quantitative real-time PCR (qPCR), and vector construction were designed using OLIGO Primer Analysis Software v.7 (Molecular Biology Insights, Inc., USA) and based on the coding sequence (CDS) of the *MaHMA2* gene (GenBank: MT444766.1) obtained from the transcriptome library. Nucleotide sequences were assembled using DNAStar software (DNAStar, USA). The BLAST online tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare and analyze sequence correlation data. Sequence comparisons were performed using the Clustal X 2.0 alignment program and DNAMAN software (version 8.0) (Thompson *et al.*, 1997). Expasy online tool (http://web.expasy.org/compute_pi/) was used to calculate the molecular masses and predict the isoelectric points of the predicted proteins. The online SWISS-MODEL prediction software (http://swissmodel.expasy.org/) was used for the 3D structure studies of *MaHMA2* (Zhao *et al.*, 2011). MEGA6 software was used to construct phylogenetic trees using the maximum likelihood (ML) method (Tamura *et al.*, 2013).

RNA isolation and cloning of the cDNA

Total RNA was extracted from fresh leaf samples using the RNAiso Plus reagent (Takara, China), according to the

manufacturer's protocol. The concentration and purity of total RNA were checked on a Nanodrop spectrophotometer, and its quality was examined by electrophoresis on 1.0% agarose gel. The extracted total RNA was reverse transcribed using PrimeScript™ RT reagent Kit (Takara, China), following the manufacturer's instructions to obtain the cDNAs.

PCR amplification procedure

The target *MaHMA2* gene amplification was performed according to the manufacturer's instructions, using the PCR Amplification Kit (Monad Biotech Co., China). The primer sequences were: *MaHMA2*-F: 5'-ATGCCTCTCGATGG-AGTCAAAGAGG-3', *MaHMA2*-R:5'-ATCCACCAGTAA-ACC-3'. The reaction mixture consisted of 4 μL cDNA, 2 μL *MaHMA2*-F (10 μM), 2 μL *MaHMA2*-R (10 μM), 25 μL 2 × Taq mixture, with ddH_2O added to a final volume of 50 μL. Amplification was carried out using the following PCR cycling conditions: denaturation at 95°C, 3 min; 35 cycles of denaturation at 95°C, 15 s; annealing at 60°C, 20 s; extension at 72°C, 1 min, then extension at 72°C for 5 min. The PCR-amplified target fragments were cloned into pMD 18-T vector using Vector Cloning Kit (Takara BioInc., Japan) following the manufacturer's instructions, and transformed into *E. coli* TOP10 receptor cells by thermal excitation. Positive colonies were selected and sequenced for verification.

qPCR reaction procedure

Three-step qPCR was performed using the FastStart Universal SYBR Green Master Mix kit (Roche, USA) in a LightCycler® 96 Real-Time PCR System (Roche, USA), according to the kit instructions. β-Actin was used as the internal reference gene (Jiang *et al.*, 2015), and primer sequences were as follows: *qMaHMA2*-F: 5'-TCACAAAGGCTGCAACTTCC-3', *qMaHMA2*-R: 5'-CATCATCAAGAAGAGACCGAA-3'. The qPCR reaction mixture consisted of 10 μL FastStart Universal® SYBR Green Master, 1 μL *qMaHMA2*-F (10 μM), 1 μL *qMaHMA2*-R (10 μM), 2 μL cDNA, and the addition of ddH_2O to make a final volume of 20 μL. Amplification was achieved using the following PCR cycling conditions: 95°C denaturation, 1 min, 35 cycles: denaturation at 95°C, 20 s; annealing at 60°C, 20 s; extension at 72°C, 30 s.

Physiological and biochemical index detection

Concentrations of soluble protein (determined by the bicinchoninic acid (BCA) assay), proline (PRO) and malondialdehyde (MDA), as well as superoxide dismutase (SOD) and peroxidase (POD) activities, were determined according to the appropriate assay kits (Comin, China). Proline, MDA and protein content, SOD and POD activities were determined as described in Liang *et al.* (2017).

Statistical analysis

Each study was replicated three times independently, and each data point shown represents the mean ± standard deviation (SD). Data were analyzed by analysis of variance (ANOVA), with multiple comparisons between samples conducted using Duncan's multiple range test. Statistical analyses were performed using Excel 2013 software (Microsoft, USA) and SPSS Statistics 19.0 software (SPSS Inc., USA).

Results

Cloning and verification of the MaHMA2 gene in mulberry

The *HMA2* gene amplified from white mulberry had a total length of 1,342 bp and an ORF of 1,194 bp, encoding a protein of 397 amino acid residues (Fig. S1), with a predicted molecular mass of 42.852 kD and an isoelectric point of 7.53, which was named *MaHMA2*. There are eight members of the mulberry HMA gene family, and we compared the nucleic acid sequences and amino acid sequences of each of the eight genes, as shown in Figs. S2 and S3. NCBI online Blast sequence comparison showed that the amino acid family encoded by the *MaHMA2* gene belonged to several families, such as the HAD-like superfamily, the zntA superfamily, and the ATPase-IB2_Cd superfamily (Fig. S4), which have certain functions in Zn ion uptake and transport, achieve the coordination of metal homeostasis in plants, and promotion of growth and development. The tertiary protein structure of *MaHMA2* was predicted by the online software SWISS-MODEL (Fig. S5). The predicted results indicated that *MaHMA2* had a high degree of structural homology with the P1B-type, ATPase family of heavy metal transport proteins.

Homology-based alignment and phylogenetic analysis of the MaHMA2

The amino acid sequence encoded by the *MaHMA2* gene of *M. alba* was compared with nine other species. The results showed up to 90% identity between this clade and *Morus atropurpurea* and *Morus notabilis*, and more than 70% with other species (Fig. S6). The *HMA2* amino acid sequences of different species were selected to construct a phylogenetic tree. Among the 21 species compared, the Guangdong mulberry species 'Da Shi' mulberry line was found to be the most similar to Sichuan mulberry (EXC27875.1 *M. notabilis*) and least similar to grape (CBI40117.3 *Vitis vinifera*), walnut (XP_018856913.1 *Juglans regia*) and wild tea tree (XP_028114667.1 *Camellia sinensis*) (Fig. 1). Based on the bioinformatics analysis, homology, and phylogeny with other plant species, the closest genetic relationship with *MaHMA2* was the *HMA2* gene of *M. notabilis* (at 97% homology) with a more distant relationship (at 97% homology) to *V. vinifera*. Other species, such as *J. regia* and *C. sinensis*, showed lower homologies, the lowest of which was with *V. vinifera*. These data suggest that the *HMA2* gene is highly conserved across species and may have evolved in response to different growing conditions and environmental factors yet had retained considerable homology with *MaHMA2*.

Expression levels of MaHMA2 under different Zn concentrations

The expression of the *MaHMA2* gene first increased and then decreased in response to increasing Zn concentration. Relative to the control (0 mg/kg), at Zn 200 mg/kg, the gene expression increased to 5.67-fold, from which point the gene expression gradually decreased as Zn concentration were further increased (Fig. 2). These findings suggest that *MaHMA2* may function in the uptake and translocation of Zn ions and that the expression of the gene was upregulated in the presence of low to moderate Zn concentrations but was

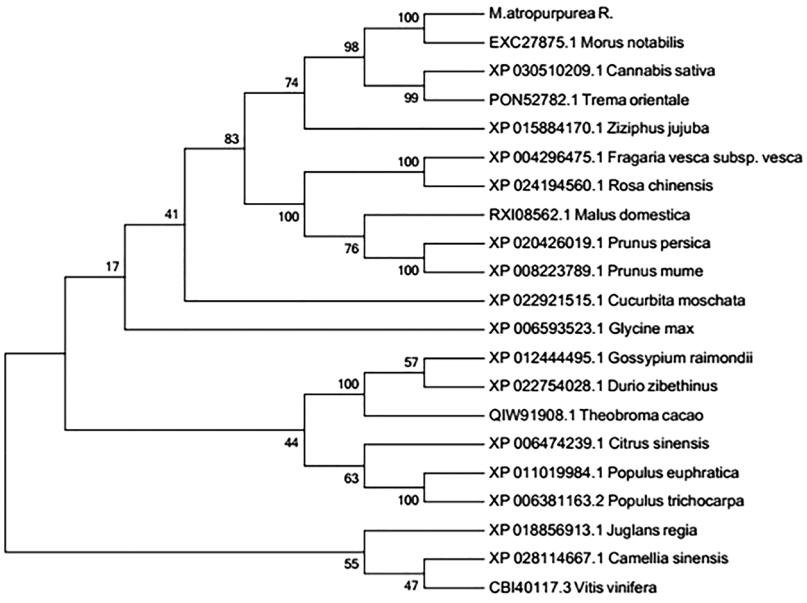

FIGURE 1. The *MaHMA2* homologous sequence phylogenetic tree.

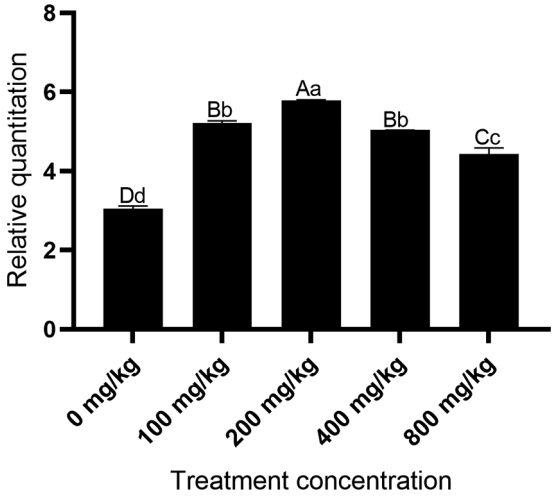

FIGURE 2. Relative levels of *MaHMA2* gene expression in response to increasing Zn concentration stress. The individual data points are mean ± standard deviation (SD) of the three biological replicates. Bars with different letters indicate a significant difference between expression levels at different concentrations ($p \leq 0.05$) on the basis of Duncan's multiple range test.

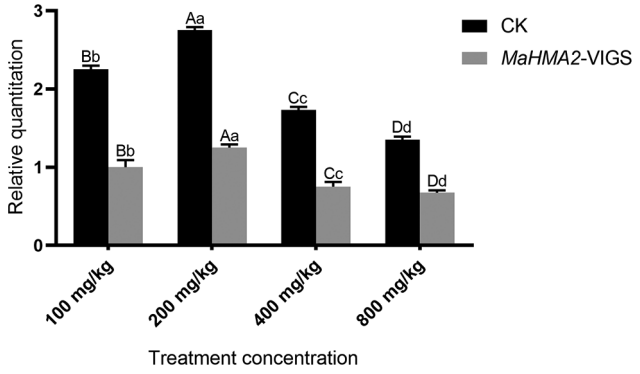

FIGURE 3. The expression level of *MaHMA2* gene under Zn stress with and without *MaHMA2* silencing. The individual data points are the mean ± standard deviation (SD) of the three biological replicates. CK means the negative control using empty pTRV2. *MaHMA2*-VIGS refers to the silencing of *MaHMA2* gene in the experimental group. Bars with different letters indicate a significant difference between expression levels at different concentrations ($p \leq 0.05$) on the basis of Duncan's multiple range test.

then downregulated once Zn ion concentrations exceeded a threshold value.

Expression level under different Zn concentrations after MaHMA2 gene silencing

The expression levels of the *MaHMA2* gene were then measured in gene-silenced VIGS transgenic plants grown under different concentrations of Zn. Empty pTRV2 vector was used as a negative control (CK) (Fig. 3). Under different concentrations of Zn stress, gene-silenced pTRV2-*MaHMA2*-VIGS plants showed a slight increase in expression relative to the 0 mg/kg VIGS plants followed by a gradual decrease with increasing Zn concentration; at 100 mg/kg Zn, expression of the gene-silenced plants was about 52% that of the empty pTRV2 plants. The expression of the *MaHMA2* gene reached its highest value at a Zn stress concentration of 200 mg/kg, indicating that Zn stress affected the expression of the *MaHMA2* gene, promoting the adaptation of mulberry to Zn ion stress.

Physiological and biochemical indexes under different Zn stress

The extent of lipid peroxidation, achieved due to the stress-induced accumulation of reactive oxygen species (ROS), was detected by assaying the concentration of MDA (Fig. 4A). As the concentration of Zn increased, plants injected with buffer as a blank control (wild type, WT) or those injected with empty pTRV2 vector as a negative CK showed no obvious increase in MDA concentration in response to increasing Zn concentration, but the gene-silenced pTRV2-*MaHMA2*-VIGS plants showed significantly higher levels of MDA which responded to increasing Zn concentration, indicating that *MaHMA2* gene silencing increased the MDA content (reflecting the stress suffered) of the transgenic plants. Proline is widely distributed in plant tissues, and it is

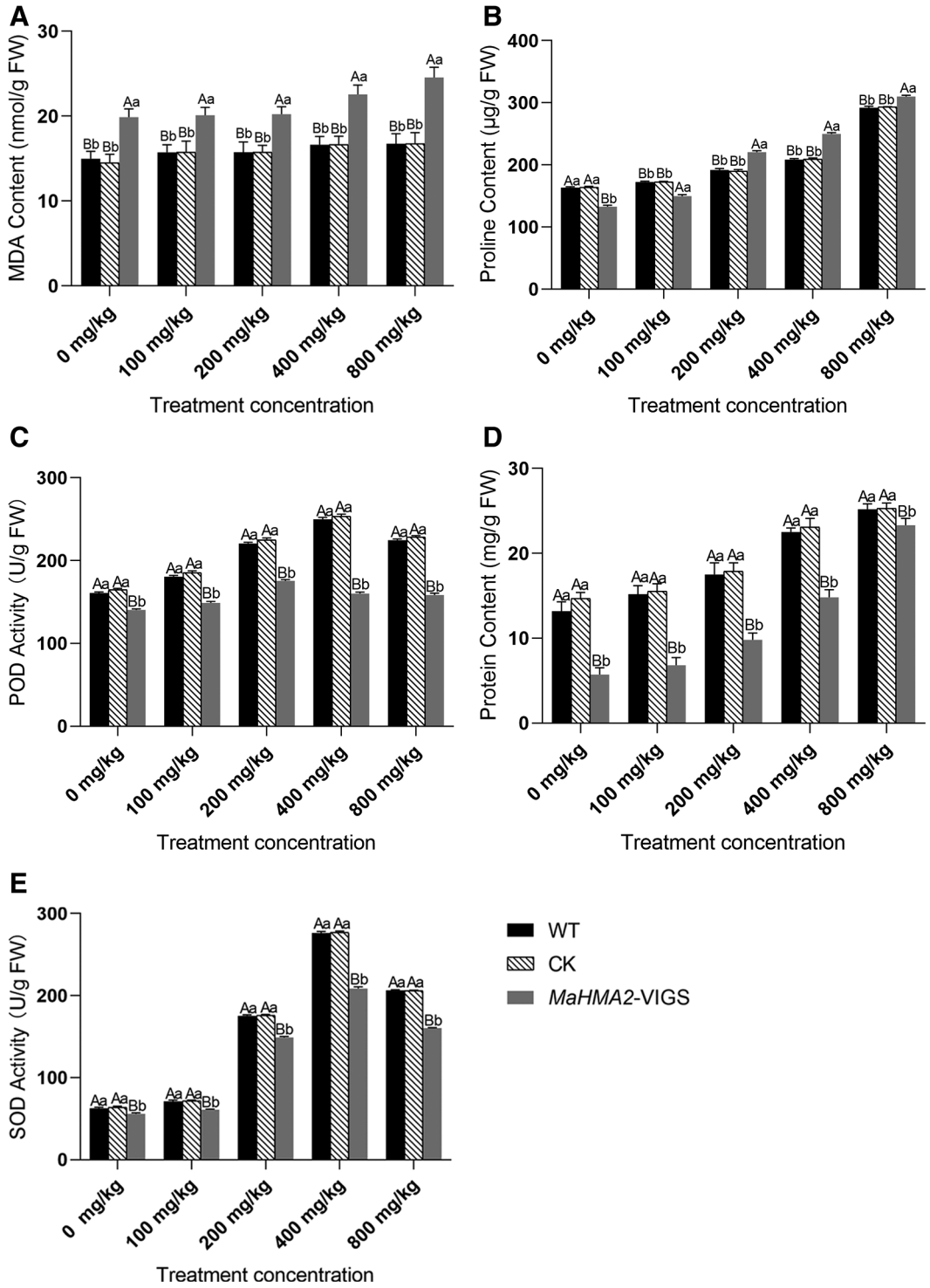

FIGURE 4. Physiological and biochemical indexes of mulberry in response to different Zn concentrations. A: MDA content; B: Proline content; C: POD activity; D: SOD activity; E: Protein content. The individual data points are mean ± standard deviation (SD) of the three biological replicates. WT (wild type) represents plants injected with buffer as the blank control, CK means negative control plants injected with empty pTRV2, and *MaHMA2*-VIGS means gene-silenced plants. Bars with different letters indicate a significant difference between expression levels at different concentrations ($p \leq 0.05$) on the basis of Duncan's multiple range test.

known that increased proline concentration reflects stress tolerance to some extent, with stress-tolerant varieties tending to accumulate more proline. Proline concentrations in the blank control, pTRV2 null-negative control, and gene-silenced pTRV2-*MaHMA2*-VIGS plants all showed an increasing trend with increasing Zn concentration. As with MDA, however, the proline concentration in the gene silenced pTRV2-*MaHMA2*-VIGS plants was significantly higher than that in the two non-silenced treatments (Fig. 4B).

Under abiotic stress, the activity of antioxidant enzymes such as POD and SOD, in plants facilitates stress tolerance by decreasing the stress-induced accumulation of ROS. As the concentration of Zn increased, the POD and (especially) SOD activity of the wild-type and blank control plants

increased, but there was a significant decrease in the activity of both enzymes in the gene-silenced pTRV2-*MaHMA2*-VIGS plants (Figs. 4C and 4D). This finding indicated that Zn stress increased oxidative stress and hence increased the activity of POD and SOD to help plants reduce the ROS levels adapt to the stress. As the Zn concentration increased, the protein concentration was significantly lower in gene-silenced pTRV2-*MaHMA2*-VIGS plants than in the blank control (Fig. 4E). The soluble protein content of control and gene-silenced pTRV2-*MaHMA2*-VIGS plants gradually increased with increasing Zn concentrations and reached a maximum at 800 mg/kg. The soluble protein concentration of the samples is commonly used to express enzyme activity.

Discussion

Plants have evolved complex molecular, cellular, and whole-plant mechanisms to cope with various abiotic stresses (Matsui *et al*., 2019). Previous studies have shown that *HMAs* play an important role in regulating plant responses to metal ion stress. Mulberry is an ecologically and economically important perennial woody plant affected by the adverse effects of various stresses on growth and productivity (Kim *et al*., 2021). Higher plants usually adapt to biotic and abiotic stresses by activating a cascade of molecular networks involving stress perception, signal transduction, and the expression of specific stress-related genes. In this study, a cDNA sequence encoding *MaHMA2* was cloned from mulberry leaves with a full length of 1342 bp and an ORF of 1194 bp, encoding a protein with 397 amino acids. The predicted protein's estimated molecular weight and isoelectric point (pI) were 42.852 kDa and 7.53, respectively. The predicted protein tertiary structure of the amino acid sequence of the *MaHMA2* gene was similar to the tertiary structure of the P-type ATPase family of Zn transporter proteins. Phylogenetic analysis showed that *MaHMA2* was closely related to *M. notabilis* and more distantly related to grape, walnut, and wild tea tree.

In the model organism *Arabidopsis thaliana*, *HMA2* is annotated as a Zn/Cd transport protein (Wong *et al*., 2009). qRT-PCR revealed that the expression of *MaHMA2* was sensitive to changes in Zn concentrations. This is in agreement with transcriptome sequencing results of Zn stress in our laboratory, which indicated a large difference in expression of the *MaHMA2* gene in the experimental group that was stressed by Zn compared with the untreated group (data not shown). The *HMA2* in arabidopsis is a Zn^{2+}-dependent ATPase that is also activated by Cd^{2+} and, to a lesser extent, by other divalent heavy metals (Pb^{2+}, Ni^{2+}, Cu^{2+}, and Co^{2+}) (Eren and Argüello, 2004). The observed increase in *MaHMA2* expression was aimed at increasing the transport of Zn to above-ground parts and provide for the study of the regulation mechanism of mulberry resistance to heavy metal stress and the cultivation of the mulberry. qRT-PCR analysis of *MaHMA2* expression in response to Zn applications showed that compared to the control, the expression of the *MaHMA2* gene was first increased, reaching the maximum value at 200 mg/kg, and then gradually decreased. However, even at 800 mg/kg, the expression was maintained higher than control levels. Partial suppression of *MaHMA2* expression was achieved after VIGS treatment of *M. alba* with pTRV2-*MaHMA2* (ca. 2-fold reduction). Interestingly, the expression pattern of *MaHMA2* in the modified seedlings (*MaHMA2*-VIGS) in response to Zn treatments was very similar to that observed in WT, although proportionately reduced at all Zn concentrations.

In our research, the mobilization of plant defense in response to Zn treatments was studied indirectly from physiological (MDA, proline, protein synthesis) and enzymatic (POD, SOD) markers of stress. The results indicated that while Zn treatments did not induce significant differences in WT MDA (indicator of oxidative stress) levels, changes in the levels of the other markers (proline, protein synthesis, POD and SOD) confirmed that Zn treatments caused a significant mobilization of plant defense responses, especially at 200–800 mg/kg. The partial repression of *MaHMA2* in *MaHMA2*-VIGS seedlings led to significant alterations in the plant response to Zn. Relative to the WT response, MDA production was elevated while protein content, POD and SOD activities were reduced at all Zn concentrations. The sensitivity of *MaHMA2* gene expression to external Zn concentrations and its effects on the mobilization of plant defense responses, suggests that *MaHMA2* is involved in the maintenance of plant Zn homeostasis to attenuate Zn stress.

In conclusion, we treated mulberry species with varied concentrations of Zn to induce Zn stress in mulberry plants. We isolated and characterized a novel *MaHMA2* gene RNA sequence technology and confirmed that Zn stress induced the gene. We found that silencing *MaHMA2* with VIGS could improve Zn tolerance by regulating physiological changes, phenotypic traits and Zn stress-related genes in mulberry. Our study revealed the function of *MaHMA2* in mulberry and laid the foundation for further understanding the mechanisms of plant responses to Zn stress.

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Availability of Data and Materials: All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Ethical Approval: This article does not contain any studies involving animals or human participants as research objects.

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1        M  P  L  D  G  V  K  E  V  T  V  I  I  A  T  R  T  V  I  V
61      CTTCATGACAACCTCCTCATTTCCCAGACTCAGATTGTAAAGGCACTAAACGAAGCAAGG
21       L  H  D  N  L  L  I  S  Q  T  Q  I  V  K  A  L  N  E  A  R
121     CTAGAAGCAAATGTTAGAATTTACGGAGGTGACAATTTCAAGAAGAAATGGCCAAGCCCT
41       L  E  A  N  V  R  I  Y  G  G  D  N  F  K  K  K  W  P  S  P
181     TACTCAATCGCTTGTGGAGTTTTGCTTTTGTTGTCGTTTCTCAAGTATGTTTACCGTCCG
61       Y  S  I  A  C  G  V  L  L  L  L  S  F  L  K  Y  V  Y  R  P
241     CTTGGGTGGTTGGCTCTTGCTGCTGTCGTTATCGGAATTTGGCCCATCTGTCTCAAAGCT
81       L  G  W  L  A  L  A  A  V  V  I  G  I  W  P  I  C  L  K  A
301     CTTGCAGCTACTCGGAATCTTAGGCTTGATATCAATATTCTCGCTATTGTTGCAGTGATT
101      L  A  A  T  R  N  L  R  L  D  I  N  I  L  A  I  V  A  V  I
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121      G  T  V  A  M  G  D  Y  V  E  A  G  T  I  V  F  L  F  T  V
421     GCCGAGTGGCTCGAGTCAAGGGCAAGCCACAAGGCAAATGTGGTAATGTCATCGCTGATG
141      A  E  W  L  E  S  R  A  S  H  K  A  N  V  V  M  S  S  L  M
481     AGCATGGTCCCTCAGAAAGCCGTCATAGCGGAAACCGGAGAAGTTGTAGATGCCGATGAG
161      S  M  V  P  Q  K  A  V  I  A  E  T  G  E  V  V  D  A  D  E
541     GTGAAGCTGAACACAGTGCTAGCAGTCAAGGCAGGGGAAGTGATACCAATTGATGGAATA
181      V  K  L  N  T  V  L  A  V  K  A  G  E  V  I  P  I  D  G  I
601     GTGGTAGAAGGAAACTGTGAAGTGGATGAGAAAACACTAACAGGAGAGTCTTTCCCAGTC
201      V  V  E  G  N  C  E  V  D  E  K  T  L  T  G  E  S  F  P  V
661     GCCAAACAGAAGGATTCTACCGTTTGGGCCGGCACCACCAATCTCAATGGCTACATTAGT
221      A  K  Q  K  D  S  T  V  W  A  G  T  T  N  L  N  G  Y  I  S
721     GTGAAGACCACGGCCTTGGCTGAAGACTGTGCCGTGGCTAAAATGGCTAAGCTTGTGGAA
241      V  K  T  T  A  L  A  E  D  C  A  V  A  K  M  A  K  L  V  E
781     GAGGCTCAAAACAGCAAGTCAAAAACTCAGAGATTCATTGACAAATGTGCTAAGTTTTAC
261      E  A  Q  N  S  K  S  K  T  Q  R  F  I  D  K  C  A  K  F  Y
841     ACCCCAGCGGTTCTGATAATATCAATTTCTGTGGCTGTGATTCCTGCGGCACTAAGACTT
281      T  P  A  V  L  I  I  S  I  S  V  A  V  I  P  A  A  L  R  L
901     CCACACCTCAGCAAATGGTTTCATTTAGCACTGGTTGTTTTGGTAAGCGCATGTCCTTGT
301      P  H  L  S  K  W  F  H  L  A  L  V  V  L  V  S  A  C  P  C
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321      G  L  I  L  S  T  P  V  A  T  F  C  A  L  T  K  A  A  T  S
1021    GGTCTTCTCATCAAAGGAGGCGATCACCTCGAGACGCTCTCCAAGATCAAGATTGCCGCC
341      G  L  L  I  K  G  G  D  H  L  E  T  L  S  K  I  K  I  A  A
1081    TTTGACAAGACCGGGACGGTCACTAGAGGCGAATTTGTGGTCTCCGATTTTCGGTCTCTT
361      F  D  K  T  G  T  V  T  R  G  E  F  V  V  S  D  F  R  S  L
1141    CTTGATGATGTTGGCTTGGAAACTTTGGTTTACTGGTGGATCCCGAATCTCTAG
381      L  D  D  V  G  L  E  T  L  V  Y  W  W  I  P  N  L  *
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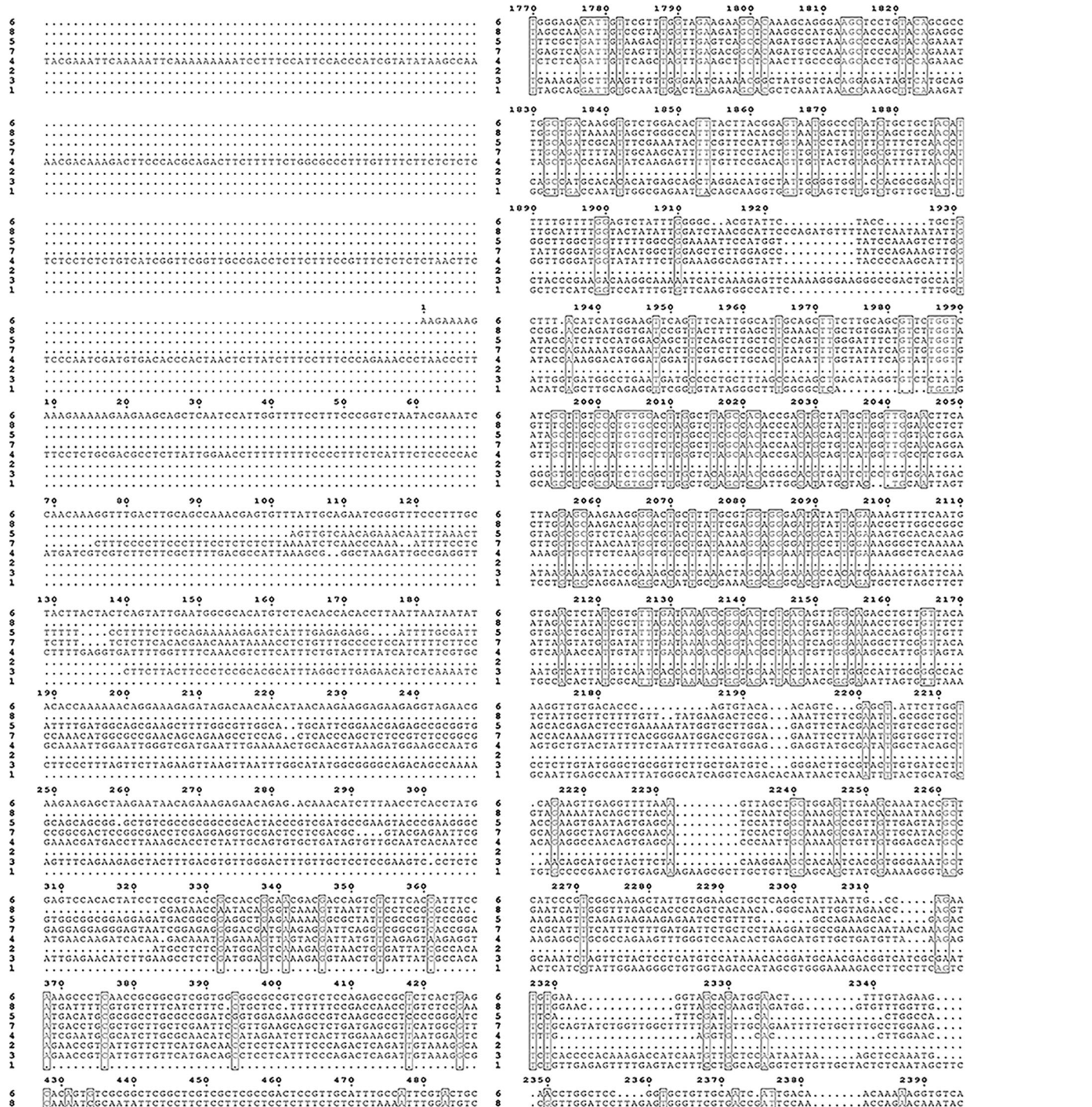
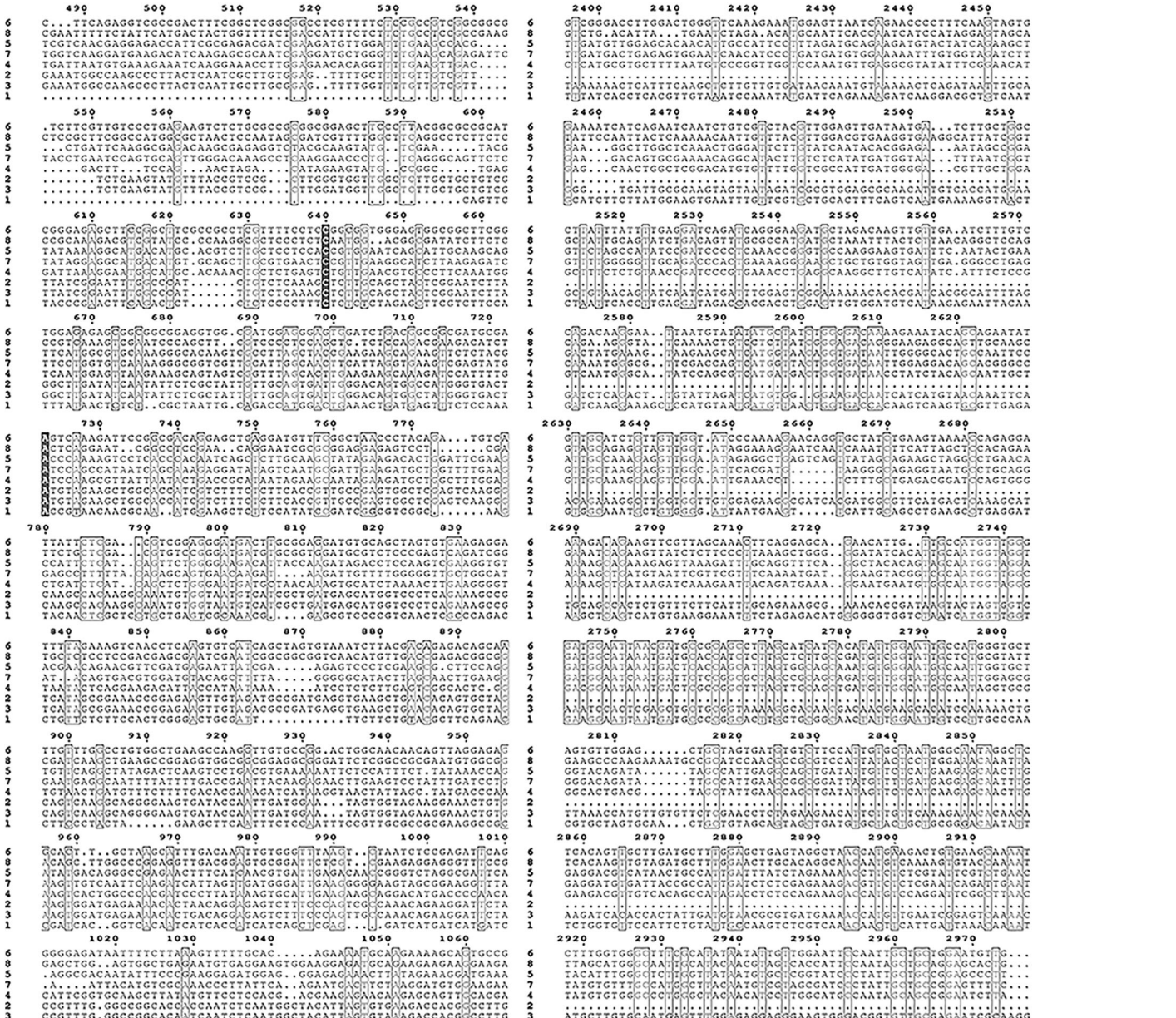
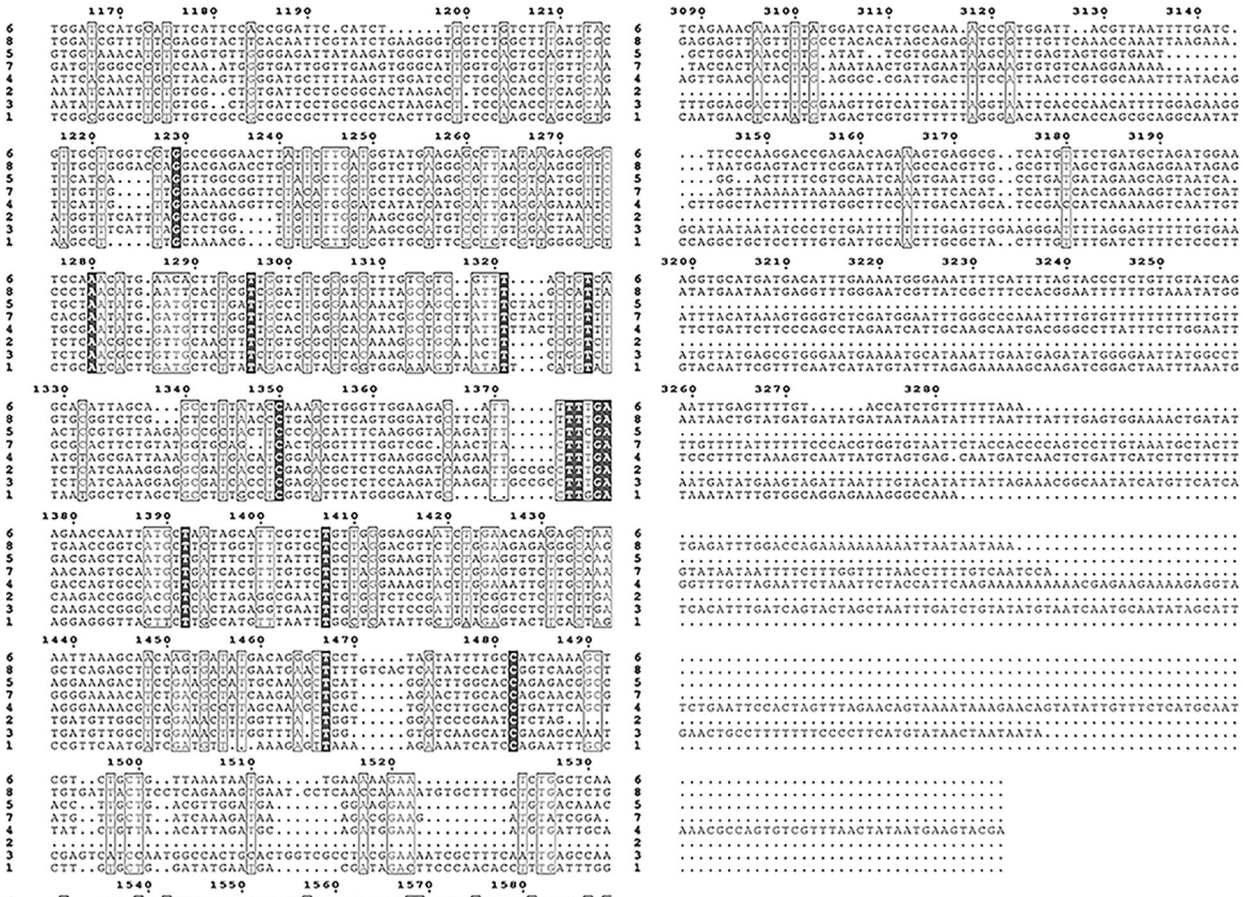
SUPPLEMENTARY FIGURE S1. The *MaHMA2* gene cDNA sequence and the predicted amino acid sequence. ATG indicates the start codon; TAG indicates the stop codon.



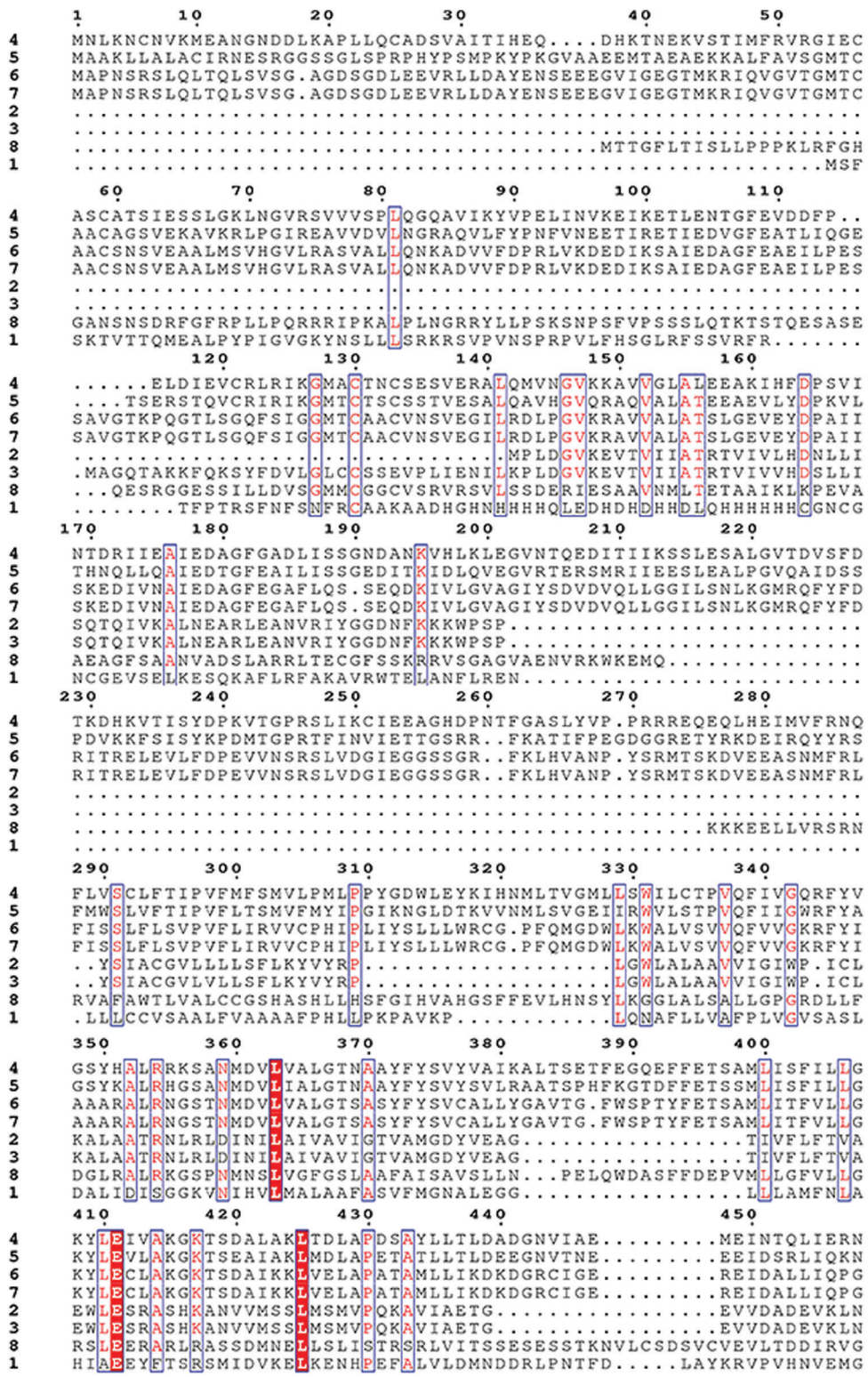
SUPPLEMENTARY FIGURE S2. (Continued)



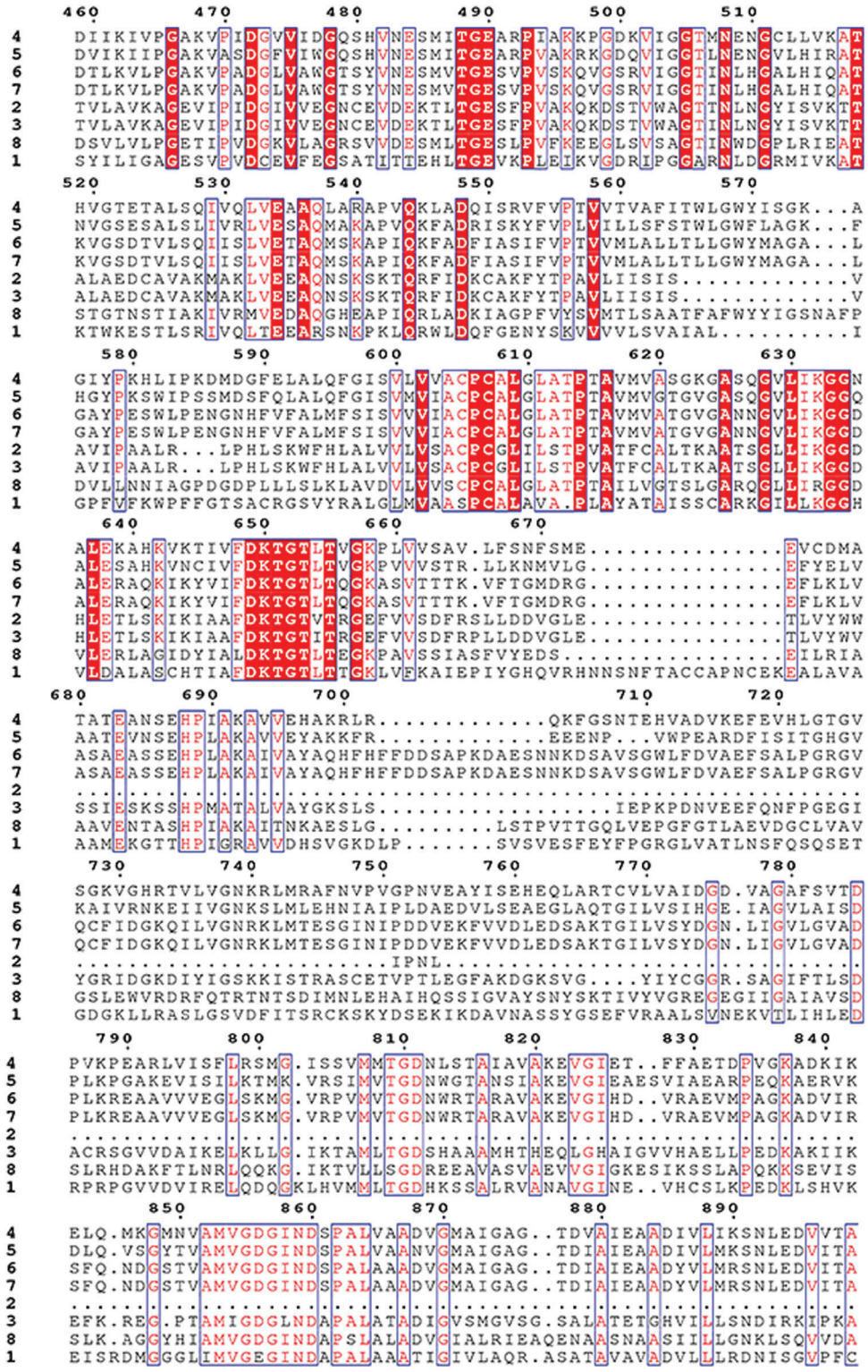
SUPPLEMENTARY FIGURE S2. (Continued)



SUPPLEMENTARY FIGURE S2. Nucleic acid sequence alignment of the *HMAs* gene family of mulberry.



SUPPLEMENTARY FIGURE S3. (Continued)



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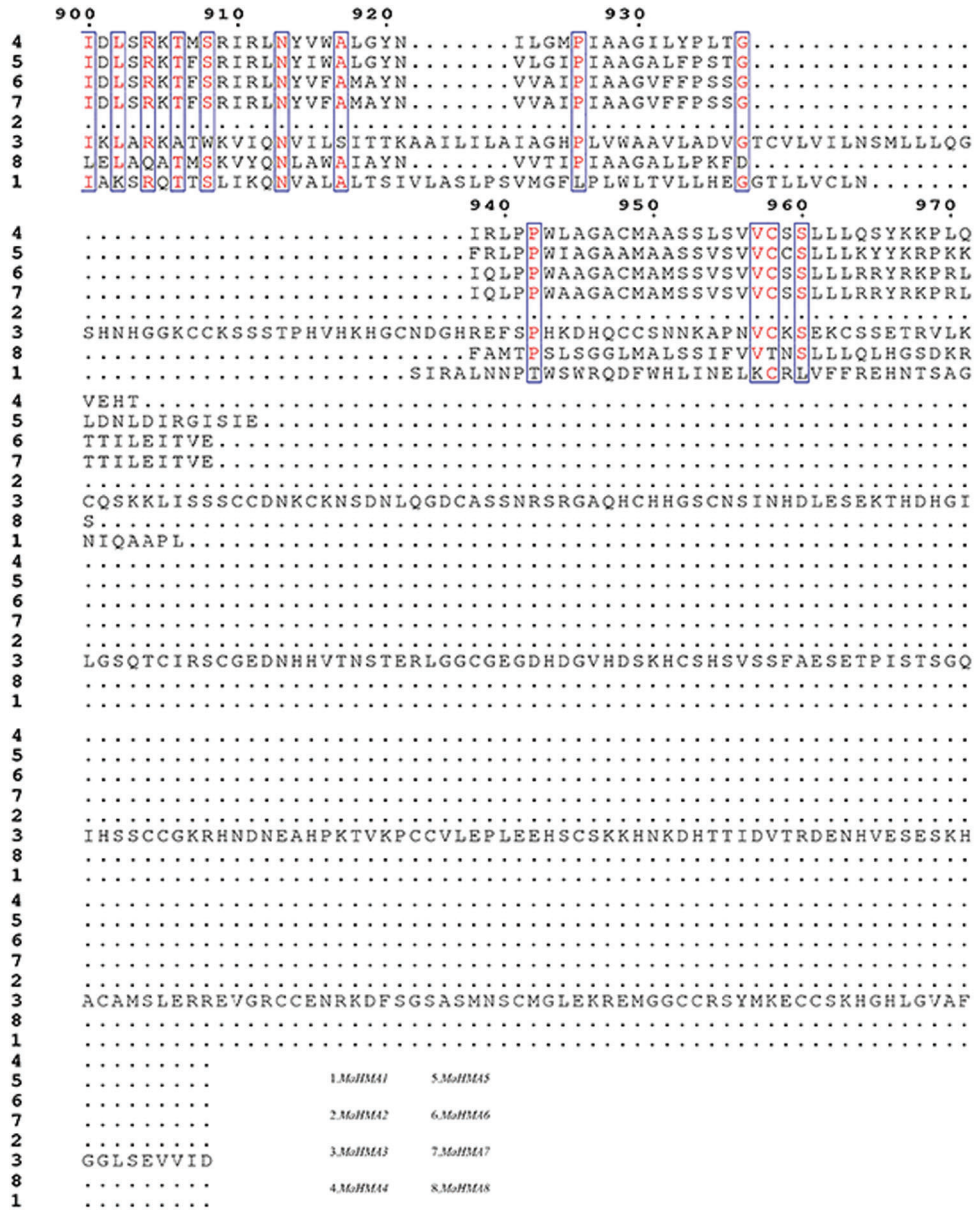
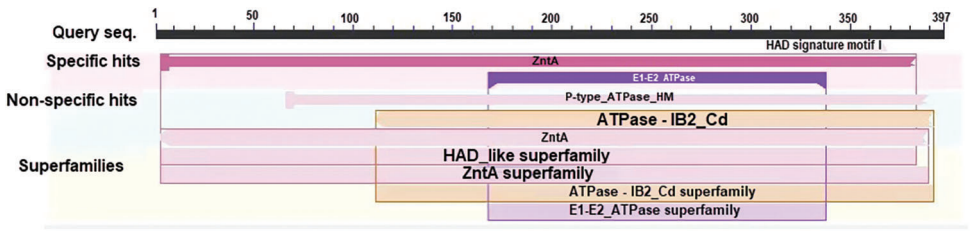
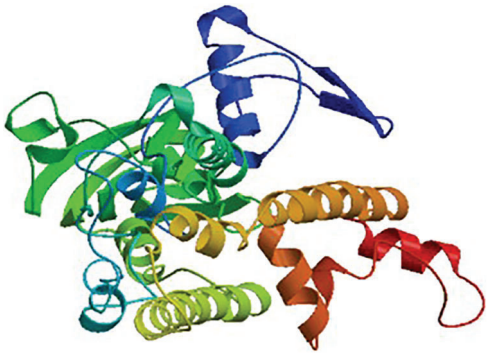


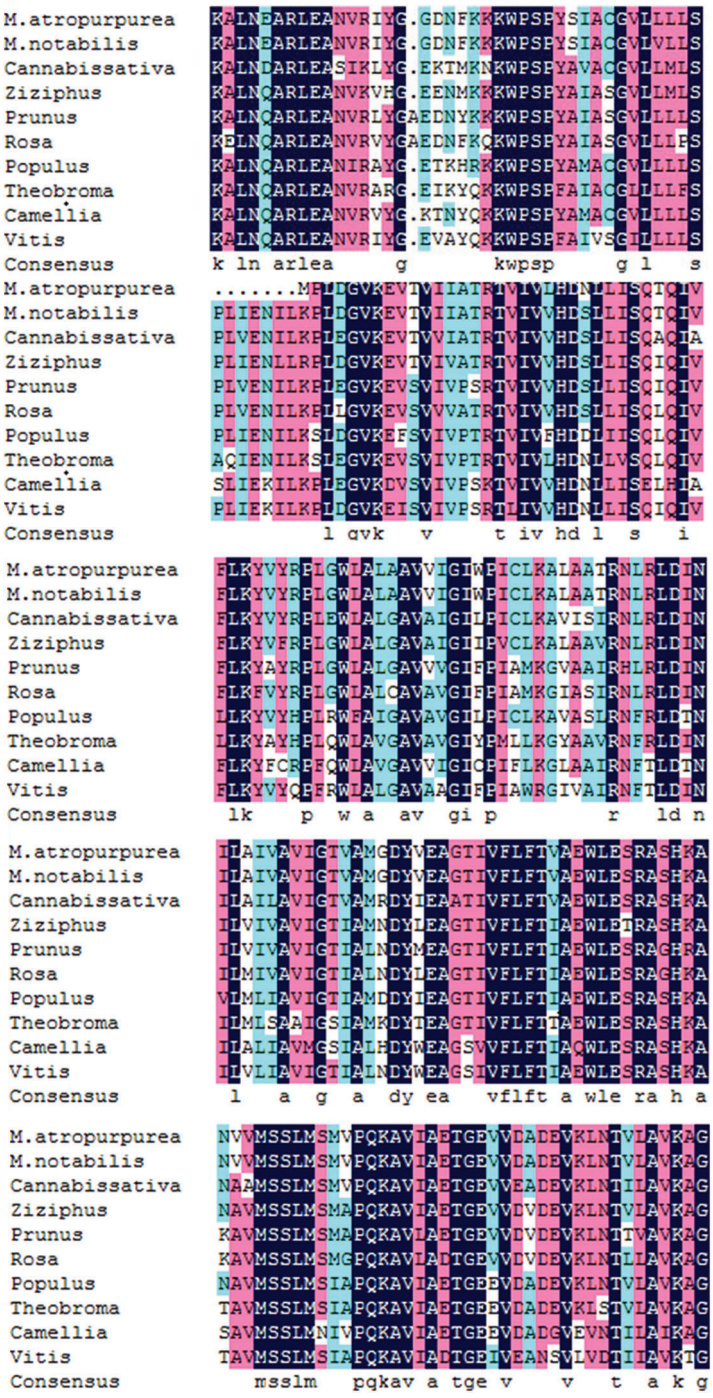
SUPPLEMENTARY FIGURE S3. Amino acid sequence alignment of the *HMAs* gene family of mulberry.



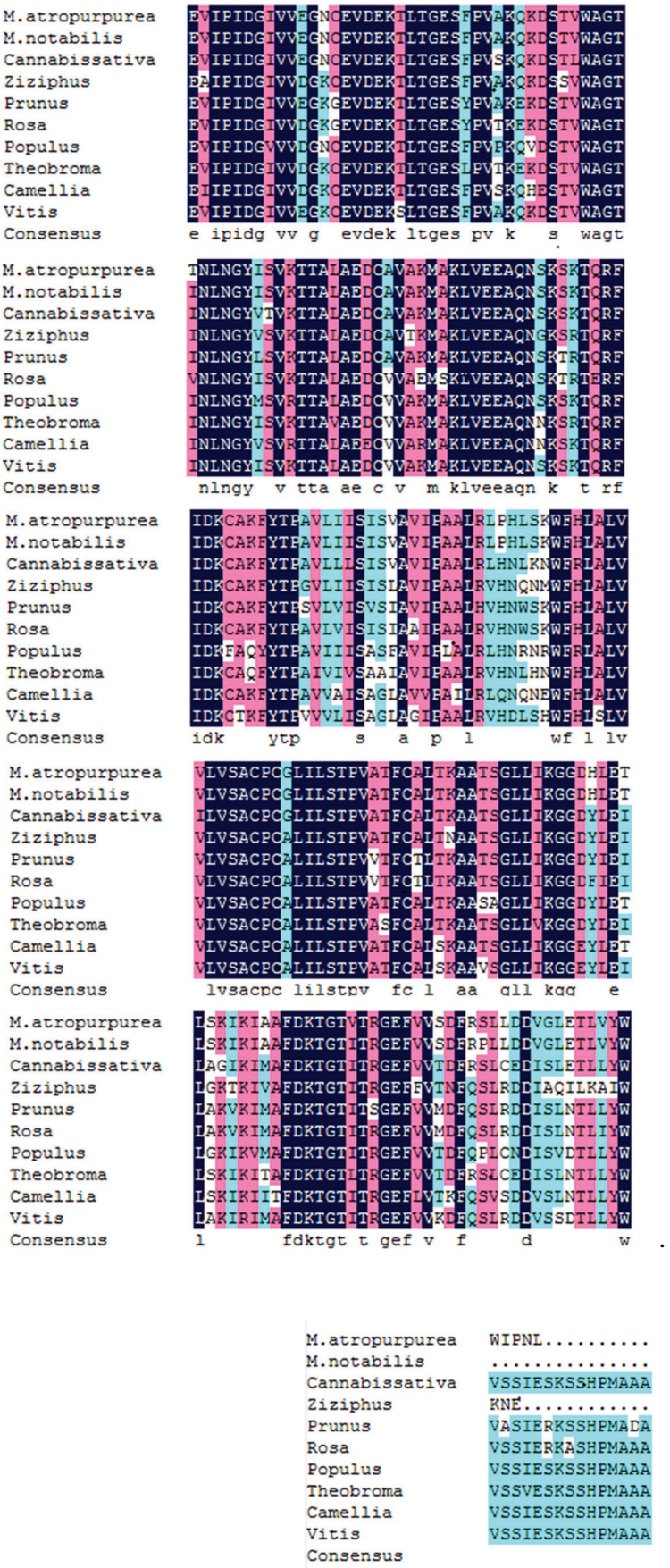
SUPPLEMENTARY FIGURE S4. The detection of conserved domains of superfamilies related with metal ion binding and transport.



SUPPLEMENTARY FIGURE S5. The *MaHMA2* protein tertiary structure.



SUPPLEMENTARY FIGURE S6. (Continued)



SUPPLEMENTARY FIGURE S6. The *MaHMA2* gene encodes a multiple sequence alignment of amino acids.