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Comparative RNA-Seq Analysis Reveals the Organ-Specific Transcriptomic Response to Zinc Stress in Mulberry

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Abstract: Mulberry (*Morus*, *Moraceae*) is an important economic plant that is considered zinc-rich. Zinc (Zn) is a micronutrient that plays vital roles in various bio-processes in plants and animals. In the present study, a comparative transcriptome analysis associated with physiological indicators was performed to reveal the potential mechanism in different organs in response to zinc toxicity in mulberry. Physiological indicators in mulberry plants treated with increasing concentrations of zinc were monitored to reveal the tolerance limits to zinc concentration. Transcriptome analysis of different organs in mulberry under excess zinc stress was performed to reveal the spatial response to zinc stress. The results show that the hormone signaling pathway and secondary metabolism including lignin biosynthesis, flavonoid biosynthesis and sugar metabolism are important for excess zinc treatment responses. In addition, the organ-based spatial response of these pathways is indicated. Lignin biosynthesis mainly responds to zinc stress in lignified tissues or organs such as stems, flavonoid biosynthesis is the main response to zinc stress in leaves, and sugar metabolism is predominant in roots. Further co-expression network analysis indicated candidate genes involved in the organ-based spatial response. Several transcription factors and genes involved in phenylpropanoid biosynthesis, cell wall biogenesis and sugar metabolism were further validated and designed as organ-based response genes for zinc stress.

Keywords: mulberry; physiology; organ-specific; secondary metabolism; transcriptome; zinc stress

Citation: Huang, S.; Kang, X.; Yu, T.; Yidilisi, K.; Zhang, L.; Cao, X.; Chao, N.; Liu, L. Comparative RNA-Seq Analysis Reveals the Organ-Specific Transcriptomic Response to Zinc Stress in Mulberry. *Forests* **2023**, *14*, 842. https://doi.org/10.3390/f14040842

Academic Editor: Chikako Honda

Received: 28 February 2023
Revised: 14 April 2023
Accepted: 17 April 2023
Published: 20 April 2023

1. Introduction

Zinc (Zn) is a micronutrient that is necessary for higher plants, animals and humans. Zinc is known to bind to various proteins and works as a cofactor involved in metabolic processes in the plant and animal kingdoms. Zinc is important for people to maintain their fitness level by affecting physical growth, the immune system, reproductive health and brain development [1,2]. Zinc deficiency in humans is a critical nutritional and health problem in the world. It affects, on average, one-third of the world's population in different countries [3]. Dietary modification with zinc-rich foods is a recommended intervention strategy to improve zinc intake for humans to keep normal healthy growth and reproduction [4].

Plants are the main sources of many food products including staples, such as rice, wheat and maize, and non-staple food, such as vegetables and fruits [5]. In addition, some plants provide specific nutrients to benefit our health. Zinc-deficient plants generally have low tissue zinc concentrations and therefore, in addition to reduced crop yields, the crop products from these plants make a lower contribution to the zinc content in the human diet [5]. Soil-plant relationships also affected the zinc content in animal food products, another human dietary source [5]. Therefore, plant zinc content is one of the main sources of human zinc intake. Increasing plant zinc content can alleviate zinc deficiency in the human

body. Zinc deficiency in crops is found in many countries and regions around the world [3]. The average total zinc concentration in soils was reported to be around 55 mg/kg [5]. On the other hand, zinc concentration in soils has gradually increased in the last decades as a consequence of human activities [6–8] including industrial processes, agriculture, increasing use of biosolids and metal mining [9,10]. The maximum zinc concentration permitted in sewage sludge-amended soils (pH 6–7) among different European countries is within the range of 100 mg/kg to 300 mg/kg zinc [11]. Zinc-polluted soils also lead to zinc toxicity in plants, which affects growth and yield [6–8].

It is possible to clean up zinc-polluted soils with zinc hyperaccumulator plants along with the production of zinc-rich foods. In plants, zinc modulates the activity of a large number of enzymes involved in the maintenance of biomembrane integrity, participates in carbohydrate metabolism and protein synthesis and plays an important role in indoleacetic acid metabolism [12,13]. Further, zinc protects cells from the damage caused by reactive oxygen species [14]. Some studies also indicated that a relatively high concentration of zinc in soil could help to reduce the cadmium content in crops [15,16]. It has been proved that the application of zinc can effectively relieve lead toxicity in *Lactuca sativa* and *Houttuynia cordata* [7,17,18]. However, beyond certain concentrations (100 mg/kg~500 mg/kg), zinc is toxic to vascular plants [19]. Mulberry is a woody plant with resistance to heavy metals, such as iron and cadmium, and is also used in medicine and food. Mulberry is capable of taking up small amounts of heavy metals and was reported to have the ability to clean up zinc-polluted soils. Mulberry planted in mines was measured to migrate 254,532.8 mg zinc every square meter of plough layer soil [20]. The contents of zinc in mulberry showed spatial differences with quite different zinc concentrations in different organs (leaf, root, bark and stem) [20]. In mulberry, the leaves and fruits are known as sites rich in zinc and are used to produce zinc-rich food [21].

The availability of the *Morus notabilis* genome and chromosome-level genome of *Morus alba* promote the transcriptome analysis of mulberry in response to various stresses [22,23]. However, little knowledge is available in terms of the response to zinc toxicity in mulberry. *M. alba* variety *Fengchi* is a new variety created by the Sericultural Research Institute, Chinese Academy of Agricultural Sciences, that is expected to spread and grow in extreme environment conditions as forage. Given the great potential of *M. alba* variety *Fengchi* to improve the ecological environment, it is also expected to be used as a heavy metal hyperaccumulator in mines. Our previous studies assessed the potential roles of lignin biosynthetic genes in response to zinc stress in *Fengchi* [24]. In the present study, we performed physiological analysis of mulberry plants under zinc stress and validated the limit of zinc concentration for mulberry tolerance. Transcriptome analysis of different organs in mulberry under excess zinc stress was performed to reveal the spatial response to zinc stress. Specifically, we address the following questions: (i) what is the concentration of zinc in soil that causes zinc stress in mulberry, (ii) how does mulberry respond to zinc stress at the transcription level and (iii) what are the organ-specific responses to zinc stress in different mulberry organs?

2. Materials and Methods

2.1. Plant Materials

The materials used in this study were obtained from the National Germplasm Resource Nursery of the Institute of Sericulture, Chinese Academy of Agricultural Sciences. Zinc treatments were reported in our previous studies [24,25]. In brief, one-year-old seedlings of *M. alba* variety *Fengchi* were transplanted into plastic pots with soil, and the potted plants were irrigated with 400 mL/kg of Murashige and Skoog (MS) medium to provide nutrients [26]. Zinc sulfate powder was applied near the roots of the mulberry trees as the excess zinc stress treatment, in which the zinc ion concentration ranges from 0 to 450 mg/kg with a gradient set every 50 mg/kg. The root, stem and leaf tissues were quickly frozen in liquid nitrogen and stored at −80 °C. This experiment was performed using three mulberry

seedlings with a similar growth status as biological replicates. These collected samples were used for both RNA-Seq and RT-qPCR (quantitative real-time PCR) analysis.

2.2. Determining the Contents of Physiological Indicators Related to Zinc Toxicity

The samples collected on day 15 were used for the determination of physiological indexes. Chlorophyll contents including Chlorophyll a (Chl a), Chlorophyll b (Chl b) and total Chlorophyll (Chl a + b) were determined with ethanol extraction followed by spectrophotometry [27]. The MDA contents in roots and stems were measured using the thiobarbituric acid method as described by Sairam and Srivastava (2001) [28]. Then, 0.5 g of the plant samples were extracted in 4.0 mL of 10% trichloroacetic acid (TCA) and centrifuged at 10,000× *g* for 10 min at 4 °C. The absorbance of the supernatant was determined at 532 and 600 nm with a spectrometer (BioTek ®Epoch 2, BioTek Instruments, Inc., Winooski, VT, USA) [28]. Proline (Pro) content was measured according to Silva et al. (2016) [29]. SOD activity was determined using a SOD measurement kit (Suzhou Keming Technology, Suzhou, China) according to the manufacturer's instructions. The MDA, proline and SOD contents in the leaves, roots and stems were measured, respectively. The zinc supply concentration resulting in zinc toxicity was determined based on both physiological indicators and plant phenotypes. Graphpad Prism8.0 was used to perform ANOVA and visualize the results. $p < 0.05$ was considered significant. All the above measurements were carried out with three biological replications.

2.3. RNA-Seq and Data Processing

The RNA-Seq dataset of mulberry containing different organs under the excess zinc treatment (450 mg/kg) was obtained using the Illumina sequencing system, and the trim galore (version-0.6.4) was used to remove the adapters and perform a quality control of the reads. The trimmed reads were further aligned to the Morus alba genome released by Jiao et al. (2020) using bowtie2 (version-2.3.2) [30]. Samtools was used to operate the bam files. The genome annotation file (.gff3) was used to calculate the expression matrix using StringTie v2.15 [31]. Differentially expressed genes (DEGs) were obtained using DEseq2 by comparing the expression levels of sample pairs [32]. A weighted correlation network analysis (WGCNA) was performed to screen the co-expressed DEGs [33]. R version 4.1.2 was used for R-package-based analyses.

2.4. Workflow for Comprehensive Transcriptome Analysis

The sample correlation was assessed using DEGs with both Principal Component Analysis (PCA) and Pearson correlation analysis. All DEGs were annotated to their orthologs in *Arabidopsis thialiana* using Blast. The DEGs involved in the response to zinc stress were further compared between different organs to reveal the possible organ-specific responses. Classification of DEGs was performed based on Venn analysis, and KEGG and GO analysis of the specific class of DEGs was performed using DAVID online tools [34]. WGCNA was performed using the DEGs as input and the treatments as conditions, and cytoscape 3.01 was used to visualize the co-expression network. TBtools v1.09876 and R version 4.1.2 were used to perform the above analysis and visualize the results [35].

2.5. RT-qPCR Analysis of Key Genes Involved in the Response to Zinc Toxicity

Total RNA extraction and cDNA synthesis were performed as in our previous report [35]. Quantitative real-time PCR (RT-qPCR) was performed using an ABI StepOnePlus™ Real-Time PCR System (USA) with actin as the reference gene [36]. Reactions were prepared in a total volume of 10 µL containing 5 µL 2× ChamQ SYBR Color qPCR Master Mix (High ROX Premixed) (Vazyme, Nanjing, China), 1 µL cDNA template and 0.3 µM of each primer. The program was set at 95 °C for 15 min, 45 cycles of 20 s at 95 °C and 60 s at 60 °C. The relative expression level was calculated using $2^{-\delta Ct}$, and the fold change (treatment/control) was calculated using $2^{-\delta\delta Ct}$. The primers are listed in Table S1. Graphpad Prism8.0 was used to perform an ANOVA and visualize the RT-qPCR results. $p < 0.05$

was considered significant. Three biological replicates, each with three technical replicates, were used for RT-qPCR.

3. Results

3.1. Mulberry Organ-Specific Physiological Responses to Zinc Treatment

As both zinc deficiency and zinc excess can lead to plant abnormal growth, several physiological indicators were selected to assess their effect on physiological processes. Malondialdehyde (MDA) contents changed slowly along with increasing zinc concentrations and showed a sharp increase at 350 mg/kg followed by a sharp decrease in MDA contents (Figure 1A). Although MDA contents showed a similar trend in the stems and roots with minor differences, the stems showed a significantly higher level than in roots. A similar bell-pattern trend in proline content was also observed in different organs, with the summit of proline content at the zinc concentration of 200 mg/kg. The proline content in leaves showed a significantly higher level than in the stems or roots (Figure 1B). SOD activity in different organs showed quite different trends with increasing zinc concentrations (Figure 1C). SOD activity increased sharply at 50 mg/kg zinc with a significant difference compared with the control, which thereafter kept stable in stems. The highest SOD activity was found in the leaves and roots at 350 and 250 mg/kg zinc, respectively, which thereafter decreased with an increase in zinc concentration. A significant difference can be observed at the summits compared with the controls. The chlorophyll content including chlorophyll A and B in the leaves showed an increase–decrease-increase fluctuation with a summit at 150 mg/kg zinc (Figure 1D). The above bell-pattern change in physiological indicators along with increasing zinc concentration was also reported in a previous study [37]. The bell-pattern change might indicate that plants experience a process including benefiting from suitable supply of zinc, suffering excess zinc toxicity and damage in the physiological response mechanism. It is obvious that a 350 mg/kg zinc supply completely induced physiologically adverse effects in all organs of mulberry. In addition, the mulberry seedlings under the >350 mg/kg zinc treatment showed growth retardation (Figure S1).

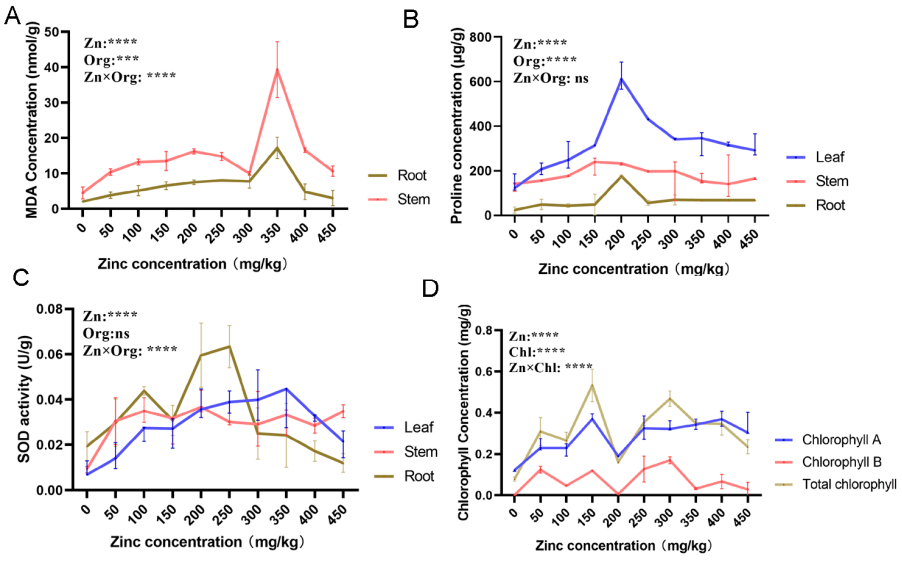

Figure 1. Change in physiological indicators under the gradient zinc concentration treatment on different organs in mulberry. (**A**). MDA contents in different organs under different zinc concentration treatments; (**B**). SOD activity in different organs under different zinc concentration treatments; (**C**). proline contents in different organs under different zinc concentration treatments; and (**D**). chlorophyll contents in different organs under different zinc concentration treatments. A two-way ANOVA was performed to analyze the difference resulting from zinc treatments and organs or types of chlorophyll. ***, $p < 0.001$; ****, $p < 0.0001$. In addition, significance analysis was also performed for every two points using ANOVA, and the results are available in Table S2.

3.2. Transcriptomic Analysis Showed Organ-Specific Differences in Response to Excess Zinc Treatments

Given the results of spatial physiological responses to zinc treatments at different concentrations, a zinc concentration >350 mg/kg would be toxic to mulberry plants and inhibit growth, as we observed (Figure S1). Therefore, the leaves, stems and roots from mulberry plants exposed to 450 mg/kg zinc were collected for a further study to explore mulberry spatial responses at transcription levels under excess zinc. A summary of the RNA-Seq datasets is available in Table S3. The Q30 values of the RNA-Seq clean data from all samples were >91%. Both the PCA and Pearson correlation analysis-based heatmaps using Fragments Per Kilobase Million values (FPKM) of DEGs showed that the replications of each sample set are clustered together (Figure 2A,B). In addition, the organ-specific difference was indicated by both the PCA and Pearson correlation analysis-based heatmaps. The difference resulting from different organs seems to be a dominant factor for distinguishing samples. Transcription-level disturbances due to excess zinc treatments in different organs are also quite different (Figure 2A,C,D). Maximum DEGs were identified in the stems (572), which also included the most number of down-regulated genes (349), while DEGs in the leaves (total DEGs: 319) and roots (total DEGs: 215) are mainly up-regulated genes (Figure 2C,D). Therefore, organ-specific differences should be considered when further analyses of DEGs involved in zinc stress responses are performed.

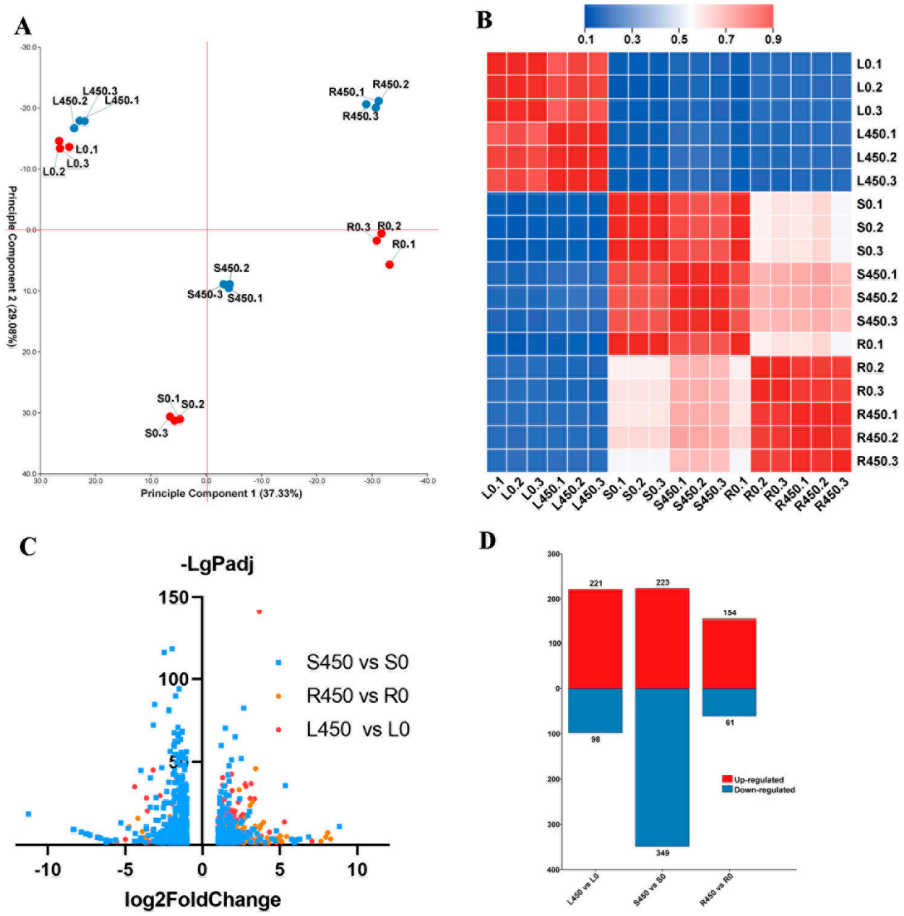

Figure 2. Comparative transcriptome analysis in different organs under excess zinc treatment. (**A**). PCA showing different organ samples under zinc treatments and control; (**B**). heatmap showing different organ samples under zinc treatments and the control based on Pearson correlation coefficients; (**C**). volcano diagram showing DEGs in different organs under zinc treatments and the control; and (**D**). up-regulated and down-regulated genes in different organs under zinc treatments and the control. L0, S0 and R0 indicate the leaves, stems and roots of mulberry without zinc treatment (controls), respectively. L450, R450 and S450 indicate the leaves, stems and roots of mulberry under the 450 mg/kg zinc treatment, respectively.

3.3. Organ-Specific DEGs in Mulberry under Zinc Stress

The Venn diagram showed that quite a large number of DEGs are organ-specific under excess zinc treatment, indicative of the organ-specific responses to zinc toxicity in mulberry (Figure 3A). The GO and KEGG pathway enrichment analysis using organ-specific DEGs showed that different biological processes were involved in the organ-specific response to zinc toxicity in mulberry. In the leaves, the hormone response and signal transduction pathways are well enriched (Figure 3B). In addition, the KEGG pathway analysis also showed that the secondary metabolic biosynthesis pathways including flavonoid biosynthesis and the indole alkaloid biosynthesis pathway are also significantly enriched (Figure 3B). Different pathways are enriched for root-specific DEGs including the jasmonic acid biosynthesis pathway and processes involved in sugar metabolites. Quite a lot of sugar metabolite-related processes including the starch and sucrose biosynthesis pathway, the carbohydrate metabolic process and the glucan catabolic process are significantly enriched (Figure 3C). These results indicate that JA and sugar metabolism participate in the response to zinc toxicity in roots. In the stems, hormone-related pathways are also significantly enriched. Phenylpropanoid biosynthesis and the following lignin biosynthesis and flavonoid biosynthesis are enriched in the stems (Figure 3D). Obviously, these results imply that there is quite a different disturbance in different organs in response to zinc toxicity in mulberry. It is possible that a more complex regulation network of genes involved in zinc stress exists in mulberry.

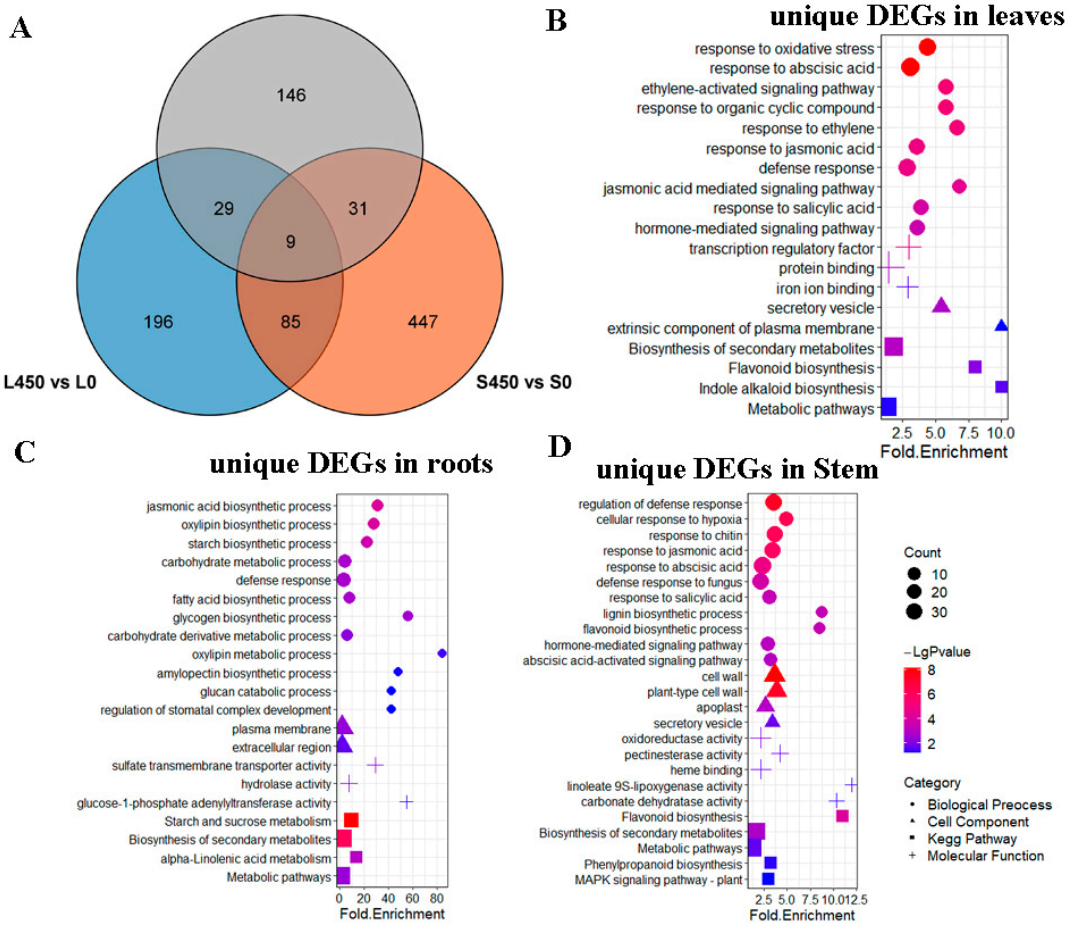

Figure 3. Organ-specific DEGs in mulberry under zinc stress. (**A**). Venn diagram showing DEGs in different organs in mulberry under zinc stress; (**B**). enrichment analysis showing unique DEGs in leaves in mulberry under zinc stress; (**C**). enrichment analysis showing unique DEGs in roots in mulberry under zinc stress; and (**D**). enrichment analysis showing unique DEGs in stems in mulberry under zinc stress.

A total of 81 transcription factors were annotated as the DEGs involved in the response to zinc toxicity including ERF (17), MYB (11), NAC (9) and WRKY (7). It is interesting that these TFs also showed organ-specific preferences. These 17 zinc toxicity-responsive ERFs mainly showed significant differences in the leaves and stems (16/17) than in the roots (1/17) (Table 1, Figure S2). The fold-change in these genes is also available in Table S5. Since most ERFs are annotated as factors involved in hormone responses and stress responses, the results match our GO and KEGG pathway enrichment analysis. In addition, zinc toxicity-responsive MYBs also showed organ-specific preferences. Among the eleven differently expressed MYBs, seven MYBs were identified as DEGs unique to stems, and two MYBs were identified as DEGs in stems and other organs (leaves or roots). Stem-specific, differentially expressed MYBs are mainly annotated as factors involved in phenylpropanoid biosynthesis or cell wall biogenesis (Table 1 and Figure S2). For example, stem-specific DEGs *MYB7* and *MYB52* were proposed to be involved in lignin biosynthesis, and *MYB66* and *MYB123* were proposed to be involved in flavonoid biosynthesis in Arabidopsis [38]. Several WRKYs, including *WRKY40*, *WRKY50* and *WRKY51* and bHLH *MYC4*, that were annotated as JA-related genes showed significantly different expressions in the roots under excess zinc treatment (Table 1). These results further suggested that organ-specific transcriptional regulation networks might be important in the response to zinc toxicity in mulberry.

Table 1. Zinc toxicity-responsive transcription factors in mulberry.

Gene ID	Organ	Ortholog	Gene Name	TF Type	Annotation
M.alba_G0008931	L	AT3G07340	*CIB3*	bHLH	Photoperiodic flowering
M.alba_G0005811	L	AT2G22850	*bZIP6*	bZIP	Vascular development
M.alba_G0006171	L	AT1G75390	*bZIP44*	bZIP	Stress response and development
M.alba_G0017047	L	AT1G27730	*STZ*	C2H2	Stress response
M.alba_G0013540	L	AT4G29190	*AtC3H49*	C3H	Cold response
M.alba_G0018475	L	AT3G47500	*CDF3*	Dof	Nitrogen responses
M.alba_G0006380	L	AT4G39780	*ERF60*	ERF	Defense response and light stimulus
M.alba_G0020260	L	AT2G44840	*ERF13*	ERF	Ethylene-activated signaling pathway
M.alba_G0020348	L	AT4G17500	*ERF-1*	ERF	Ethylene-activated signaling pathway
M.alba_G0003406	L	AT5G51190	*ERF105*	ERF	Response to cold stress
M.alba_G0016087	L	AT2G47520	*ERF71*	ERF	Response to hypoxia stress
M.alba_G0016369	L	AT1G50640	*ERF3*	ERF	Ethylene-activated signaling pathway
M.alba_G0001958	L	AT3G02550	*LBD41*	LBD	Stress response
M.alba_G0017051	L	AT3G49940	*LBD38*	LBD	Cellular metal ion homeostasis
M.alba_G0012646	L	AT4G37260	*MYB73*	MYB	Stress responses and leaf senescence
M.alba_G0006600	L	AT1G01720	*ANAC002*	NAC	Response to wounding and abscisic acid
M.alba_G0007251	L	AT1G69490	*ANAC029*	NAC	Leaf senescence
M.alba_G0013712	L	AT1G01720	*ANAC002*	NAC	Response to wounding and abscisic acid
M.alba_G0019459	L	AT4G27410	*ANAC072*	NAC	ABA-mediated dehydration response
M.alba_G0007498	L	AT1G13260	*EDF4*	RAV	Response to low temperature
M.alba_G0011100	L	AT1G80840	*WRKY40*	WRKY	Fungus defense
M.alba_G0009278	R	AT4G01500	*NGA4*	B3	Leaf, stigma development
M.alba_G0005063	R	AT4G17880	*MYC4*	bHLH	Activate JA-responses
M.alba_G0010656	R	AT4G20970	*NA*	bHLH	Dehydration stress memory
M.alba_G0018432	R	AT3G47640	*PYE*	bHLH	Regulating response to iron deficiency

Table 1. *Cont.*

Gene ID	Organ	Ortholog	Gene Name	TF Type	Annotation
M.alba_G0015523	R	AT5G28770	*bZIP63*	Bzip	Circadian phase in response to sugars
M.alba_G0001737	R	AT5G39660	*CDF2*	Dof	Photoperiodic flowering response
M.alba_G0003407	R	AT4G17500	*ERF-1*	ERF	Ethylene-activated signaling pathway
M.alba_G0004215	R	AT3G13040	*γ-YB2*	G2-like	Phosphate starvation
M.alba_G0016414	R	AT2G31180	*MYB14*	MYB	Cold or wound stress
M.alba_G0006667	R	AT5G64530	*ANAC104*	NAC	Xylem development
M.alba_G0013320	R	AT1G80840	*WRKY40*	WRKY	Photosynthesis and Iron Homeostasis
M.alba_G0013615	R	AT5G26170	*WRKY50*	WRKY	Defense response to fungus, JA response
M.alba_G0018966	RLS	AT3G18960	*REM7*	B3	Tissue development
M.alba_G0018963	RLS	AT5G23090	*NF-YB13*	NF-YB	NA
M.alba_G0002019	RS	AT5G16770	*MYB9*	MYB	Suberin biosynthesis and transport
M.alba_G0006489	RS	AT5G64810	*WRKY51*	WRKY	Jasmonic acid-inducible defense responses
M.alba_G0013321	RS	AT1G80840	*WRKY40*	WRKY	Jasmonic acid-inducible defense responses
M.alba_G0001053	S	AT4G29930	*NA*	bHLH	NA
M.alba_G0004848	S	AT1G32640	*MYC2*	bHLH	Activate JA-responses
M.alba_G0012659	S	AT1G72210	*BHLH96*	bHLH	Regulation of RNA polymerase II
M.alba_G0018293	S	AT4G20970	*NA*	bHLH	Defense response to fungus
M.alba_G0019112	S	AT4G25440	*ZFWD1*	C3H	Development
M.alba_G0003582	S	AT2G40140	*SZF2*	C3H	Response to biotic and abiotic stresses
M.alba_G0005725	S	AT4G38960	*BBX19*	DBB	Photomorphogenesis and flowering
M.alba_G0005394	S	AT5G51990	*CBF4*	ERF	Drought stress and abscisic acid treatment
M.alba_G0012399	S	AT1G21910	*DREB26*	ERF	Response to JA and SA, abiotic stress
M.alba_G0016407	S	AT2G31230	*ERF15*	ERF	Stress response
M.alba_G0005395	S	AT5G51990	*CBF4*	ERF	Drought stress and abscisic acid treatment
M.alba_G0014394	S	AT1G50420	*SCL-3*	GRAS	Response to gibberellin
M.alba_G0013518	S	AT2G22840	*GRF1*	GRF	Leaf development.
M.alba_G0019709	S	AT3G61890	*HB-12*	HD-ZIP	Leaf and stem development
M.alba_G0013465	S	AT4G37540	*LBD39*	LBD	Cell wall biogenesis
M.alba_G0011537	S	AT5G35550	*MYB123*	MYB	Anthocyanin biosynthesis
M.alba_G0012042	S	AT1G17950	*MYB52*	MYB	Lignin, xylan and cellulose biosynthesis
M.alba_G0018463	S	AT5G61420	*MYB28*	MYB	Seed development and aliphatic glucosinolate biosynthesis
M.alba_G0018447	S	AT3G47600	*ATMYB94*	MYB	Cuticular wax biosynthesis
M.alba_G0011536	S	AT2G16720	*MYB7*	MYB	General phenylpropanoid and lignin R2R3-MYB repressors
M.alba_G0018280	S	AT5G14750	*MYB66*	MYB	Anthocyanin production and differentiation of trichome cells
M.alba_G0013188	S	AT1G75250	*ATRL6*	MYB	Signal transduction
M.alba_G0019458	S	AT3G15510	*ANAC056*	NAC	System development
M.alba_G0009218	S	AT3G04070	*ANAC047*	NAC	Response to flooding
M.alba_G0009713	S	AT5G63790	*ANAC102*	NAC	Mediating response to low oxygen stress

Table 1. *Cont.*

Gene ID	Organ	Ortholog	Gene Name	TF Type	Annotation
M.alba_G0011705	S	AT4G14540	*NF-YB3*	NF-YB	Response to heat, response to water deprivation
M.alba_G0006285	S	AT4G24660	*ATHB22*	ZF-HD	Embryo development ending in seed dormancy
M.alba_G0007224	S	AT1G69600	*ATHB29*	ZF-HD	Early responsive to dehydration stress.
M.alba_G0013466	SL	AT1G27730	*STZ*	C2H2	Stress response
M.alba_G0015192	SL	AT3G46080	*NA*	C2H2	Transient stress
M.alba_G0003253	SL	AT5G52020	*DREB*	ERF	Glucosinolate metabolic process
M.alba_G0003254	SL	AT5G51990	*CBF4*	ERF	Drought stress and abscisic acid treatment
M.alba_G0003536	SL	AT2G40340	*AtERF48*	ERF	Response to abscisic and acid stress
M.alba_G0005396	SL	AT5G51990	*CBF4*	ERF	Response to drought stress and abscisic acid treatment
M.alba_G0017242	SL	AT4G34410	*ERF109*	ERF	Retarding programmed cell death under salt stress
M.alba_G0019814	SL	AT1G19210	*ERF17*	ERF	JA, defense to biotic stresses
M.alba_G0000389	SL	AT5G48150	*PAT1*	GRAS	Callus formation, photomorphogenesis, red, far-red light phototransduction
M.alba_G0011734	SL	AT4G17230	*SCL13*	GRAS	Cellular response to hypoxia, heat
M.alba_G0004071	SL	AT5G04760	*DIV2*	MYB	Negative roles in salt stress and is required for ABA signaling in Arabidopsis
M.alba_G0014170	SL	AT3G44350	*ANAC061*	NAC	Response to salt stress
M.alba_G0005182	SL	AT4G11070	*WRKY41*	WRKY	ABA defense response
M.alba_G0014899	SL	AT2G38470	*WRKY33*	WRKY	Stress response
M.alba_G0019631	SL	AT2G46400	*WRKY46*	WRKY	ABA signaling and auxin homeostasis in response to abiotic stress

Note: The symbols were the gene names given to the orthologs in *Arabidopsis thaliana* and the function annotation refers to the information in TAIR.

3.4. Network of Genes Involved in the Response to Zinc Toxicity in Different Organs

WGCNA showed that modules MEbrown, MEgreen and MEturquoise had significant correlations with different organs, which indicated that DEGs in these modules are organ-specific responses under zinc stress (Figure 4A). Further analysis showed that the top co-expressed genes (nodes of the top 500 connections with correlation coefficients > 0.6) in the leaves are involved in hormone signaling. Several transcription factors including *EFR-1*, *EFR13*, *EFR71*, *ANAC002* and *WRKY40* and jasmonate signaling-related genes *JAZ1* and *JAZ8* were co-expressed (Figure 4C). Co-expressed genes in the stems are also hormone signaling-related genes (Figure 4B). The co-expression network of zinc-response genes in the roots was quite different from the networks in the leaves and stems. Several sugar metabolism-related genes such as *CWINV1*, *BE3*, *GBSS1* and *ADG1* showed a significant correlation and interacted with other stress response genes including *PYE*, *JMT* and *AVP* (Figure 4D). In addition, another module, MEblues, showed a significant correlation with the treatments, which indicated these DEGs might be generally important genes involved in the response to zinc toxicity. Repressor *BAN* and activator *MYB52* together with a series of genes involved in lignin biosynthesis and flavonoid biosynthesis comprised a regulation network of phenylpropanoid biosynthesis and cell wall biogenesis involved in the response to excess zinc. In addition, *ANAC104* involved in suberin biosynthesis and *MYB9* involved in xylem development were also proposed to be involved in cell wall biogenesis. The connections between phenylpropanoid biosynthesis and other stress response genes were

also shown in the network. *ANAC072* and *PIP2;1* were reported to be involved in the ABA-mediated dehydration response.

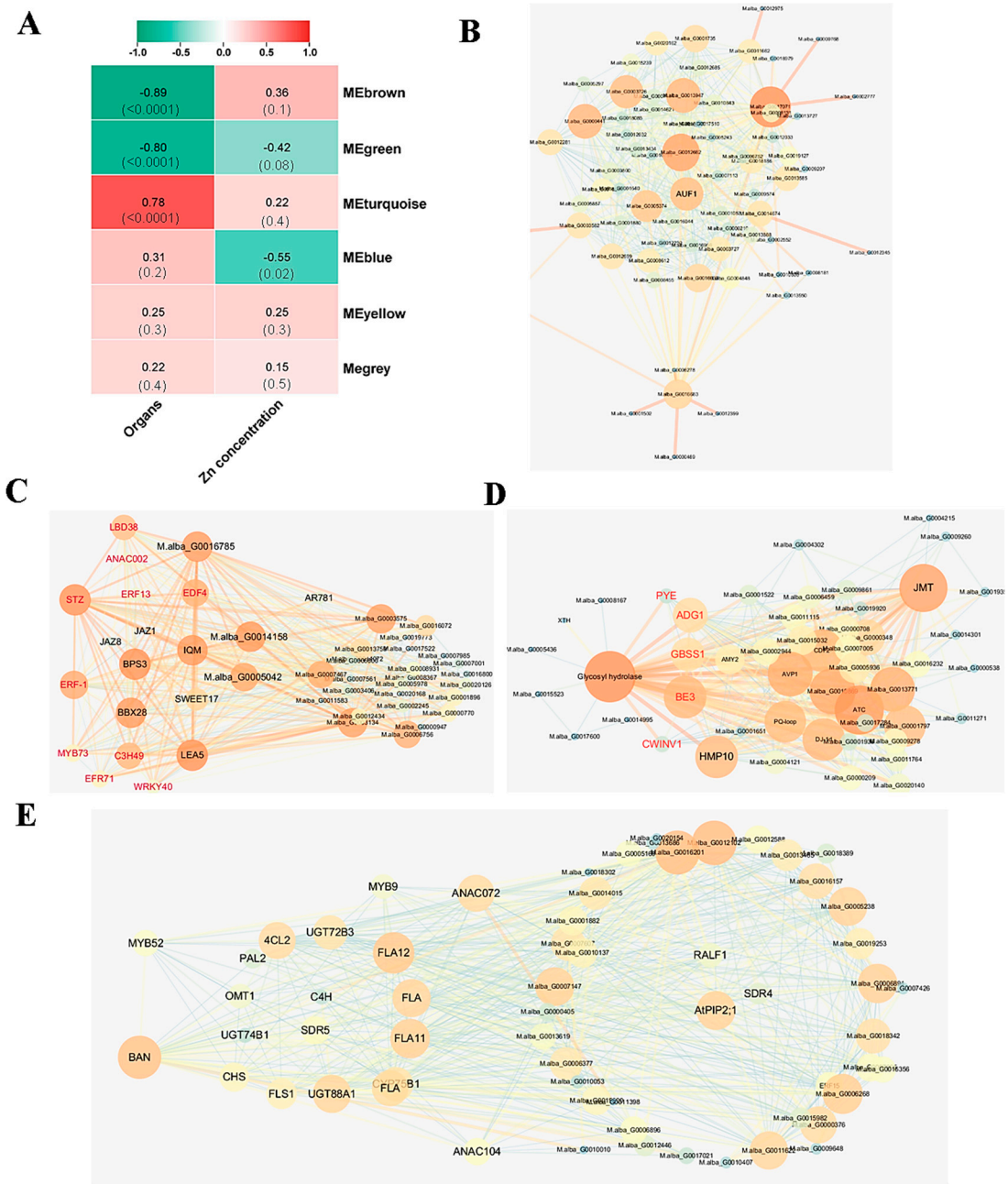

Figure 4. Co-expression of DEGs in different organs in mulberry under zinc stress. (**A**). Heatmap of module-trait associations based on WGCNA; (**B**). co-expression network showing DEGs in the stems of mulberry under zinc stress; (**C**). co-expression network showing DEGs in the leaves of mulberry under zinc stress; (**D**). co-expression network showing DEGs in the roots of mulberry under zinc stress; and (**E**). co-expression network showing DEGs in both the stems and roots of mulberry under zinc stresses. Co-expressed genes were clustered in the same-colored modules such as MEbrown, MEblue, etc.

3.5. Validation of Transcription Levels of Key Genes Responsive to Zinc Toxicity

Several key genes responsive to zinc toxicity based on our WGCNA and co-expression networks were further validated using RT-qPCR. The detailed information for these selected genes is provided in Table S4. The RT-qPCR results correspond well with the RNA-Seq

results except for *BAN*, which showed no significantly different expression in all detected organs of mulberry exposed to zinc stress (Figure 5 and Table S4). Genes involved in phenylpropanoid biosynthesis, such as *PAL2*, *CHS* and *4CL2* (Figure 5A–C), genes involved in sugar metabolism, such as *CWINV1*, *GBSS1* and *ADG1* (Figure 5D–F), and transcription factors involved in cell wall biogenesis, such as *MYB9* and *ANAC 104* (Figure 5H,I), show quite similar organ-specific expression change comparing with the results of the transcriptome analysis. These genes are considered organ-specific zinc stress response genes.

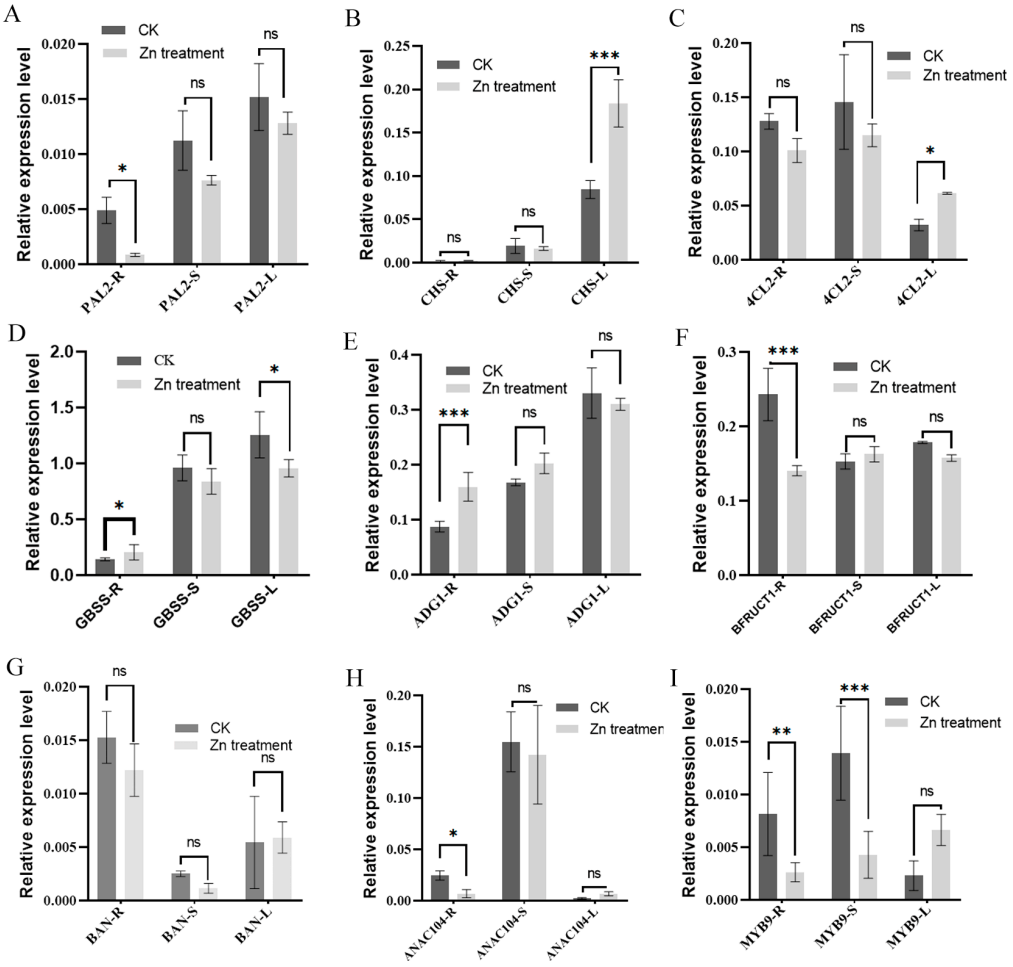

Figure 5. RT-qPCR showing the selected DEGs in response to zinc stress in mulberry. (**A**–**I**), relative expression levels of selected DEGs in different organs under zinc stresses. the gene names were marked in each subfigure and R, S and L indicated roots, stems and leaves respectively. Error bars represent SE. Asterisks indicate significant differences as determined with Student's *t*-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

4. Discussion

4.1. Limitation of Excess Zinc Supply in Soil for Mulberry

Some heavy metals such as zinc are known as micronutrients that are essential to plant growth and survival [1]. However, plants would suffer zinc toxicity if the zinc concentration in the soil is beyond the limitation of zinc concentration in the soil. The threshold total zinc values from the literature for zinc in sensitive plant species is 150~200 mg/kg zinc, and 100–500 mg/kg zinc is regarded as the range of zinc contents at which the yield of many crops might be reduced by 25% due to toxicity [39]. Zinc toxicity function disrupts key enzymatic reactions in many cellular processes including carbon fixation and metabolism [40–42]. Different plants had quite different capacities to deal with zinc toxicity, and the limitation in zinc supply that resulted in zinc toxicity varied for different plant species [39]. Toxicity symptoms usually become visible at >300 mg/kg zinc in leaf DW, although some crops show toxicity symptoms at <100 mg/kg zinc

in leaf DW [5,43,44]. Mandarin orange (*Citrus reticulata* Blanco) seedlings supplied with 5 mM (~325 mg/kg) zinc are considered zinc sufficient and induced prolific growth and sprouted abundantly, while plants that received more than 10 mM (~650 mg/kg) of zinc suffered zinc toxicity with prime features of growth retardation, defoliation and sluggish root growth [27]. Wheat under 14 mg/kg zinc treatment in the soil can help to deal with drought stress and result in zinc-mediated alleviation of drought stress [45]. Some plants such as *Thlaspi caerulescens* are zinc hyperaccumulators that show zinc-hypertolerant capacity. Mulberry is also reported to have an outstanding ability to uptake zinc and survive in zinc-polluted mines [20]. A previous study revealed that a 50 mg/kg zinc treatment can not only promote plant growth but also alleviates the adverse effects of lead stress in *Morus alba* [46]. Heavy metal toxicity also can be reflected by the change in physiological indicators levels such as MDA, SOD, proline and chlorophyll [7,27,37,46]. In the present study, the limitation of excess zinc supply in the soil for mulberry was identified by referring to physiological indicators. A 350 mg/kg zinc supply completely induced physiological adverse effects in all organs of mulberry, and beyond this limitation, possible damages that cannot be alleviated by physiological responses occurred when supplying excess zinc (Figure 1). The bell-pattern change might indicate that plants experience a process including benefiting from a suitable supply of zinc, suffering excess zinc toxicity and damage in the physiological response mechanism. Similar physiological indicator change patterns were also reported in the previous study [37]. In fact, mulberry plants with 400 mg/kg or 450 mg/kg zinc supply can still survive but only with slow growth (Figure S1). The maximum zinc concentration permitted in sewage sludge-amended soils (pH 6–7) is in the range of 100 mg/kg to 300 mg/kg zinc in some countries [11]. Therefore, mulberry is a potential plant that can be used as a bio-cleaner in zinc-polluted areas.

4.2. Organ-Specific Responses to Zinc Toxicity

Zinc homeostasis showed a spatial-temporal distribution, and a spatial response to acute zinc deficiency in Sorghum has been reported [47]. The distribution of zinc in mulberry also showed spatial differences with quite different zinc concentrations in different organs (leaf, root, bark and stem) [20]. Therefore, organ-specific responses to zinc toxicity should be evaluated. Our results and previous reports have indicated that hormone signaling pathways and several secondary metabolite-related pathways, such as lignin and flavonoids, are important for zinc stress responses [8,19,24,47–49]. These pathways were also identified in the present study, and organ-specific responses were also revealed. Hormone signal pathways participate in the response to zinc toxicity in all organs, but some hormones may play dominant roles in specific organs of mulberry. For example, in the roots, only JA-related pathways were enriched, including its biosynthesis pathway and alpha-linolenic acid metabolism. Secondary metabolism is another important biological process involved in the response to zinc toxicity in all organs, but genes involved in specific secondary metabolites were enriched in specific organs such as the flavonoid biosynthesis pathway in leaves, sugar metabolism in roots and lignin biosynthesis in stems. The causes of organ-specific responses to zinc toxicity in mulberry are various. The distribution of zinc in organs, organ preference expression of key genes involved in pathways and roles of different organs in response to stresses can together result in organ-specific responses. For example, genes involved in the lignin biosynthesis pathway prefer expression in lignified tissues, which may lead to this pathway mainly responding to zinc toxicity in stems or lignified roots [24]. Sugar homeostasis in vascular tissue is important for the response to various stresses including drought, oxidative stress and stresses resulting from heavy metals [50,51]. Our results also indicated that the sugar metabolic pathway participates in the response to zinc toxicity in the roots of mulberry.

4.3. Molecular Regulation Network of Genes in Response to Zinc Exposure

The current understanding of plant zinc homeostasis regulation mechanisms is mainly based on studies on *Arabidopsis*. The transcriptional level regulation of zinc homeostasis based on *bZIP19* and *bZIP23* in response to changes in cellular zinc status was reported

in *Arabidopsis* [8]. However, the molecular mechanism of the transcriptional regulation network in response to zinc toxicity is little reported for woody plants. In the present study, a total of 88 transcription factors including ERFs, MYBs bHLHs and WRKYs were screened as possible regulators in response to zinc toxicity using transcriptome analysis. AP2/ERF proteins have important functions in the transcriptional regulation of a variety of biological processes and are known as regulators involved in hormone responses such as the ABA response, JA response in chlorophyll degradation and both the biotic and abiotic stress responses [52–57]. ERFs were the most predominant TFs identified in this study. These ERFs mainly show different expression levels in the leaves and participate in regulating a network involved in hormone-signaling pathway-mediated stress responses. EFR-1 and EFR13 were reported to be involved in the ethylene-activated signaling pathway, and ERF71 was identified as a regulator involved in the response to hypoxia stress [55]. The present study indicated that these EFRs were possibly important regulators involved in the response to zinc toxicity through the hormone signaling pathway in mulberry leaves. Other hormone response genes such as *JAZ1* and *IAZ8* were also co-expressed with these *ERF*s to comprise the regulation network in mulberry leaves (Figure 4B) In addition to the hormone signaling pathway, the secondary metabolic pathway is also important for the response to zinc toxicity. In mulberry roots, a possible regulation network including sugar metabolism-related genes was also built, indicating their important roles in response to zinc toxicity (Figure 4D). Our previous study reported the genes involved in the lignin biosynthesis pathway positively respond to zinc toxicity in lignifying tissues [24]. In the present study, a possible regulation network of phenylpropanoid biosynthesis and cell wall biogenesis was identified, and their roles in the response to zinc toxicity were primarily revealed. Repressor BAN, activator MYB52 as well as ANAC104 and MYB9 were identified as regulators in this network.

5. Conclusions

Finally, a schematic diagram was summarized (Figure 6). Mulberry seedlings show different growth statuses under different concentrations of zinc treatments. Mulberry seedlings show growth retardation when suffering > 350 mg/kg zinc supply in the soil, and the physiological indicators show a bell-pattern change with a summit at high zinc concentrations. Different pathways are enriched in different organs in response to excess zinc treatment indicating an organ-specific response in mulberry. Flavonoids in leaves, lignin and cell wall in stems and sugar metabolism in roots are important for response to zinc toxicity in specific organs.

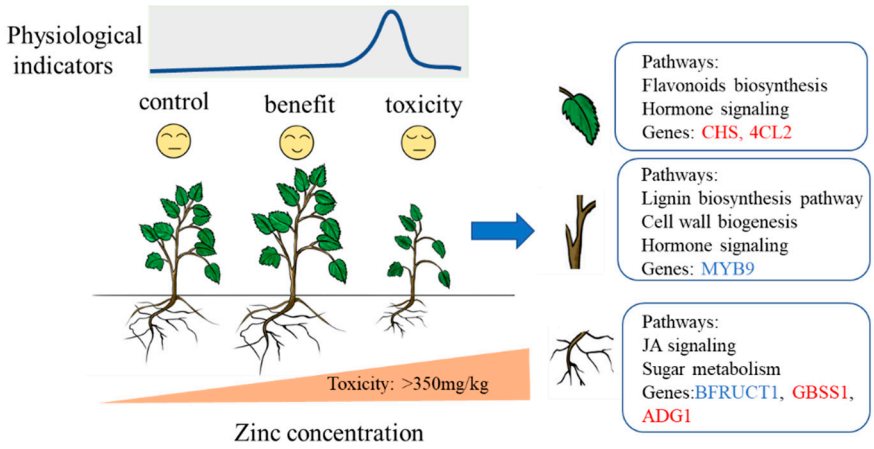

Figure 6. Schematic diagram showing the organ-based response to zinc toxicity in mulberry.

Mulberry seedlings show different growth statuses under different concentrations of zinc treatments. Plants show growth retardation when suffering > 350 mg/kg zinc supply in the soil, and the physiological indicators show a bell-pattern change with a summit at

high zinc concentrations. Different pathways are enriched in different organs in response to excess zinc treatment, indicating an organ-specific response in mulberry. The up-regulated genes are colored red, and the down-regulated genes are colored blue.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14040842/s1, Table S1: Primers used in this study; Table S2: Detailed statistics significance analysis results for the change in physiological indicators; Table S3: Summary of the RNA-Seq dataset; Table S4: Detailed information and fold change in selected genes for RT-qPCR; Table S5: DEGs in different organs; Figure S1: Growth status of mulberry seedlings after 15-day zinc treatments; Figure S2: Distribution of different expressed transcription factors (DETFs) in different organs. R, unique DETFs in roots; S, unique DETFs in stems; L; unique DETFs in leaves; RS, DETFs in both roots and stems; SL, DETFs in both stems and leaves.

Author Contributions: L.L. and N.C. guided this work and provided advice; L.Z. design the experiments; S.H., X.K., T.Y. and K.Y. performed the experiments and analyzed the data; N.C. analyzed the data, organized the figures and wrote the manuscript. X.C. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was jointly supported by the Natural Science Foundation of Jiangsu Province (BK20210879 to Nan Chao), the National Natural Science Foundation (NSF 32201526 to Nan Chao), the Crop Germplasm Resources Protection Project of the Ministry of Agriculture and Rural Affairs of the People's Republic of China (19190172), the National Infrastructure for Crop Germplasm Resources (NICGR-2019-43), and the China Agriculture Research System of MOF and MARA.

Acknowledgments: We thank Feng Jiao at Northwest University of Agriculture and Forestry who provided us with the genome annotation file for *M. alba*.

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

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