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Waterlogging-responsive Genes Revealed by Transcriptome Sequencing in Leaves of Two Crabapple Species with Contrasting Waterlogging Tolerance

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Abstract. Crabapples (*Malus* sp.) are ornamental woody plants that belong to the Rosaceae family. Flooding has severely hampered the growth and development of crabapple, and little is known about the molecular responses of crabapple to waterlogging tolerance. Cuttings of waterlogging-tolerant *Malus hupehensis* and waterlogging-intolerant *Malus halliana* received flooding treatment of 30 days and regular planting, respectively. Using transcriptome sequencing, we isolated 5703 and 2735 waterlogging-responsive genes from waterlogging-treated *M. hupehensis* and *M. halliana* leaves. Among these differentially expressed genes (DEGs), only 746 were shared by both. Several variables may explain the greater waterlogging tolerance of *M. hupehensis*: there were more waterlogging response genes related to carbohydrate and energy metabolism; signal transduction; antioxidation; lipid metabolism; protein and amino acid metabolism; and polysaccharide, cell wall, and cytoskeleton metabolism pathway in the waterlogged leaves of *M. hupehensis* than in *M. halliana.* In particular, the number of DEGs related to anaerobic metabolism, fatty acid metabolism, protein phosphorylation and dephosphorylation, γ-aminobutyric acid metabolism and cellulase, pectinase metabolism pathway in the flooded leaves of *M. hupehensis* was more than that in *M. halliana.* The alterations in gene expression patterns of the two crabapple species induced by waterlogging varied substantially. These outcomes pave the way for further studies into the functions of genes that may be involved in waterlogging tolerance in crabapples.

Under the present abnormal global climate, rainstorms and flood disasters frequently occur in some areas, and flooding has become one of the principal stresses suffered by plants (Tanoue et al. 2016). Based on water depth, there are two forms of flooding: soil waterlogging and submersion (Fukao et al. 2019; Nishiuchi et al. 2012). A lack of oxygen is one of the direct repercussions of floods (Voesenek and Bailey-Serres 2015). Too much water hinders the gas exchange between plants and the atmosphere, causing anoxia in flooded tissues, which significantly impacts plants' physiologic metabolism, leading to the inhibition of plant growth and development and even death (Kozlowski 1984). As an ornamental tree, crabapples (*Malus* sp.) are widely planted in Eurasia and North America. China is a vital germplasm resource center for crabapple. In the flood-prone areas of the Yangtze River and Yellow River in southern China, waterlogging has become one of the main limiting factors for the use and promotion of ornamental crabapple (Zhang et al. 2019). Therefore, it is significant to study its flood tolerance for producing, developing, and propagating new cultivars of ornamental crabapple.

Plants will adjust their morphologic structure and physiologic and molecular metabolism processes to minimize stress damage. This adaptation mechanism can be summarized as follows: 1) when the floods are too deep or ephemeral, the growth of axial roots and lateral roots stops, the formation of new leaves becomes inhibited, and the energy and material consumption (including the downregulation of respiration and limited stimulation of fermentation) are reduced to extend survival time. For example, the leaf and internode elongation, chlorophyll degradation, and carbohydrate consumption are inhibited in the *Oryza sativa Submergence-1*, which submerged by water, while the activities of the enzymatic activities of pyruvate decarboxylase and alcohol dehydrogenase in leaves were significantly higher than those of the intolerant *O. sativa* M202 (Fukao et al. 2006). 2) Under relatively shallow and long-term flooding stress, the formation of adventitious roots or other aerenchyma, the rapid elongation of the apical meristem, the accelerated elongation of the stem, and the formation of the air membrane in the upper stratum corneum occur in the flooded plant, and new tissues are exposed to air as soon as possible to alleviate hypoxia stress. For example, under waterlogging stress, the internode of deep-water *O. sativa* rapidly elongates and lasts for several months to keep the leaf tip always above the water surface, so that gas exchange can be carried out (Bailey-Serres and Voesenek 2008). 3) After the waterlogging stress is relieved, the transportation of oxygen from the above-ground parts of plants to root tips is accelerated to alleviate the stress of root hypoxia. Spraying nitric oxide donor sodium nitroprusside can enhance the compensatory growth of *Gossypium*

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hirsutum after waterlogging relief, thereby significantly reducing yield losses caused by waterlogging stress (Zhang et al. 2022).

The molecular mechanisms driving these activities, however, remain poorly understood. Adaptations in the expression of genes are required for plants to withstand waterlogging stress. Alcohol dehydrogenase (*Adh*) was the earliest anaerobic gene isolated and can prolong the survival time of plants under hypoxic conditions; this process represents one of the survival strategies of terrestrial plants responding to hypoxia stress (Crawford 1992). The activity of ADH in *Zea mays* seedlings primary roots were found to increase continuously under flooding stress, and the expression of *Ahd1* and *Adh2* increased significantly, which enhanced submergence tolerance (Freeling 1973). *Pyruvate decarboxylase* (*Pdc*) is the primary anaerobic inducible gene. The transcript levels of *Pdc* increased under conditions of O_2 shortage in *Z. mays* root tips and in more mature root zones (Drew 1997); and the high expression of *Pdc1* and *Pdc2* in roots and stems helps improve the waterlogging tolerance of *Arabidopsis thaliana* (Ismond et al. 2003). Moreover, *Adh* and *Pdc* genes have been reported in *O. sativa* shoots (Hossain et al., 1996; Mujer 1993), *A. thaliana* leaves and roots (Dolferus et al. 1997), *Z. mays* seedling roots and shoots (Gerlach et al. 1982; Thelen et al. 1999), and other plants under hypoxia stress.

The gene transcription abundance of ethylene biosynthesis enzymes 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and 1- aminocyclopropane-1-carboxylic acid (ACC) oxidase (1-aminocyclopropane-1-carboxylic acid oxidase, ACO) was found to increase in *A. thaliana* shoots (Rauf et al. 2013), promoting the largescale synthesis of ethylene, enhancing the activity of cellulase, accelerating cell division and programmed cell death in some cells of roots, loosening the cell arrangement, enlarging the tissue gap, and forming the aerenchyma (Yin et al. 2012). Ethylene response factors (ERFs) induce the expression of related genes and regulate the formation of adventitious roots under flooding stress. In *A. thaliana*, *Hypoxia Responsive ERF1* (*HRE2*), a member of group VII ethylene response factors also regulates adventitious root formation. Overexpression of *HRE2* can significantly increase the density of adventitious roots (Eysholdt-Derzsó and Sauter 2019).

M. halliana and *M. hupehensis* have a long history of cultivation in China and have great importance in the world's garden construction. Both *M. halliana* and *M. hupehensis* are native to China. *M. halliana* is mainly distributed in southwest, south-central, and eastern China, and north America and Japan. *M. hupehensis* is distributed in central, western, and southern China. The distribution areas of *M. halliana* and *M. hupehensis* overlap, but *M. hupehensis* is distributed more widely in China. *M. halliana* originated in Gansu (arid and semi-arid regions), China, and *M. hupehensis* originated in Hubei (humid regions), China. *M. hupehensis*, especially *M. hupehensis* var. mengshanensis, has stronger waterlogging resistance than *M. halliana* (Cheng and Li 1990) and is widely used as an apple (*Malus domestica*) rootstock. Both *M. halliana* and *M. hupehensis* can be propagated by cutting, but the potted *M. halliana* rots easily under flooding, and the resistance of *M. hupehensis* is better, which can be used as the rootstock of apple. With the deterioration of environmental conditions, the phenomenon of waterlogging in the middle and lower reaches of the Yangtze River is becoming more and more serious, and the crabapple planting industry is seriously affected by waterlogging; however, few studies on flooding stress in crabapples have been reported. In this work, we further develop our views on *M. halliana* and *M. hupehensis* waterlogging tolerance by examining waterlogging-induced changes in the transcriptomics for the leaves of these two crabapple species with distinct waterlogging tolerance. We identified potential genes for waterlogging resistance in crabapples by examining the genes that are responsive to waterlogging.

Materials and Methods

Plant materials. One-year-old crabapple cuttings of *M. halliana* and *M. hupehensis* were purchased from Yizhou crabapple cuttings breeding base in Linyi, Shandong, China, which were used as experimental materials and planted at Henan Agriculture University, Zhengzhou, Henan, China, throughout the trial period. The cuttings of *M. halliana* and *M. hupehensis* are taken from the one plant and collected from clonally propagated material, respectively. Crabapple cuttings are planted in small pots (10 cm i.d., 15 cm high), one plant per pot; the depth of soil in the pot was 12 cm and the soil used was horticultural nutrient soil (Henan Yetong Agricultural Technology Co., Ltd, Zhengzhou, Henan, China). After the plant growth stabilized, an experimental study was carried out. The cuttings of *M. halliana* and *M. hupehensis* with the same growth trends (the difference in the number of true leaves of each crabapple cutting is not more than two, and the difference in plant height of each crabapple cuttings is not more than 3 cm, which is considered that these crabapple cuttings have the same growth trend) were selected and divided into two groups: 15 cuttings in the waterlogging treatment group and 15 cuttings in the control group. The waterlogging treatment group simulated flooding stress: the waterlogging treatment is to put eight crabapple cuttings with small pots into large pots (20 cm high × 60 cm long × 30 cm wide), and add water to cover the soil surface of crabapple cuttings by 3 cm, and the water level is about equal to the opening of the small pot. The water was changed every 3 days to prevent the water quality from deteriorating. The control group was planted under standard cultivation management. After 30 days of treatment, all functional leaves (including the fourth to seventh leaves from the meristem) were collected at noon on a sunny day. First, we divided 15 crabapple cuttings into three groups, five in each group, and then collected the functional leaves of five cuttings in each group, cut them up, mixed them evenly, and put them into three tinfoil bags, as three biologic duplicates. Three biologic replicates from the waterlogging treatment (YS) of *M. hupehensis* (HU) were named HUYS-1, HUYS-2, and HUYS-3, and the controls (CK) of *M. hupehensis* were named as HUCK-1, HUCK-2, and HUCK-3. Three biologic replicates from the waterlogging treatment of *M. halliana* (HA) were named HAYS-1, HAYS-2, and HAYS-3, and the controls of *M. halliana* were named as HACK-1, HACK-2, and HACK-3. All replicates were frozen in liquid N_2, and then stored at −80°C in a refrigerator for RNA extraction.

Complementary DNA preparation and RNA sequencing. Using a bioanalyzer (2100; Agilent Technologies, Palo Alto, CA, USA), RNA quality was evaluated. Eukaryotic messenger RNA (mRNA) was enriched using Oligo(dT) beads after total RNA extraction. After fragmenting the enriched mRNA into small pieces through a fragmentation buffer, the mRNA was reverse-transcribed into complementary DNA (cDNA) using random primers. Using DNA polymerase I, RNase H, deoxy-ribonucleoside

triphosphate, and a buffer, second-strand cDNA was generated. The cDNA fragments were further purified using a QiaQuick Polymerase Chain Reaction (PCR) extraction kit (Qiagen, Venlo, The Netherlands), end repaired, and ligated to Illumina sequencing adapters. The products from ligation were size-selected using agarose gel electrophoresis, amplified by PCR, and sequenced on a sequencing platform (NovaSEq. 6000; Illumina, Inc., San Diego, CA, USA) by Gene Denovo Biotechnology Co. Ltd. (Guangzhou, Guangdong, China), ultimately giving 150-base pair paired-end reads.

Analysis of transcriptome data. Initial processing of raw RNA sequencing (RNA-seq) data in the fastq format was performed using in-house Perl scripts. All downstream analyses relied on high-quality, adapter-, and/or ploy-N-free reads produced by eliminating low-quality sequences and adapter- and/or ploy-N-containing reads. The number of bases with sequenced base quality value above Q-value ≥20 and percentage in Clean Data (Q20), the number of bases with sequenced base quality value above Q-value ≥30 and percentage in clean data (Q30), and the percentage of filtered sequence base GC contents of these clean reads were estimated in the interim. Bowtie 2.2.8 (Langmead and Salzberg 2012) was used to generate a reference genome index, and HISAT2.2.4 (Kim et al. 2015) was used to align paired-end clean reads to the *M. domestica* genome. The DESeq2R package (Love et al. 2014) was used for differential expression analysis. Genes were considered differentially expressed with a false discovery rate (FDR) of less than 0.05 and an absolute fold change of at least two. The Gene Ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (Ashburner et al. 2000; Ogata et al. 2000) were used to designate the functional categories of genes.

Quantitative real-time PCR (QRT-PCR). As previously mentioned, 24 DEGs (11 of *M. halliana* and 13 of *M. hupehensis*) were isolated from the leaves. Three biologic duplicates were produced for each therapeutic modality. The primers used for qRT-PCR are listed in Supplemental Table S1. As a control, Actin expression was used, with each 20-μL reaction solution containing 10 μL SYBR Premix Ex Taq mix (Vazyme, Nanjing, Jiangsu, China). Then, the following amplification protocol was used: initial denaturation at 95°C for 30 seconds, followed by 40 denaturation cycles at 95°C for 10 seconds, and 30 seconds of annealing at 60°C. The $2^{-\Delta\Delta Ct}$ technique was used to determine relative expression degrees, and three to five replicates were included in each study.

Statistical analysis. Using a totally random design, 15 cuttings were assigned to each treatment. The tests were conducted in groups of three to five. The outcomes reflected the mean ± *SE*. Four treatment combinations were compared using analysis of variance testing to determine their differences (two treatments × two species). At $P < 0.05$, novel Duncan's multiple range tests were used to distinguish among the four means.

Results

RNA-seq and de novo assembly. Twelve libraries containing three biologic replicates of *M. halliana* HA and *M. hupehensis* for control and waterlogging treatments were constructed and sequenced. As indicated in Table 1, the ranges of raw reads, clean reads, and clean bases acquired from each library were 37,919,470 to 45,373,898, 37,860,072 to 45,291,362, and 5.66 to 6.77 Gb, respectively. The higher percentages of Q30 (93.76% to 94.70%), Q20 (97.92% to 98.30%), and clean reads (99.36% to 99.50%),

Table 1. Summary of the RNA sequencing data collected from the leaves of control and waterlogged *Malus halliana* and *Malus hupehensis*.

Sample and treatment[i]	Raw data, n[ii]	Clean data, n (%)[iii]	Adapter, n (%)[iv]	Low quality, n (%)[v]	polyA, n (%)	N, n (%)[vi]	Q20, %[vii]	Q30, %[viii]	GC, %[ix]
M. halliana									
HACK-1	40,496,896	40,415,298 (99.80)	9156 (0.02)	72,442 (0.18)	0 (0.00)	0 (0.00)	98.25	94.60	45.57
HACK-2	45,373,898	45,291,362 (99.82)	9204 (0.02)	73,332 (0.16)	0 (0.00)	0 (0.00)	98.26	94.60	45.60
HACK-3	42,371,700	42,294,588 (99.82)	8788 (0.02)	68,324 (0.16)	0 (0.00)	0 (0.00)	98.23	94.50	45.58
HAYS-1	38,920,900	38,846,234 (99.81)	7770 (0.02)	66,896 (0.17)	0 (0.00)	0 (0.00)	98.20	94.48	45.44
HAYS-2	41,623,870	41,541,384 (99.80)	8838 (0.02)	73,648 (0.18)	0 (0.00)	0 (0.00)	98.11	94.27	45.39
HAYS-3	42,347,834	42,257,624 (99.79)	9816 (0.02)	80,394 (0.19)	0 (0.00)	0 (0.00)	97.92	93.76	45.41
M. hupehensis									
HUCK-1	37,919,470	37,860,072 (99.84)	6194 (0.02)	53,204 (0.14)	0 (0.00)	0 (0.00)	98.30	94.70	45.63
HUCK-2	40,851,310	40,782,340 (99.83)	8650 (0.02)	60,320 (0.15)	0 (0.00)	0 (0.00)	98.26	94.61	45.62
HUCK-3	44,363,242	44,276,346 (99.80)	11838 (0.03)	75,058 (0.17)	0 (0.00)	0 (0.00)	98.23	94.53	45.60
HUYS-1	38,233,740	38,153,756 (99.79)	7790 (0.02)	72,194 (0.19)	0 (0.00)	0 (0.00)	98.18	94.38	45.78
HUYS-2	43,648,974	43,560,296 (99.80)	8794 (0.02)	79,884 (0.18)	0 (0.00)	0 (0.00)	98.21	94.52	45.72
HUYS-3	42,238,408	42,146,166 (99.78)	9834 (0.02)	82,408 (0.20)	0 (0.00)	0 (0.00)	98.17	94.36	45.74

[i] Three biologic replicates from the waterlogging treatment (YS) of *M. hupehensis* (HU) were named HUYS-1, HUYS-2, and HUYS-3, and the controls (CK) of *M. hupehensis* (HU) were named as HUCK-1, HUCK-2, and HUCK-3. Three biologic replicates from the waterlogging treatment of *M. halliana* (HA) were named HAYS-1, HAYS-2, and HAYS-3, and the controls (CK) of *M. halliana* (HA) were named as HACK-1, HACK-2, and HACK-3.
[i] Total base number of offline data.
[ii] Total number and percentage of high-quality data bases after filtering.
[iii] Number and percentage of reads containing adapter and percentage.
[iv] Number and percentage of reads with more than 50% base mass value Q-value ≤20 in single-end reads.
[v] Number and percentage of high filtering reads with N ratio.
[vi] Bases with sequenced base quality value above Q-value ≥20 and percentage in clean data.
[vii] Bases with sequenced base quality value above Q-value ≥30 and percentage in clean data.
[viii] Percentage of filtered sequence base GC.

the lower percentages for the error rate (0.03%), low-quality sequences (0.14% to 0.19%), poly-N reads (0.00%), and adaptor reads (0.02% to 0.03%) showed that RNA-seq data were of good quality and acceptable for further investigation. Here, 54.18% to 56.27% (33.78% to 35.57%) of the clean reads obtained in *M. halliana* and *M. hupehensis* leaves were mapped uniquely (multiply) to the *M. domestica* genome (Sun et al. 2020) (Table 2). A total of 59,179 known and 4383 novel genes were identified in *M. halliana* and *M. hupehensis* leaves (Table 2).

Waterlogging-responsive genes. Using an absolute value for the $\log_2$ ratio of at least 1 and an FDR threshold of less than 0.05, we detected the DEGs in the leaves of waterlogged *M. halliana* and *M. hupehensis*. Based on these two criteria, we identified 1409 upregulated and 1326 downregulated DEGs in waterlogging-treated *M. halliana* leaves and 3395 upregulated and 2308 downregulated DEGs in waterlogging-treated *M. hupehensis* leaves (Fig. 1A, Supplemental Table S2). Correspondingly, only 1989 and 4957 DEGs were found in the leaves of waterlogged *M. halliana* and *M. hupehensis*, respectively accounting for 72.72% and 86.92% of DEGs determined in their control group (Fig. 1B, Supplemental Table S2). Only 746 DEGs were discovered in the leaves of waterlogged *M. halliana* and *M. hupehensis* (Fig. 1B, Supplemental Table S3). Moreover, there was greater variation in gene expression in the leaves of waterlogged *M. hupehensis* compared with that in the leaves of waterlogged *M. halliana*, as indicated by a lower proportion of DEGs with an absolute value of a $\log_2$ ratio of at most two in the leaves of waterlogged *M. hupehensis* (47.43%) compared with the leaves of waterlogged *M. halliana* (67.68%) (Supplemental Table S2).

In *M. halliana* and *M. hupehensis* leaves, waterlogging altered the transcription factors (TFs) and genes involved in carbohydrate and energy metabolism; polysaccharide, cell wall, and cytoskeleton metabolism; antioxidation and detoxification; lipid, protein, and amino acid metabolism, signal transduction; and hormone metabolism (Supplemental Tables S4–S10). The leaves of waterlogged *M. halliana* exhibited 57 downregulated and 89 upregulated TFs, whereas *M. hupehensis* leaves had 102 downregulated and 73 upregulated TFs, as shown in Supplemental Table S4. As shown in Supplemental Table S5, 299 downregulated and 388 upregulated genes related to signal transduction were recovered from the leaves of waterlogged *M. hupehensis*, whereas 132 downregulated and 240 upregulated genes related to signal transduction were obtained from the leaves of waterlogged *M. halliana*. We discovered that the leaves of waterlogged *M. hupehensis* had more upregulated (209) than downregulated (159) genes associated with energy and carbohydrate metabolism, and the leaves of waterlogged *M. halliana* had more downregulated (122) than upregulated (76) genes. We detected 211 upregulated and 160 downregulated genes related to antioxidation in the leaves of waterlogged *M. hupehensis* and 129 upregulated and 70 downregulated genes in the leaves of waterlogged *M. halliana* (Supplemental Table S7). We isolated 125 downregulated and 115 upregulated and 80 downregulated and 35 upregulated polysaccharide and cell wall metabolism genes, respectively, from the leaves of waterlogged *M. hupehensis* and *M. halliana* (Supplemental Table S8). In this study, we isolated 83 downregulated and 96 upregulated genes and 49 downregulated and 57 upregulated genes, respectively, associated with lipid and fatty acid metabolism from the leaves of waterlogged *M. hupehensis* and *M. halliana* (Supplemental Table S9). The leaves of waterlogged *M. halliana* exhibited 381 downregulated and 375 upregulated genes associated with protein metabolism, whereas *M. hupehensis* leaves had 502 downregulated and 848 upregulated genes, as shown in Supplemental Table S10.

Validation of qRT-PCR. We analyzed the correlation between RNA-seq data and qRT-PCR data, in which the R^2 of *M. halliana* was 82.33%, and that of *M. hupehensis* was 68.62%. The results of all DEGs obtained via qRT-PCR analysis were strongly related to the data produced by RNA-seq (Fig. 2, Supplemental Table S1). Thus, the RNA-seq data were reliable.

Discussion

Response of TFs to waterlogging stress. The leaves of waterlogged *M. halliana* exhibited 57 downregulated and 89 upregulated TFs, whereas *M. hupehensis* leaves had 102

Table 2. Summary of clean reads and genes mapped to the reference genome from the leaves of control and waterlogged *Malus halliana* and *Malus hupehensis*.

Sample and treatment[i]	Total reads, n	Total mapped, n (%)	Multiple mapped, n (%)	Unique mapped, n (%)	Known genes, n (%)	Novel genes, n	Total genes, n
			M. halliana				
HACK-1	40,393,378	36,670,026 (90.78)	14,110,968 (34.93)	22,559,058 (55.85)	45,628 (50.41)	3,496	49,124
HACK-2	45,263,034	41,386,921 (91.44)	16,098,092 (35.57)	25,288,829 (55.87)	46,320 (51.18)	3,521	49,841
HACK-3	42,273,794	38,643,679 (91.41)	14,855,313 (35.14)	23,788,366 (56.27)	45,755 (50.55)	3,473	49,228
HAYS-1	38,819,570	34,492,538 (88.85)	13,279,061 (34.21)	21,213,477 (54.65)	44,999 (49.72)	3,426	48,425
HAYS-2	41,510,482	36,776,540 (88.60)	14,285,827 (34.41)	22,490,713 (54.18)	45,503 (50.28)	3,441	48,944
HAYS-3	42,230,144	37,439,728 (88.66)	14,495,918 (34.33)	22,943,810 (54.33)	45,618 (50.40)	3,470	49,088
			M. hupehensis				
HUCK-1	37,820,532	33,902,649 (89.64)	12,841,670 (33.95)	21,060,979 (55.69)	45,574 (50.35)	3,302	48,876
HUCK-2	40,741,206	36,489,868 (89.57)	13,797,689 (33.87)	22,692,179 (55.70)	45,758 (50.56)	3,312	49,070
HUCK-3	44,231,722	39,590,126 (89.51)	14,950,940 (33.80)	24,639,186 (55.70)	45,990 (50.81)	3,347	49,337
HUYS-1	38,029,220	33,578,540 (88.30)	12,847,915 (33.78)	20,730,625 (54.51)	46,339 (51.20)	3,431	49,770
HUYS-2	43,417,052	38,228,088 (88.05)	14,770,610 (34.02)	23,457,478 (54.03)	46,995 (51.92)	3,496	50,491
HUYS-3	42,008,768	37,082,504 (88.27)	14,158,490 (33.70)	22,924,014 (54.57)	46,683 (51.58)	3,436	50,119

[i] Three biologic replicates from the waterlogging treatment (YS) of *M. hupehensis* (HU) were named HUYS-1, HUYS-2, and HUYS-3, and the controls (CK) of *M. hupehensis* (HU) were named as HUCK-1, HUCK-2, and HUCK-3. Three biologic replicates from the waterlogging treatment of *M. halliana* (HA) were named HAYS-1, HAYS-2, and HAYS-3, and the controls (CK) of *M. halliana* (HA) were named as HACK-1, HACK-2, and HACK-3.

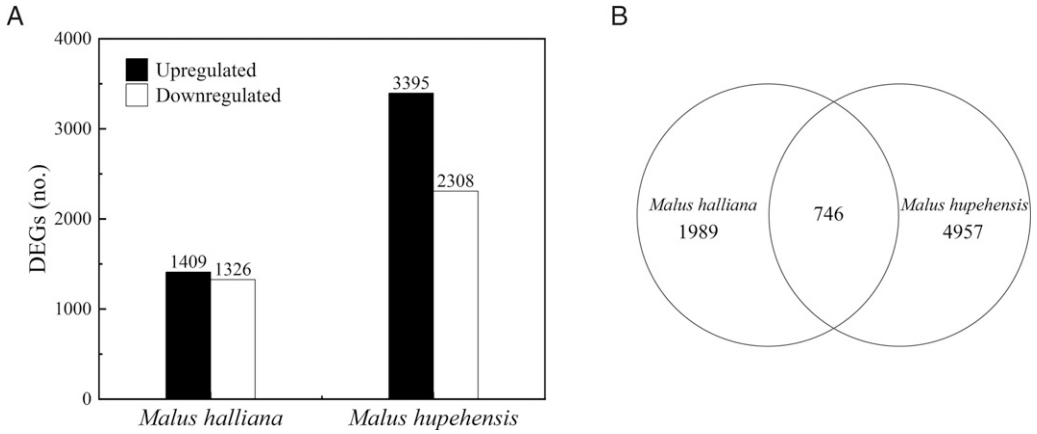

Fig. 1. Differentially expressed genes (DEGs) identified in the leaves of waterlogged *Malus halliana* and *Malus hupehensis*. (**A**) Upregulated and downregulated genes in the leaves of waterlogged *M. halliana* and *M. hupehensis*. (**B**) Venn diagram analysis of waterlogging-responsive genes in *M. halliana* and *M. hupehensis* leaves.

downregulated and 73 upregulated TFs, as shown in Supplemental Table S4. Most TFs were members of the myeloblastosis (MYB), Apetala2 (AP2)/ERF, zinc finger, WRKY, homeobox, helix-loop-helix (bHLH), and NAC families. In waterlogged *Rehmannia glutinosa* roots (Wang et al. 2017) and *Actinidia chinensis* roots (Zhang et al. 2015), comparable outcomes were obtained.

Hypoxia stress caused by flooding seriously affects plant growth. MYBs are broadly implicated in controlling plant secondary metabolism throughout the physical development of flora and play a crucial role in plant responses to hypoxia, drought, high salt, heat, cold, UVB radiation, hormone induction, and other environmental factors (Liu et al. 2008). *AtMYB2* was the first TF confirmed to regulate hypoxia response and is the key regulator of the hypoxia-induced *ADH1* gene in the leaves of *A. thaliana*, *Nicotiana tabacum*, and *Pisum sativum* (Hoeren et al. 1998). In this study, we isolated 16 and 12 upregulated MYB transcription factors in the leaves of waterlogged *M. halliana* and *M. hupehensis*, which accounted for 70.83% and 41.38% of the total number of differentially expressed MYBs in the waterlogged *M. halliana* and *M. hupehensis*, respectively, indicating that MYB TFs might be involved in the waterlogging tolerance of *M. halliana* and *M. hupehensis*. The formation of the ERF-VII subfamily is a key regulator involved in plant hypoxia tolerance or submergence tolerance, which is widely involved in regulating energy metabolism-related pathways under hypoxia (Fukao et al. 2012). Licausi et al. (2010) found that *HRE1* and *HRE2* enhance the submergence tolerance of *A. thaliana* seedlings by increasing the expression of anaerobic metabolism-related genes. Here, we identified seven upregulated ERFs from the leaves of waterlogged *M. hupehensis* and five upregulated ERFs from the leaves of waterlogged
M. halliana. In addition, the waterlogging-induced upregulation of ERFs was more significant in the leaves of waterlogged *M. hupehensis* than in the leaves of waterlogged *M. halliana*, indicating that ERFs may play a role in the greater waterlogging tolerance of *M. hupehensis*.

Recent studies have suggested that members of the WRKY family participate in hypoxia stress caused by waterlogging responses in *Diospyros kaki* fruit (Zhu et al. 2019), *O. sativa* coleoptile and roots (Mohanty et al. 2016; Shiono et al. 2014), and *A. thaliana* leaves and stems (Raineri et al. 2015). For example, waterlogging can induce the expression of *WRKY22*, thereby enhancing disease resistance in *A. thaliana* (Hsu et al. 2013). In this study, we isolated 38 upregulated WRKYs from the leaves of waterlogged *M. halliana* and eight upregulated WRKYs from the leaves of waterlogged *M. hupehensis*. Waterlogging stress can also cause some NAC transcription factors to respond. For

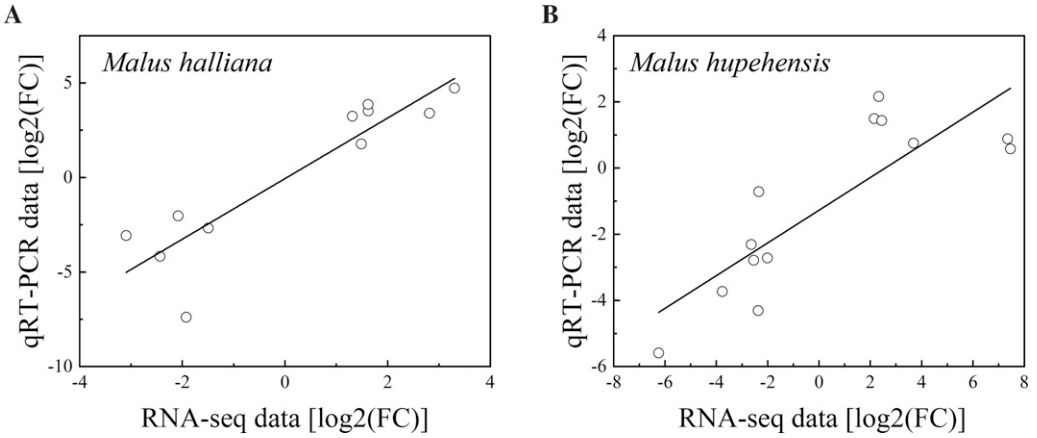

Fig. 2. Correlation analysis of RNA sequencing (RNA-Seq) and quantitative real-time PCR (qRT-PCR) data from the leaves of waterlogged *Malus halliana* (**A**) and *Malus hupehensis* (**B**). FC = fold change.

example, *ANAC102* can activate multiple hypoxia response genes in roots and stems of *A. thaliana* and regulate the normal germination of seeds under waterlogged conditions (Christianson et al. 2009). We identified seven upregulated NACs from the leaves of waterlogged *M. hupehensis* and 17 upregulated NACs from the leaves of waterlogged *M. halliana*. Although the waterlogging-induced downregulation of WRKYs and NACs was the most pronounced in the leaves of waterlogged *M. hupehensis*, this response was inadequate to distinguish the waterlogging tolerance between the two kinds of crabapple. Waterlogging-induced TF downregulation may play an essential role in the waterlogging tolerance of *M. hupehensis*; however, the precise regulatory mechanism must be investigated in more detail.

The considerable differences in waterlogging-induced changes of TF expression patterns between the two crabapple species shows that TFs may play a role in the waterlogging tolerance of *M. hupehensis*.

Genes related to signal transduction. As shown in Supplemental Table S5, 299 downregulated and 388 upregulated genes were recovered from the leaves of waterlogged *M. hupehensis*, and 132 downregulated and 240 upregulated genes were obtained from the leaves of waterlogged *M. halliana*. Approximately half of these DEGs encoded protein kinases, followed by genes that may be involved in hormone-mediated signal transduction and protein dephosphorylation. Protein phosphorylation is the most basic, standard, and crucial posttranslational modification of proteins when plants encounter abiotic stress. Phosphorylation plays a crucial role in the control of environmental stress defense systems among flora (Li et al. 2020). We isolated 28 downregulated and 57 upregulated genes that may be associated with protein phosphorylation and one downregulated and four upregulated gene(s) that may be associated with dephosphorylation from the leaves of waterlogged *M. hupehensis*, but only nine downregulated and 35 upregulated genes from the leaves of waterlogged *M. halliana*. This result may be connected to the waterlogging tolerance of *M. hupehensis*. To identify the accurate functions of these kinases and phosphatases, further studies are required.

Hormones have a crucial role in controlling plant responses to waterlogging (Shen et al. 2022). For example, abscisic acid is a negative regulator of adaptive mechanisms such as internode elongation and adventitious root formation under waterlogging stress (Zhao et al. 2021). As shown in Supplemental Table S5, 85 DEGs that may be associated with signal transduction mediated by hormones were upregulated in the leaves of waterlogged *M. hupehensis*, signifying that hypoxia due to waterlogging may activate signal pathways mediated by hormones, thereby increasing the waterlogging tolerance *of M. hupehensis*. In contrast, we discovered 40 downregulated and 71 upregulated genes from the leaves of waterlogged *M. halliana*, suggesting that hormone-mediated signal pathways may be altered among these leaves, thereby influencing the waterlogging tolerance of both *M. hupehensis* and *M. halliana*.

Genes related to carbohydrate and energy metabolism. Because oxygen is the ultimate electron acceptor of mitochondrial respiration, hypoxia produced by floods delays or entirely suppresses this essential activity (Bailey-Serres and Voesenek 2008). As shown in Supplemental Table S6, we discovered eight downregulated and 24 upregulated genes that may be associated with aerobic glycolysis from the leaves of waterlogged *M. hupehensis* but only eight upregulated and six downregulated genes from the leaves of waterlogged *M. halliana*. Experimentation demonstrated conclusively that sensitive plants lose soluble sugars after a few days of waterlogging, whereas tolerant species retain large quantities of carbohydrates in roots (Ferner et al. 2012; Martínez-Alcántara et al. 2012). Therefore, enhanced glycolysis in the leaves of waterlogged *M. hupehensis* may enable the resistance of *M. hupehensis* to waterlogging by sustaining basic respiration and attaining greater energy demands. This is consistent with the results of a transcriptome comparison of the roots of two oak species (*Quercus robur* and *Quercus petreae*) with differing flood tolerance (Le Provost et al. 2012).

The plant tricarboxylic acid cycle is limited by aerobic metabolism, which is weakened under waterlogging stress. As a result, the critical enzyme activities of PDC and ADH involved in anaerobic metabolism increase, and anaerobic respiration becomes an essential source for plants to obtain ATP (Christianson et al. 2010); however, anaerobic respiration requires the consumption of many carbohydrates, which can easily cause "sugar starvation" in plants. In addition, anaerobic respiration accumulates ethanol, acetaldehyde, CO_2, and lactic acid, leading to irreversible deterioration of the mitochondria and even cell injury or death (Agarwal and Grover 2006). As shown in Supplemental Table S6, we identified three upregulated and one downregulated gene-encoded ADHs related to anaerobic respiration in the leaves of waterlogged *M. hupehensis* but only one downregulated gene encoded ADH in the leaves of waterlogged *M. halliana*. Similar results were obtained in *Populus ×canescens* roots (Kreuzwieser et al. 2009), but there was no transcript abundance change in the leaves, the specific reasons need to be further explored. We discovered that the leaves of waterlogged *M. hupehensis* had more upregulated (209) than downregulated (159) genes that may be associated with energy and carbohydrate metabolism, whereas the leaves of waterlogged *M. halliana* had more downregulated (122) than upregulated (76) genes. *M. hupehensis* leaves exhibited better energy and carbohydrate metabolic adaptations to waterlogging than *M. halliana* leaves.

Genes related to antioxidation. Waterlogging stress efficiently induces plants to produce excessive reactive oxygen species (ROS), breaking the metabolic balance of ROS (Zhang et al. 2015). ROS removal is facilitated by plant enzymes such as superoxide dismutase, glutathione reductase, and peroxidase, as well as reducing chemicals such as ascorbic acid and glutathione (Asada 1999). Here, we isolated seven upregulated and two downregulated gene-encoded peroxidases from the leaves of waterlogged *M. hupehensis*. In contrast, we obtained eight upregulated and seven downregulated genes from the leaves of waterlogged *M. halliana* (Supplemental Table S7). To resist the toxicity of ROS under flooding stress, in addition to antioxidant enzymes, terrestrial plants can also activate nonenzymatic antioxidant protection mechanisms to maintain the balance of ROS metabolism (Yu 2018). Flavonoids have been postulated to function as a secondary ROS-scavenging pathway in plants (Agati et al. 2011; Fini et al. 2011). The waterlogging tolerance of *Chrysanthemum morifolium* leaves and inflorescences and *Isatis tinctoria* roots when antioxidant enzymes are inactivated by severe or prolonged stress has been linked to flavonoids (Tan et al. 2008; Zhou et al. 2018). We detected seven upregulated and five downregulated genes that may relate to the flavonoid biosynthesis pathway in the leaves of waterlogged *M. hupehensis* and eight upregulated and two downregulated genes in the leaves of waterlogged *M. halliana* (Supplemental Table S7). The expression of genes related to the flavonoid

biosynthesis pathway was elevated in the leaves of waterlogged *M. halliana,* and the specific regulatory mechanism needs further study.

Genes related to polysaccharide, cell wall, and cytoskeleton metabolism. Hypoxia stress caused by flooding also affects plant cell wall metabolism. For example, the morphologic adaptation of plants under hypoxia is accompanied by the activation of cell wall–degrading enzymes such as cellulase and pectinase (Xu et al. 2013). We isolated 125 downregulated and 115 upregulated and 80 downregulated and 35 upregulated genes that may relate to polysaccharide and cell wall metabolism, respectively, from the leaves of waterlogged *M. hupehensis* and *M. halliana* (Supplemental Table S8), indicating that polysaccharide and cell wall metabolism was less impaired in the leaves of waterlogged *M. hupehensis* than in the leaves of waterlogged *M. halliana*.

Numerous studies have shown that the formation of aerenchyma and adventitious roots after flooding is related to the accumulation of ethylene (Rasmussen et al. 2017; Wany et al. 2017). Flooding conditions were found to promote the largescale synthesis of ethylene, enhance the activity of cellulase, accelerate cell division and programmed cell death of some cells, loosen the arrangement of cells, enlarge the tissue gap, and form aerenchyma in roots (Yin et al. 2012). We identified four downregulated and 15 upregulated genes and five downregulated and 10 upregulated genes, respectively, that may be associated with cellulase, pectinase, and cell division in leaves of *M. hupehensis* and *M. halliana* subjected to waterlogging (Supplemental Table S8), suggesting that increased pectinase and cell division affect the waterlogging tolerance of *M. halliana*. Xyloglucan endoglycosidase/hydroxylase (XTH) is a cell wall relaxation factor that can change the shape and size of cells, affect the regeneration and differentiation of cells, and participate in the expansion and degradation of cell walls. Under flooding stress, *XTH13*, *XTH32*, *XTH8*, *XTH9*, and *XTH23* all participate in the formation of *Z. mays* cell aerenchyma in roots (Thirunavukkarasu et al. 2013). Here, we identified three upregulated and one downregulated XTH gene(s) from the leaves of waterlogged *M. hupehensis*, but two upregulated and one downregulated XTH gene(s) from the leaves of waterlogged *M. halliana*, suggesting that the upregulation of XTHs plays a role in the waterlogging tolerance of *M. hupehensis* and *M. halliana* leaves.

Soluble sugar is a vital osmoregulation substance in plants. Plant cells maintain the balance of osmotic potential by regulating soluble sugar content under flooding conditions. This phenomenon is widely regarded as a plant adaptation and protection mechanism in the face of adversity (Zhang et al. 2006). For example, the content of soluble sugar, reduced sugar, and sucrose in leaves was found to increase after *Ginkgo biloba* was subjected to flooding stress (Sarkar and Das 2000). Here, we determined 15 upregulated and three downregulated genes may be involved in fructose and sucrose biosynthesis in the leaves of waterlogged *M. hupehensis* and four upregulated and eight downregulated genes in the leaves of waterlogged *M. halliana*. Thus, an increase in soluble sugar may play an essential role in the waterlogging tolerance of *M. hupehensis.*

Genes related to lipid metabolism. Many kinds of plant lipids play an essential role in various stress responses. For example, to cope with hypoxia stress caused by flooding, plants maintain the optimal function of their membranes by remodeling their lipid composition, including the contents of various lipids, the length of the carbon skeleton, and fatty acid saturation (Kurokawa et al. 2018; Li et al. 2014). In this study, we isolated 83 downregulated and 96 upregulated genes and 49 downregulated and 57 upregulated genes, respectively, that may be associated with lipid and fatty acid metabolism from the leaves of waterlogged *M. hupehensis* and *M. halliana* (Supplemental Table S9). According to the transcriptome analysis of different plant-related tissues under hypoxic stress, plant lipid biosynthesis and catabolism are two of the most abundant signal pathways for differential enrichment (Xie et al. 2015). *A. thaliana* flooded for 2 days had induced gene expression in the fatty acid degradation pathway but inhibited gene expression related to fatty acid biosynthesis in roots (Xie et al. 2015). Similar results were observed in our study. We isolated seven downregulated and three upregulated genes and two downregulated and one upregulated gene(s) that may relate to fatty acid synthesis, respectively, from the leaves of waterlogged *M. hupehensis* and *M. halliana*. Both upregulated genes are involved in fatty acid degradation in the leaves of waterlogged *M. hupehensis* and *M. halliana*. Changes to and remodeling of plant lipid dynamics are essential in regulating hypoxia perception.

Genes related to protein and amino acid metabolism. In plant responses to diverse stressors, amino acids play crucial functions. The isolation of waterlogging-responsive genes associated with protein metabolism from the leaves of waterlogged *M. hupehensis* (1350) and *M. halliana* (756) (Supplemental Table S10) may explain the increased waterlogging tolerance of *M. hupehensis*.

Under hypoxic conditions, the biosynthesis of normal proteins is inhibited, and anaerobic peptides are induced to synthesize in *Z. mays* roots (Sachs et al. 1980). In the leaves of waterlogged *M. hupehensis* and *M. halliana*, we respectively determined 22 downregulated and 10 upregulated, as well as 11 downregulated and three upregulated, protein synthesis–related genes. The lower downregulation of protein synthesis–related genes may be responsible for the lower waterlogging tolerance of *M. halliana*. γ-Aminobutyric acid (GABA) is an amino acid that also plays a role in plant flooding response mechanisms (Gilliham and Tyerman 2016). Salah et al. (2019) showed that the exogenous application of

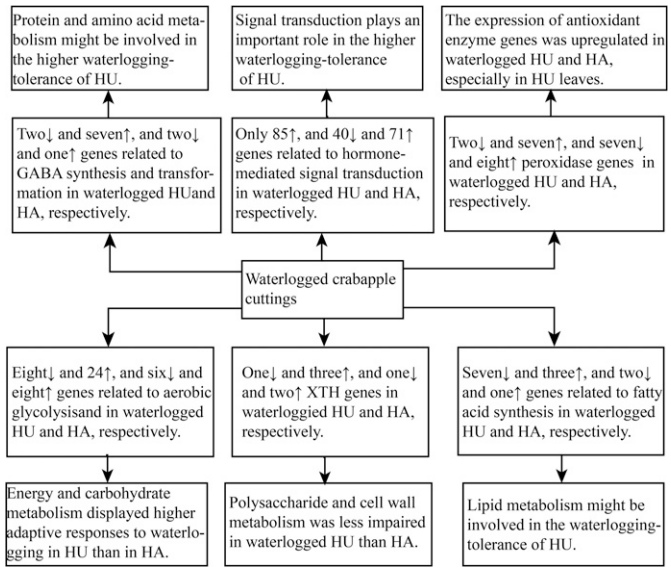

Fig. 3. A potential response of crabapple cuttings to waterlogging stress. HU = *Malus hupehensis*; HA = *Malus halliana*; GABA = γ-aminobutyric acid; XTH = xyloglucan endoglycosidase/hydroxylase. ↑ = upregulation of gene expression, ↓ = downregulation of gene expression.

GABA can promote the content of GABA in *Z. mays* leaves and improve their photosynthesis and antioxidant capacity. Seven upregulated and two downregulated, as well as two upregulated and one downregulated, GABA production and transformation-associated genes were identified from the leaves of waterlogged *M. hupehensis* and *M. halliana*, respectively (Supplemental Table S10), suggesting that amino acid metabolism may be implicated in the greater waterlogging resistance of *M. hupehensis*.

Conclusions

Using RNA-seq, we isolated 5703 and 2735 waterlogging-responsive genes from waterlogging-treated *M. hupehensis* and *M. halliana* leaves. Among these DEGs, only 746 were shared by both. Through the integration of the present findings and the available data in the previous reports, a model for the adaptive responses of crabapple cuttings to waterlogging was proposed (Fig. 3). There were common and unique mechanisms for waterlogging tolerance in crabapple plants. Several variables may explain the greater waterlogging tolerance of *M. hupehensis*: there are more waterlogging response genes related to carbohydrate and energy metabolism, signal transduction, antioxidation, lipid metabolism, protein and amino acid metabolism, and polysaccharide, cell wall, and cytoskeleton metabolism pathways in the waterlogged leaves of *M. hupehensis* than in *M. halliana*. In particular, the number of DEGs related to anaerobic metabolism, fatty acid metabolism, protein phosphorylation and dephosphorylation, GABA production and transformation, and cellulase, pectinase metabolism pathway in the flooded leaves of *M. hupehensis* was more than that in *M. halliana.* The alterations in gene expression patterns of the two crabapple species induced by waterlogging varied substantially. These outcomes pave the way for further studies into the functions of genes that may be involved in waterlogging tolerance in crabapples.

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