

Genome-Wide Identification of Halogenated acid dehalogenase Genes in Medicago

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Abstract

Halogenated acid dehalogenases (HADs) hydrolyze a variety of organic phosphate substrates. They have been shown to play important roles in various physiological processes (e.g. phosphorus metabolism and stress tolerance) by few studies. In this study, 24 and 29 HAD proteins were identified in *Medicago truncatula* and *M. sativa*, respectively. Conserved motif analysis showed that most HAD proteins contain acyltransferases and phosphatase domains, and all HAD proteins have a common conserved motif (DXD). Gene duplication analysis showed that tandem duplication is the main driver of HAD gene evolution with intense purification pressure. Moreover, cis-acting element analysis for promoter region implied that HAD gene expression in Medicago is closely related to phytohormones, light, and various stresses. Available transcription data revealed that specific HAD genes were highly expressed in leaf, flower and mature seed. HAD genes responding to phosphorus deficiency, low temperature, high temperature, dehydration and salt stress were found. Meanwhile, members of HADs that may be involved in nodule organogenesis and symbiosis with *arbuscular mycorrhizal* fungi were identified. These results provide a basis for further functional studies of the HAD gene family and potential applications for genetic improvement in the context of stress and plant-microbe interactions.

1. Introduction

Halogenated acid dehalogenases (HADs) could catalyze carbon or phosphoryl group transfer reactions on a variety of substrates ^[1]. They form an enzyme superfamily consisting of ATPases, epoxide hydrolases, dehalogenases, phosphonotases, phosphomutases, and other phosphatases ^[2-3]. In plants, HADs participate in kinds of metabolic reactions involving sugars ^[4], sugar alcohols ^[5], lipids ^[6-7], amino acids ^[8] and other important compounds ^[9-10]. Although HAD protein members have low sequence similarity (15%-30%) with each other, they all have a core catalytic domain, a unified Rossmannoid fold. There are four highly conserved motifs found in HAD proteins (motif I, II, III and IV). Both motifs I and IV have two conserved aspartic acid residues, motif II a serine or threonine residue and motif III a lysine residue. The motifs I and IV are involved in coordinating Mg^{2+} at the active site, and the motifs II and III contribute to stabilizing intermediates in the hydrolysis reaction. Interestingly, of four motifs, only motif I owns core catalytic group (the two conserved aspartic acid residues mentioned above) ^[11]. Available studies have shown that HADs should be closely involved in plant growth, development and stress adaptation ^[12-14]. However, the functions of most HADs in plants are still unknown.

Medicago is an important legume forage crop with a high yield, good nutritional composition, and excellent adaptability to adverse environments ^[15]. Research focusing on the gene functions in Medicago is the basis for breeding new variety. To date, the advance of genome sequencing in Medicago ^[16] and the establishment of a research system on the model plant *Medicago truncatula* ^[17] greatly facilitated the discovery of gene functions in Medicago. To explore the role of HADs in Medicago growth, we developed a hidden Markov model to identify all HAD genes in Medicago, and further confirmed them based on key protein domains and motifs. Results showed that 24 and 29 typical HAD proteins were identified in *M.*

truncatula and *M. sativa*, respectively. Protein structure, gene chromosomal location, and evolutionary relationships were investigated using bioinformatics approaches. Expression profiles of these *HAD* genes were summarized from available reports and specific databases^[18–20] to reveal their potential functions. Finally, *M. sativa* plants were cultivated and treated by various adverse environmental factors, and the *HAD* genes expression levels were analyzed by RT-qPCRs. All this work lead to the discovery of key *HAD* genes in specific growth conditions or in response to stresses.

2. Results

2.1. Identification of *HAD* Genes in *M. truncatula* and *M. sativa*.

The whole genome sequences of *M. truncatula* and *M. sativa* were downloaded from the Phytozome database (<https://phytozome-next.jgi.doe.gov/>) and Figshare database (https://figshare.com/articles/dataset/Medicago_sativa_genomic_fa_zip/12859889), respectively. The hidden Markov models of *HAD* obtained from the Pfam protein database (PF12710) were used to find *HAD* proteins in *Medicago*. Then the specific model for *Medicago* was constructed based on found *HAD* domains. The result was further verified by HMMER web server. Finally, 26 and 31 putative *HAD* proteins were found in *M. truncatula* and *M. sativa*, respectively.

Since the two core Asp residues of motif I are necessary for catalyzing ability of enzymes, the proteins without such residues were excluded. Finally, 24 proteins in *M. truncatula* were identified as *HAD* (named MtrHAD1-24), and 29 proteins in *M. sativa* (named MsaHAD1-29). Analysis of basic protein information showed that the lengths of *HAD* proteins ranged from 89 aa (MsaHAD23) to 777 aa (MtrHAD7), molecular weights 9.7kDa (MsaHAD3) to 84.9kDa (MtrHAD7), and isoelectric points 3.81 (MtrHAD17) to 9.93 (MsaHAD28) (Table S1). This demonstrated that there are significant differences between *HAD* protein members.

2.2 Protein and Gene Structure Analysis of *HAD*

To study the evolutionary relationship between *HAD*s, protein sequences were aligned using Fast Tree software, and a phylogenetic tree was constructed with the maximum likelihood method and JTT model. Overall, 53 proteins were divided into three major groups (I, II, and III) (Fig. 1), group I comprising 19 MtrHAD and 22 MsaHAD proteins, group II 3 MtrHAD and 4 MsaHAD proteins, and group III 2 MtrHAD and 3 MsaHAD proteins. The conserved protein motifs were analyzed with MEME software. The results showed that the motif compositions varied among *HAD* groups. The number of motifs in each *HAD* protein ranges from 0 to 14, which confirms that there is a great divergence between *HAD* proteins. Meanwhile, *HAD* proteins in the same group in the phylogenetic tree commonly have similar motif compositions (Fig. 1). By annotation with the UniProt database, twenty motifs with the lowest E-values are listed in Table 1. Interestingly, seven motifs (motif 2, 4, 6, 9, 14, 16, 18) were annotated 2,3-diketo-5-

methylthio-1-phosphopentane phosphatase. The motif 5 is unknown in function. The others are all annotated as glycerol-3-phosphate acyltransferase.

As noted above, HAD proteins were defined by four highly conserved motifs. They were also found in the identified *Medicago* HAD proteins (Fig. 2). Given that several HAD proteins have been reported to function extracellularly, we use the software SignalP-4.1 to predict whether HAD proteins have the ability to be secreted. The results showed that only 3 HAD proteins (MtrHAD1, MtrHAD19, MsaHAD2) contained the signal peptide needed for protein secretion (Table S2).

Table 1
Information of motifs found in HAD proteins in *M. truncatula* and *M. sativa*.

Motif	Sequence	Annotation
Motif1	CPEGTTCREPYLLRFSALFAELTDEIVPVA	Glycerol-3-phosphate acyltransferase
Motif2	IIDCSDSNWVVDLGFTDLFNQLLPTMPWN	2,3-diketo-5-methylthio-1-phosphopentane phosphatase
Motif3	GDGLGDECPSLTRKDKYFVLP	Glycerol-3-phosphate acyltransferase
Motif4	MDRMMELHSNGKTIEEIEKVLQRIPIHPR	2,3-diketo-5-methylthio-1-phosphopentane phosphatase
Motif5	AIKSAYALGCDLRIVSDANTFFIETILKHL	-
Motif6	RKNFPVWDLICKDPSLVKAEIHGWSDGEE	2,3-diketo-5-methylthio-1-phosphopentane phosphatase
Motif7	CPPNMVKGFLKEYJQ	Glycerol-3-phosphate acyltransferase
Motif8	VSMFYGTTASGLKCLDPFFFFMNPRPVYEI	Glycerol-3-phosphate acyltransferase
Motif9	NZEGRLFICPHHDLL	2,3-diketo-5-methylthio-1-phosphopentane phosphatase
Motif10	AVTYSISKVSEIISPIKTVRLTRDREQDAE	Glycerol-3-phosphate acyltransferase
Motif11	PKPJIFHDGRLVQRPTPLKALLMFLWLPIG	Glycerol-3-phosphate acyltransferase RAM2
Motif12	EMVSRAVLPKFYMEDLNLEAWEVFSSCGKR	Glycerol-3-phosphate acyltransferase RAM2
Motif13	KDSALKKYFGDEKPDJGJGDRCTBHDFMSS	Glycerol-3-phosphate acyltransferase
Motif14	EQVLHQLINKISMEEHVQFISTDCKLQTLS	2,3-diketo-5-methylthio-1-phosphopentane phosphatase
Motif15	DGTLLKSRSSFYPYFMLVAFEAGSLLRAFIL	Glycerol-3-phosphate acyltransferase
Motif16	GDDVCFTEINTNPGY	2,3-diketo-5-methylthio-1-phosphopentane phosphatase
Motif17	CAGGKSSIEVANYIQ	Glycerol-3-phosphate acyltransferase
Motif18	NIVVVFDFDKT	2,3-diketo-5-methylthio-1-phosphopentane phosphatase
Motif19	YLFISEELGJKILIFITFAGL	Glycerol-3-phosphate acyltransferase
Motif20	YYAYKLLGIKVIVKGTPPPPP	Glycerol-3-phosphate acyltransferase

Note: the MEME Suite 5.4.1 was used for motif search, 20 motifs with the lowest E-values were listed and annotated with UniProt.

2.3. Chromosomal Localization of HAD Gene in *M. truncatula* and *M. sativa*.

Through the visualization of HAD gene localization on chromosomes, we found that HAD genes are unequally distributed on the chromosomes of *M. truncatula* and *M. sativa* (Fig. 3). Due to the accuracy of the Medicago genomes available, 2 HAD genes, *MtrHAD7* and *MsaHAD21*, could still not be located on any chromosome. Interestingly, HAD genes were present on all chromosomes except the chromosome 6. By comparing HAD gene localization between *M. truncatula* and *M. sativa*, we found that both chromosome 3 and chromosome 7 have more HAD genes in *M. sativa* than in *M. truncatula*, and the seemingly additional genes in chromosome 3 and chromosome 7 in *M. sativa* are close in distance.

2.4. Collinearity (Duplication) Analysis of HAD Genes in *M. truncatula* and *M. sativa*.

BLASTp and MCScanX software were used to analyze the gene collinearity relationship between the two species. The results were visualized using Circos software. There was no HAD gene collinearity found in *M. truncatula* and only two pairs of HAD genes were collinear in *M. sativa*. Between *M. truncatula* and *M. sativa*, the collinearities of 18 pairs of HAD genes were found (Fig. 4). Further, gene duplication was considered. A total of 20 pairs of gene duplications were identified. They all belonged to segmental duplication, and the evolutionary driving force was mainly tandem duplication.

The synonymous substitution rate (Ks) and non-synonymous substitution rate (Ka) demonstrate selection pressure in evolution. Using KaKs_Calculator2.0 to calculate Ks and Ka, we found that the Ka/Ks values of paralogous HAD gene pairs were all < 1 (Table S3), suggesting that purifying selection processes existed during HAD gene evolution.

2.5. Analysis of cis-Acting Elements in the Promoter Regions of HADs

The cis-acting elements in the promoter region (length 1500 bp) of HAD genes were analyzed using PlantCARE software. It was found that cis-acting elements present in the promoter regions of HAD genes are associated with responses to light, stress, phytohormone, and growth development (Fig. 5). Specifically, 231 and 274 light-responsive elements, 20 and 29 growth and development-related elements, 201 and 239 stress-responsive elements, 133 and 145 phytohormones responsive elements were found in HAD gene promoter regions in *M. truncatula* and *M. sativa*, respectively. They include light responsive element (G-box, etc.), meristem regulatory element (CAT-box), temperature responsive element (STRE + CCAAT-box, LTR), defence and wound-responsive element (TC-rich repeats, W-box + WUN-motif), drought-responsive element (MYC + MBS), anaerobic induction element (ARE), auxin responsive element (TGA-element and AuxRR-core), gibberellin-responsive element (TATC-box, GARE-motif, and P-box), salicylic acid-responsive element (TCA-element), MeJA- responsive element (CGTCA-motif and TGACG-motif),

ethylene-responsive element (ERE) and ABA response element (ABRE) (Fig. 5, Table S4). These indicate that HAD genes are likely involved in various important physiological processes.

2.6. Expression Patterns of MtHADs in Medicago

The spatial expression profile was analyzed based on microarray data from the ArrayExpress database (Athare et al. 2019). In total, 16 *MtrHAD* genes were found to be expressed in flower, leaf, vegetative bud, nodule, petiole, pod, root, stem and seed (Fig. 6). About half of the *MtrHAD* genes were expressed in different organs, such as *MtrHAD11*, *MtrHAD23* and *MtrHAD19* were highly expressed in stem, petiole and leaf. In contrast, *MtrHAD15* and *MtrHAD14* were specifically expressed in leaf, *MtrHAD13* in mature seed (36 d after pollination) and *MtrHAD24* in flower (Fig. 6). All these indicated that HAD genes were closely related to Medicago growth and development.

2.7. Expression Patterns of HAD genes in Medicago in response to various factors.

To confirm the function of *HAD* genes in Medicago, previously reported transcriptome data (e.g. MtrExpressV2) in *M. truncatula* and *M. sativa* were reanalyzed. The results demonstrated that under the treatment of small signaling peptide CEP that can enhance the uptake of nitrates and phosphates, except *MtrHAD10*, *MtrHAD8*, *MtrHAD22*, *MtrHAD24* and *MtrHAD21*, the expression levels of the other *MtrHAD* genes were all reduced, indicating that *MtrHADs* were associated with plant nitrate and phosphate uptake (Fig. 7A). In response to darkness, only the expression level of the *MtrHAD20* gene was promoted by darkness for 2, 4, and 6 days. The expression levels of the other *MtrHAD* genes decreased after 2 days and 4 days; among them, several *MtrHAD* genes (e.g. *MtrHAD9*, *MtrHAD13*) were upregulated to express (Fig. 7B) after 6 days. Under salt stress (150 mM NaCl), the expression levels of *MtrHAD5*, *MtrHAD4*, *MtrHAD24* and *MtrHAD11* were continuously increased for 6 days, indicating that they were positively responsive to salt stress. In terms of phytohormone response, we found that nearly all *MtrHAD* genes responded positively or negatively to IAA (Fig. 7C). Interestingly only a few *MtrHAD* genes responded to BAP, such as *MtrHAD24* and *MtrHAD9*. Data from the treatment of AM fungi showed that most *MtrHAD* genes were up-regulated to express after Rhizophagus colonization (Fig. 7D). When plants were symbiotic with the nitrogen-fixing bacterium *Sinorhizobium meliloti*, *MtrHAD* genes were highly expressed in five different nodule zones. Specifically, *MtrHAD5*, *MtrHAD10* and *MtrHAD11* were highly expressed in the zone of cell differentiation or infection (zone IZ); *MtrHAD16*, *MtrHAD20* and *MtrHAD9* were highly expressed in the zone of nitrogen fixation (zone ZIII); and *MtrHAD3* and *MtrHAD12* were highly expressed in both zone IZ and zone ZIII. These indicate that they should participate in the building of symbiotic relationships and related reactions of nitrogen fixation (Fig. 7E). In phosphorus deficiency treatment, expression levels of several *MtrHAD* genes (*MtrHAD3*, *MtrHAD5*, *MtrHAD8*, *MtrHAD9*, *MtrHAD13-15*, *MtrHAD21*) increased and recovered after resupply of macronutrients both in shoots and roots (Fig. 7F). *MsaHAD* genes showed the same trend as *MtrHAD* genes under phosphorus deficiency (Fig. 7G). After

selenium fertilization, the expression levels of genes *MsaHAD7-8*, *MsaHAD14-18* and *MsaHAD22* in leaves changed significantly (Fig. 7H). The above analysis showed that the of HAD gene expression involves in various physiological processes, such as response to phytohormone, light, salt stress, fungus colonization, and nitrogen fixation.

In order to verify the expression profiles of HAD genes, *M. sativa* plants were cultivated and subjected to various stresses. The MsaHAD genes significantly responding to stress described above were selected for RT-qPCR analysis (Fig. 8). The results showed that *MsaHAD18* was up-regulated significantly to express in response to low temperature. In response to high temperature, MsaHAD7 was significantly up-regulated to express. In contrast, *MsaHAD9*, *MsaHAD14*, *MsaHAD16*, *MsaHAD17*, and *MsaHAD18* were inhibited to express. During dehydration treatment, the expression levels of MsaHAD7, *MsaHAD11*, *MsaHAD25* and *MsaHAD29* were significantly promoted. Under salt stress, *MsaHAD17* was highly up-regulated to express. These results confirmed that HAD genes were closely involved in the response to various stresses in *Medicago*.

3. Discussion

The HAD superfamily is found in almost all organisms, which are mainly composed of ATPase and acid phosphatase^[29]. HADs share low overall sequence similarity (15–30%). Using blast tools is often not effective in finding them, but they can be unambiguously identified due to the presence of four short conserved sequence motifs^[3]. In this study, proteins containing the HAD domain were searched using hidden Markov models of HAD and the specific species model was constructed using the obtained HAD domains. Among the conserved motifs of HAD proteins, a mutation of aspartic acid in motif I would result in a significant reduction or even loss of enzyme activity^[30]. Therefore, the proteins identified by MEME Suite software were further manually screened, resulting in 24 MtrHAD and 29 MsaHAD genes finally found in *M. truncatula* and *M. sativa*, respectively (Table S1). Further analysis confirmed that there are significant differences in sequence length, isoelectric point, and molecular weight among these HAD proteins. They could be clustered into three groups in the phylogenetic tree and a total of 20 conserved motifs were identified in *Medicago* HAD proteins. The conserved motifs of HAD varied widely among different groups. Interestingly, the motif16, encoding 2,3-diketo-5-methylthio-1-phosphopentane phosphatase, was present in almost all HAD proteins. Consistent with the analysis of conserved motifs, HAD gene structures in *M. truncatula* and *M. sativa* were also quite different. HAD genes are not evenly distributed across chromosomes and have a low degree of intraspecific collinearity.

The potential functions of HAD genes were investigated in the study. First, cis-acting elements in promoter regions were analyzed. The results showed that HAD gene expression should be regulated by various factors, including light, phytohormone, and stress. Moreover, the reported transcriptome data demonstrated that the expression levels of most HADs were relatively low in seed, exceptionally, *HAD13* was specifically up-regulated at the late stage of seed development in *M. truncatula*. This indicated that *MtHAD13* may be involved in seed maturation. Although there is no direct evidence of HAD function in seed development, Lee et al. (2021) found that in *Arabidopsis*, *AtHAD1* was induced by drought and ABA

and was found in dry seeds, its mutant exhibited increased ABA-sensitivity in germination, seedling growth, and stomatal closure^[31]. *MtHAD13* might also be involved in ABA-regulated seed maturation in *M. truncatula*, which needs further investigation to confirm. In addition, the HAD24 gene was found to be specifically expressed in flowers. Its function is also worth exploring in future.

In transcriptome analysis of different regions of co-rooted nodules, HAD genes showed specific expression profiles, such as *MtrHAD1*, *MtrHAD4*, *MtrHAD22* and *MtrHAD24* genes were specifically expressed in the apical region of nodules. Substantial studies have shown that HADs are involved in effective nodule organogenesis, nitrogen fixation, and Pi metabolism in nodules^[32–34]. Therefore, it is not difficult to understand why HAD genes are highly expressed nodules in Medicago. At the same time, transcriptome analysis revealed that HAD genes participate in many important physiological processes, including nutrition uptake, phytohormone response, and stress adaptation. This implies that an in-depth study of HAD genes should provide a train of thought for the breeding of Medicago variety. In fact, studies have already shown that transgenic plants, with gain of HAD function, altered plant phosphatase activity rates and influenced Pi acquisition and utilization in soybean^[35] and rice^[36], as well as improved tolerance to salt, osmotic and oxidative stress in Arabidopsis^[37].

It is interesting that most HAD genes were negatively responsive to light. To date, no studies have reported how light affects HAD in terms of gene expression and enzyme activity. This question also needs to be answered in the future. In addition, a majority of HADs were highly expressed in symbiosis with *arbuscular mycorrhizal* fungi. Previous studies have revealed that a set of hydrolase genes were symbiosis-inducible to AM, including some members of HAD in plants^[38]. For example, Li et al. (2019) found that the gene *GmPAP33*, one member of HADs, participates in arbuscular degeneration during AM symbiosis by participating in phospholipid hydrolysis in soybean^[39].

To verify whether the selected genes were responsive to various stresses, RT-qPCRs were performed. The HAD genes that were specifically up-regulated to express under low temperature, high temperature, dehydration, and salt stress were found. Numerous studies have shown that various special sugars, lipids, amino acids and other complex compounds are accumulated under abiotic and biotic stresses^[40–42]. This may be due to the contribution of HAD enzymes under stress conditions. Therefore, exploring the relationship between special metabolites and the function of HAD enzymes under stress is worth more attention in the future.

4. Materials And Methods

4.1. Identification of MtrHAD Genes in M. truncatula and Medicago sativa.

The whole protein sequences of *M. truncatula* and *Medicago sativa*. were downloaded from the JGI database (Phytozome (doe.gov)) and Figshare database(figshare - credit for all your research)^[43]. Hidden

Markov models (HMMs) of the HAD family were obtained from the Pfam protein database (Pfam: Home page (xfam.org))^[44]. After ClusterW alignment analysis, the HAD domain sequences that extracted from *M. truncatula* and *M. sativa*. protein sequences were used to construct a species-specific HMM model. the HMM model of the HAD domain was used to search against the *M. truncatula* and *M. sativa*. Protein sequence data by HMM search (<http://hmmer.org/>) with the parameter E-value = 0.5^[45]. The identified proteins were further examined manually to verify the presence of conserved motif I in HAD proteins. The final sequences were defined as HAD proteins and submitted to Bioperl to calculate the isoelectric point and molecular weight. Conserved motifs of the identified proteins were predicted by MEME Suite 5.4.1(<https://meme-suite.org/meme/index.html>) using the following parameters: the search window of motif was 2–30 amino acids, and the motif number was set as 20^[46].

4.2. Phylogenetic analysis

The full-length protein sequences of *M. truncatula* and *M. sativa*. were aligned by Fast Tree. The unrooted maximum likelihood method was adopted, the phylogenetic tree was constructed using the Jones-Taylor-Thornton (JTT) model^[47].

4.3. Chromosomal Distribution, gene structure and Synteny Correlation Analysis

The corresponding gene loci of the identified proteins were obtained from the genome annotation file. Almost all the gene loci were mapped on the *M. truncatula* and *M. sativa*. chromosomes with Mapchart^[48]. Gene collinearity relationships were analysed by MCScanX^[49]. The collinear relationship of HAD genes from *M. truncatula* and *M. sativa*. was finally drawn by Circos^[50]. Introns and exons of all the gene loci were extracted from the *M. truncatula* and *M. sativa* genome annotation file and drawn with TBtools. The synteny correlation analysis of HAD genes between the homologs in *M. truncatula* and *M. sativa* were verified and visualized using TBtools software^[51]. The synonymous substitution rate (Ks) and non-synonymous substitution rate (Ka) of the MtrHAD gene pairs were calculated using KaKs_Calculator2.0^[52].

4.4. Analysis of cis-elements in the promoters of MtrHAD and MsaHAD genes

The promoter sequences (1500 bp upstream of the transcription start site) of all MtrHADs were extracted from the *M. truncatula* genome file and analyzed using PlantCARE software (PlantCARE, a database of plant promoters and their cis-acting regulatory elements (ugent.be))^[53].

4.5. Expression Profile of HADs in Different Organs

Microarray data on the expression profile of MtrHAD genes in different organs and growth stages were extracted from the ArrayExpress database (<https://www.ebi.ac.uk/arrayexpress/>, ArrayExpress ID: E-MEXP-1097)^[54–55]. The heatmap was drawn using the TBtools software.

4.6. Expression profile of HADs in response to various factors

The RNAseq data on the expression profile of MtrHAD genes in responses to abiotic factors and biotic stress were extracted from the MtExpress V2 (Expression Atlas Server (inra.fr))^[56]. The heatmap were drawn using the TBtools software with row scale. *M. sativa* seedlings were grown in a greenhouse (16 h irradiation/8 h darkness, 25°C) for five weeks as previously described. The plants were subjected to low temperature (4°C, 3h) or high temperature (38°C, 3h), or immersed by 150 mM NaCl for 1 d (only roots were immersed), or dehydrated to loss water (when the fresh weight was about 50% of origin fresh weight, samples were collected). Seedlings were frozen in liquid nitrogen and used for extraction of total RNA and synthesis of cDNA using the Biospin Plant Total RNA Extraction Kit (BIOER, Hangzhou, China) and High speed-strand cDNA Synthesis Plus Kit (Coffit, HongKong, China), respectively, as described by the manufacturer's instructions. Primers were designed using the Primer 5. and listed in Table S5. qRT-PCR was conducted using ABI Quant Studio 1. The PCR reaction mix was consisted of diluted cDNA as template, 500 nM forward and reverse primers and 10 µL 2×NovoStart[®] SYBR qPCR SuperMix Plus (Novoprotein, Shanghai, China). The *MtActin7* (*Medtr3g095530*) gene was amplified as the internal control to normalize the amount of the template. Relative expression was calculated using the normalized ($2^{-\Delta\Delta C_t}$) value. The expression levels in the control plants(wt) without treatment (0 h) were normalized to 1^[57].

5. Conclusion

In this study, 24 and 29 HAD proteins were identified in *M. truncatula* and *M. sativa*, respectively. The protein structure and gene localization in chromosome were analyzed. It was found that tandem duplication was the crucial driving power for HADs evolution in Medicago. In addition, HAD proteins have undergone intense purification pressures during their evolution. The cis-acting elements in the promoter region of HAD genes demonstrated that the expressions of HAD genes are responsive to stresses, light, growth development and phytohormones. Gene expression data showed that HAD genes in Medicago were closely related to plant growth and development, especially they all were responsive to phosphorus deficiency. The genes closely related to seed maturation (*MtHAD13*), flower development (*MtHAD24*), nodule organogenesis (e. g. *MtHAD1* and *MtHAD4*) and AM Fungi in inoculation (*MtHAD18* and *MtHAD13*) were identified. Meanwhile, several specific HAD genes (*MsaHAD7*, *MsaHAD11*, *MsaHAD17*, *MsaHAD18*, *MsaHAD29*) were found to respond significantly to abiotic stress, for example, high temperatures, low temperature, water loss, and salt stress. The study provides valuable information for further investigation on biological functions of HADs, especially in stress adaptation.

Declarations

Author Contributions: Q.Z and Y.L. designed and wrote the article; Y.C. performed the data analysis, J.W. prepared Table 1 and Figure 1-5, S.Z. prepared Figure 6-8, Q.F. was responsible for finishing the

supplementary material. All authors read and agreed to the published version of the manuscript.

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Availability of data and materials: The Sequences of *M. truncatula* and *M. sativa* were downloaded from the Phytozome database (<https://data.jgi.doe.gov/refine-download/phytozome?organism=Mtruncatula&expanded=285>) and Figshare database (https://figshare.com/articles/dataset/Medicago_sativa_genomic_fa_zip/12859889). The related gene expression data in this article can be found from Array Express (<https://www.ebi.ac.uk/biostudies/arrayexpress/studies/E-MEXP-1097>) and MtExpress V2 (https://lipm-browsers.toulouse.inra.fr/pub/expressionAtlas/app/v2/zz_complete_dataset, obtained by searching the gene locus). All data generated or analyzed during this study are included in this article and its supplementary information files. The datasets used and analyzed in this study are available from the corresponding author upon reasonable request.

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Figures



Figure 1

Phylogenetic tree, conserved protein motifs, and gene structures of HADs in *M. truncatula* and *M. sativa*.
 Note: in gene structure, green represents untranslated 5'-and 3' -regions, and yellow represents exons.

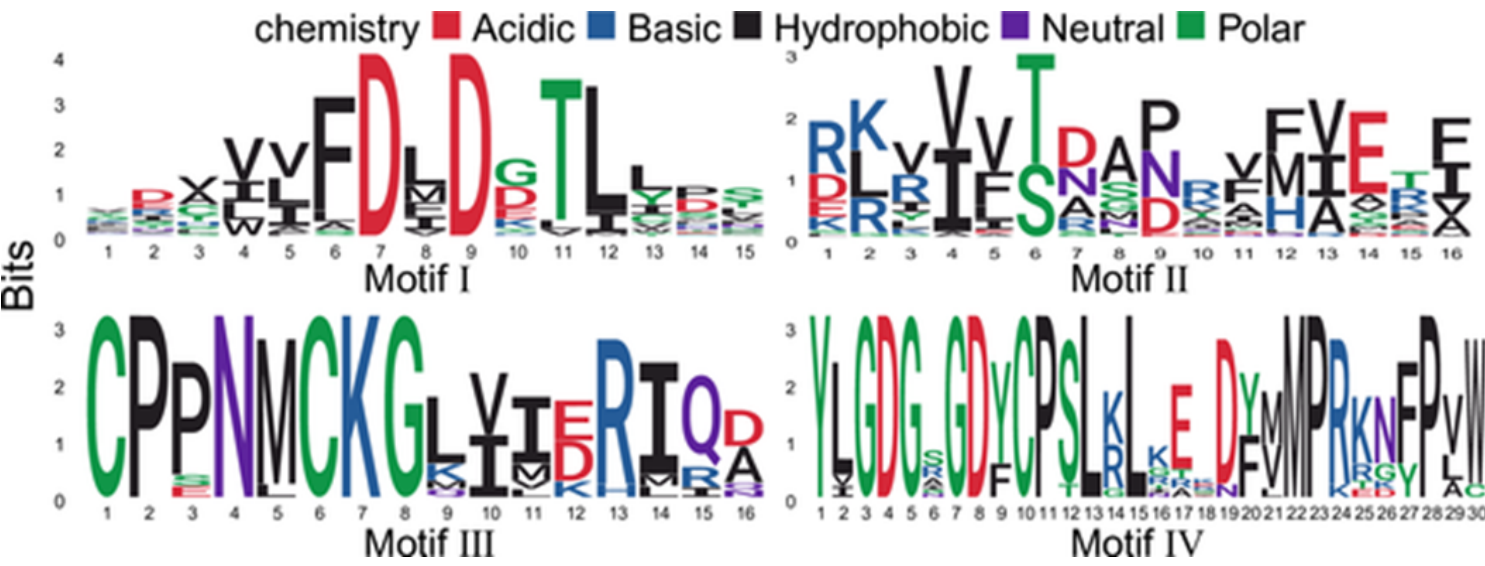


Figure 2

The four conserved motifs of HAD proteins in *M. truncatula* and *M. sativa*.
 Note: signatures of the motif I: DXD; motif II: S/T; motif III: K; motif IV: GDXXXD.

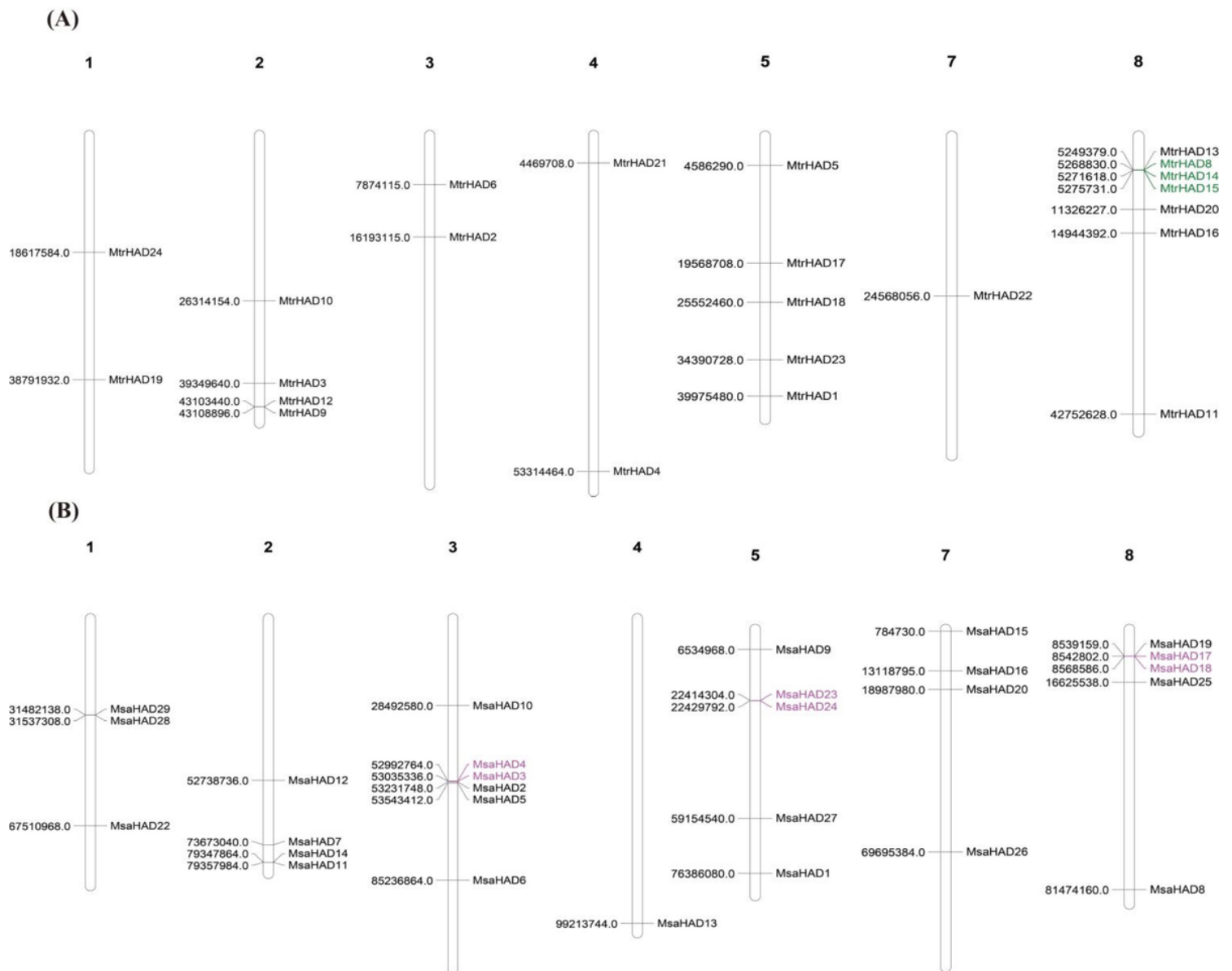


Figure 3

Chromosomal location of the HAD genes in *M. truncatula* and *M. sativa*. (A) Chromosomal location of the HAD genes in *M. truncatula*, the tandem repeat genes are shown in green. (B) Chromosomal location of the HAD genes in *M. sativa*, the tandem repeat genes are shown in purple.

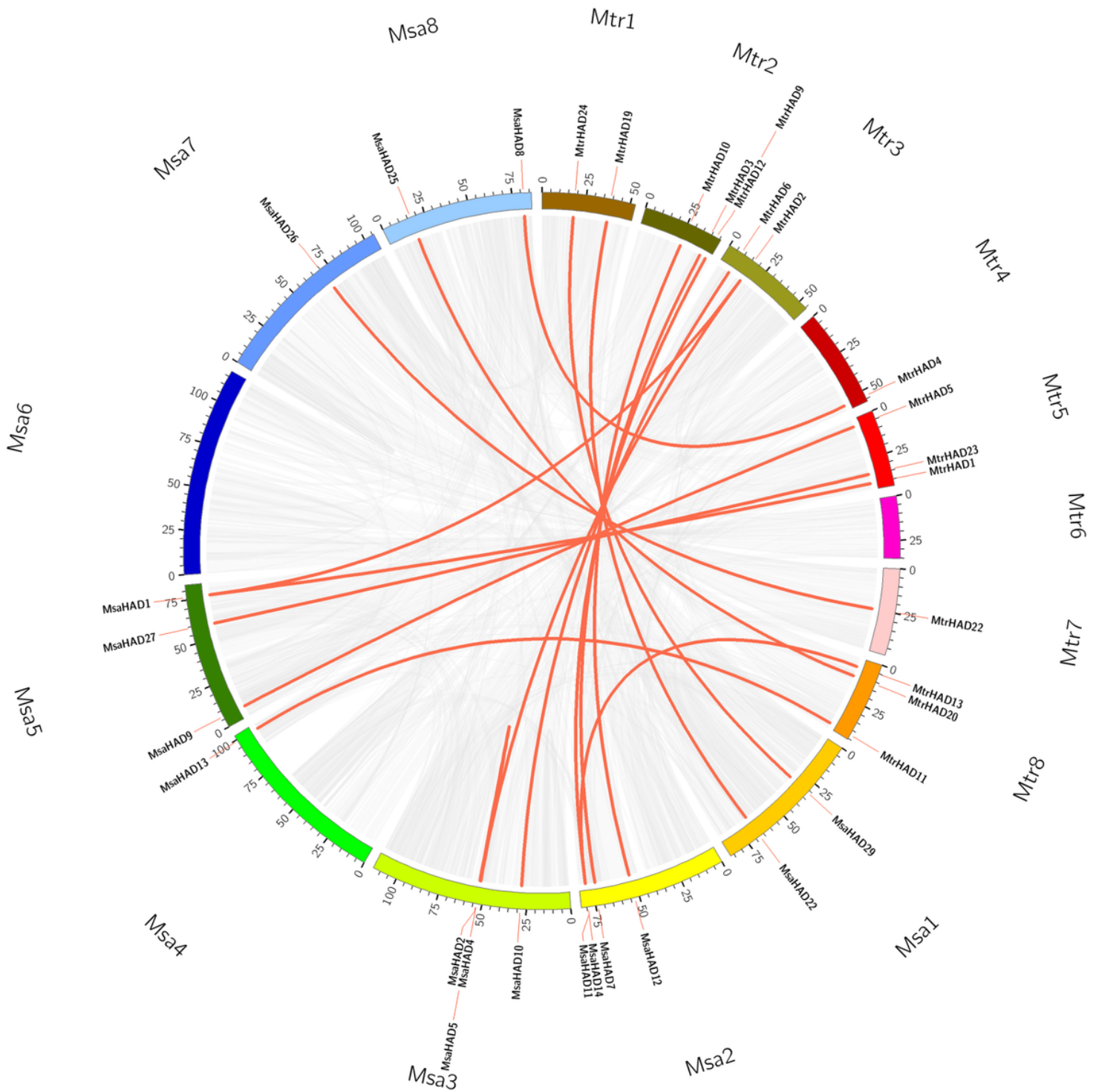


Figure 4

Chromosomal distribution and inter chromosomal relationship between *HAD* family genes in *M. truncatula* and *M. sativa*. The transparent lines indicate all the collinear blocks in the *M. truncatula* and *M. sativa* genomes. The red lines indicate duplicated *HAD* gene pairs. The chromosome names are marked on the outermost edge of the circle. The collinearity relationship of the *HAD* genes not localized on chromosome are not shown in the figure.

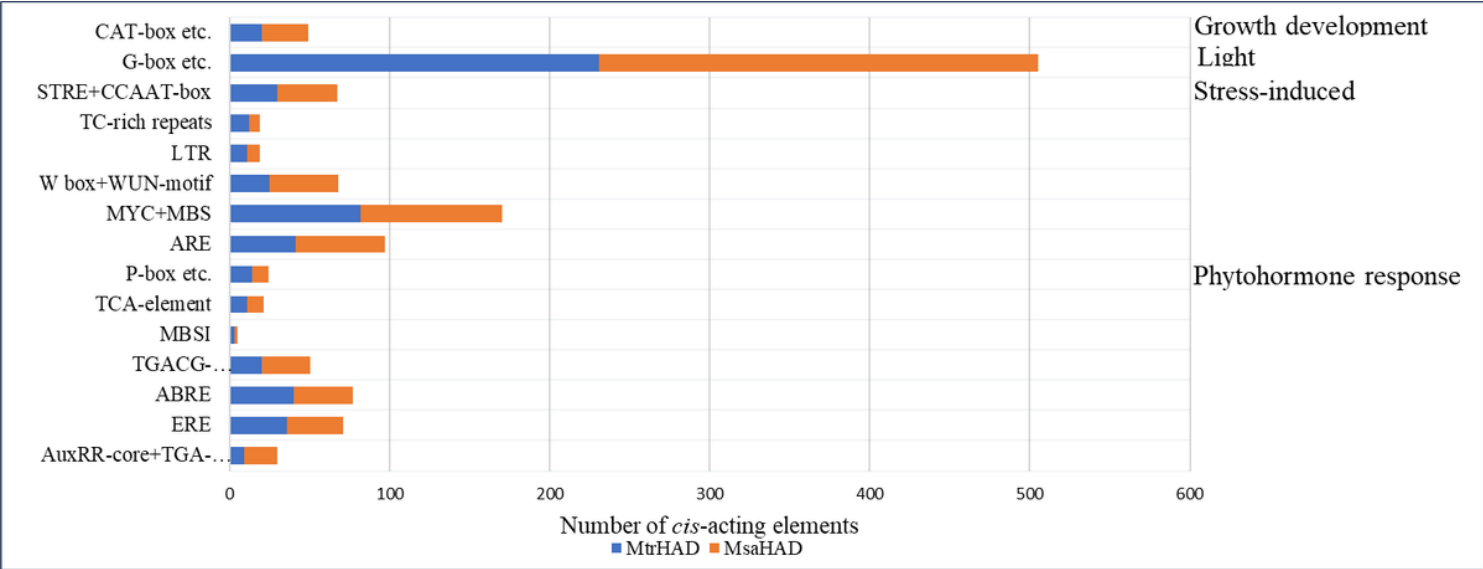


Figure 5

The number of *cis*-acting elements in the promoter regions of HADs.

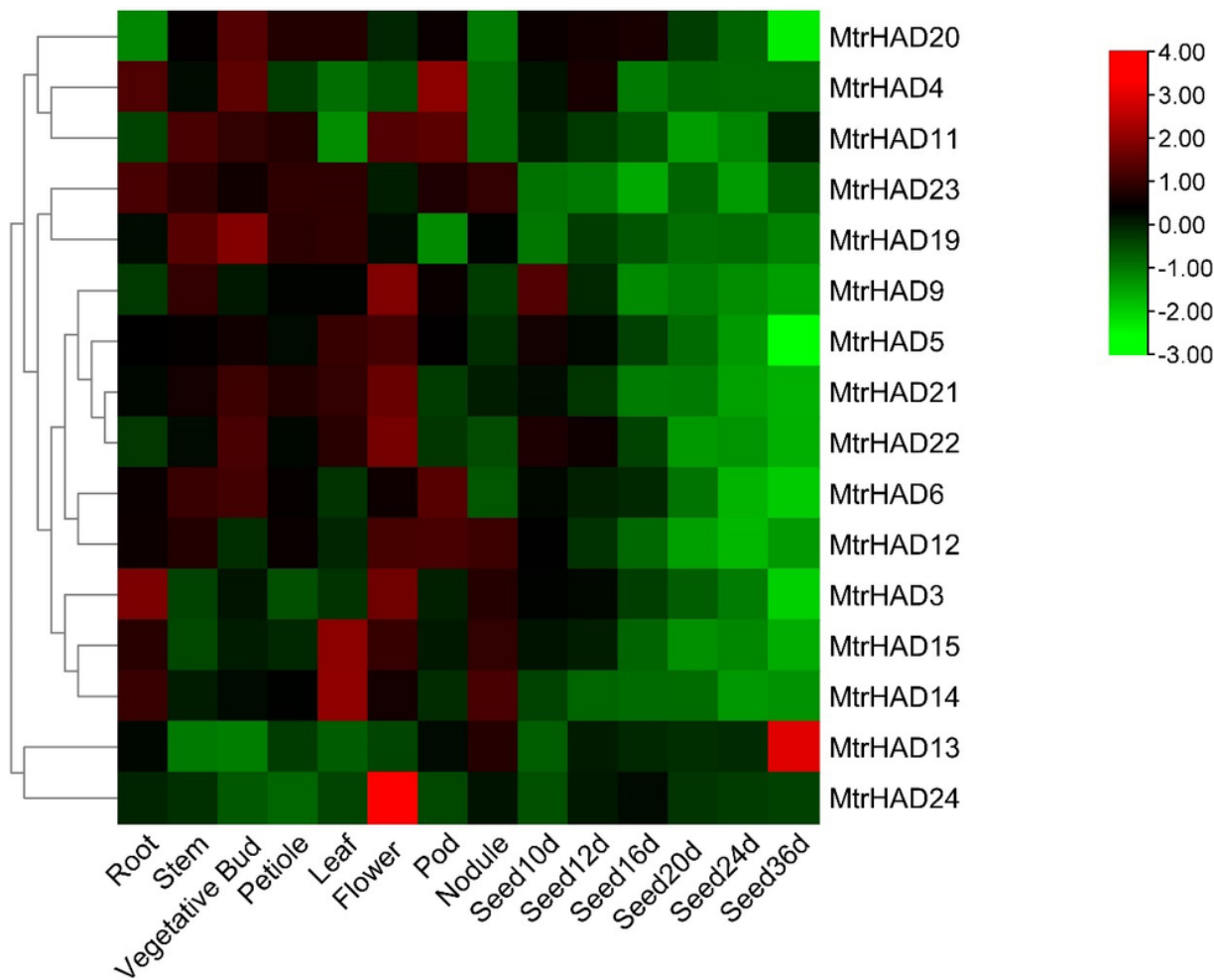


Figure 6

Heat map of gene expression of MtrHADs in different organs. The data were collected from ArrayExpress database. The relative expression levels in heat map was characterized by $\log_2(x+1)$ transformed

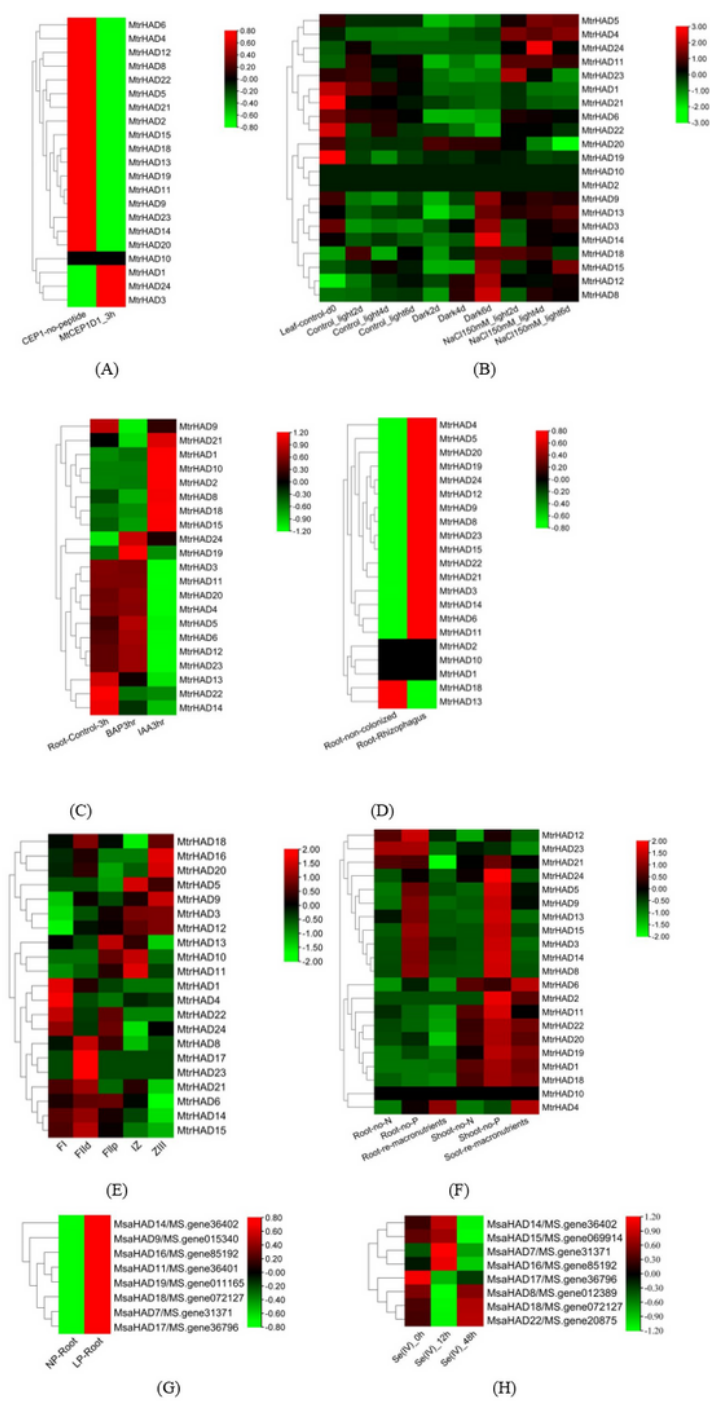


Figure 7

Heat map of HADs expression in response to CEP-inducibility, darkness and salt, phytohormones, AM fungus, macronutrients and drought stress. (A) Root treated at 0 h with no peptide (Root-CEP1-no-

peptide), Root-CEP1D1_3h represent root that were treated by CEP1 domain 1 synthetic peptide (CEPD1) [21]. (B) The five-week-old R108 *M. truncatula* was light-treated for 0, 2, 4 and 6 days, respectively, darkness or 150 mM NaCl with light treatment for 2, 4, 6 days [22]. (C) Treat the root parts of seedlings with BAP and IAA for 3 h, respectively, 3 days after germination (BAP3hr, IAA3hr), treated with DMSO as a control for the same time (Root-Control-3h) [23]. (D) Roots colonized with AM Rhizophagus or not (Root-Rhizophagus, Root-non-colonized) [24]. (E) Nodules were harvested at 10 or 15 dpi for laser dissection analyses. FI, FIId, FIIf, IZ and ZIII represent the five different zones of the mature nodules, respectively [25]. (F) Roots and shoots are subjected to nitrogen deficiency (Root-no-N, Shoot-no-N), phosphorus deficiency (Root-no-P, Shoot-no-P) and recovery of constant elements (Root-re-macronutrients, Shoot-re-macronutrients) treated *M. truncatula* 22 days after germination [26]. The above data are all represented in the heatmap using the TMM value after the log2 transformation. (G) 18-day-old seedlings were separately exposed to Pi-replete (500 $\mu\text{mol/L}$ KH_2PO_4) and severe Pi-deficient (5 $\mu\text{mol/L}$ KH_2PO_4) conditions for 12 days. NP-Root (roots under Pi-replete conditions), LP-Root (roots under Pi-deficient conditions), NP-Shoot (shoots under Pi-replete conditions) and LP-Shoot (shoots under Pi-deficient conditions) [27]. Data are presented in the heatmap using log2 (x+1) transformed TPM expression values. (H) Se (IV)_0h, Se (IV)_12h and Se (IV)_48h represent spraying Se (IV) at different times (0, 12, and 48 h) [28]. Data are presented in the heatmap using log2 (x+1) transformed FPKM expression values.

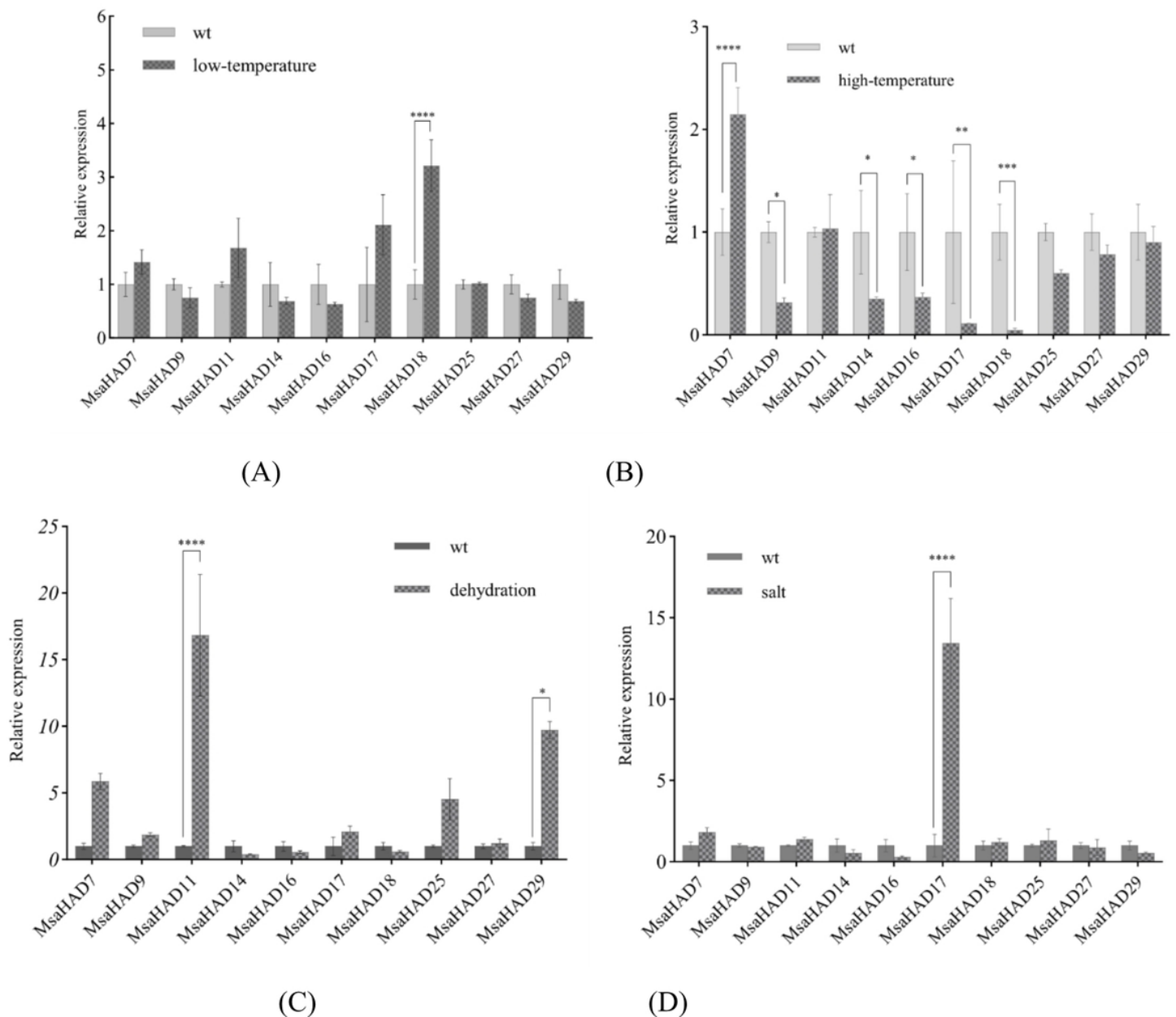


Figure 8

Relative expression of MsaHADs in response to cold treatment (A), heat treatment (B), dehydration treatment (C) and salt treatment (D). five-week-old seedlings were placed in a growth chamber at 4°C, 38°C, or treated with 150mM NaCl for 3 h or water loss to 1/2 of fresh weight. Mean values and standard errors were calculated from three biological replicates. *, **, ***, **** indicate significant difference between cold treatment, heat treatment, dehydration treatment, 150mM NaCl treatment and control using one-way ANOVA at $p \leq 0.05, 0.01, 0.001, 0.0001$.

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