Genome-wide Identification and Function Analysis of *HMAD* Gene Family in Cotton (Gossypium Spp.)

Qinqin Wang
Xinjiang Agricultural University
Xuke Lu
CAAS CRI: Chinese Academy of Agricultural Sciences Cotton Research Institute
Xiugui Chen
CAAS CRI: Chinese Academy of Agricultural Sciences Cotton Research Institute
Lanjie Zhao
CAAS CRI: Chinese Academy of Agricultural Sciences Cotton Research Institute
Mingge Han
CAAS CRI: Chinese Academy of Agricultural Sciences Cotton Research Institute
Shuai Wang
CAAS CRI: Chinese Academy of Agricultural Sciences Cotton Research Institute
Yuexin Zhang
CAAS CRI: Chinese Academy of Agricultural Sciences Cotton Research Institute
Yapeng Fan
CAAS CRI: Chinese Academy of Agricultural Sciences Cotton Research Institute
Wuwei Ye (yew158@163.com)
Chinese Academy of Agricultural Sciences Cotton Research Institute

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Abstract

Background: Soil salinized and heavy metal toxicity has become a major threat to sustainable crop production worldwide. Previous studies revealed that halophytes were supposed to tolerate other stress including heavy metal toxicity. Though *HMAD* (heavy-metal-associated domain) was reported to play various important functions in different plants, little is known in *Gossypium*.

Results: A total of 169 *G. hirsutum* genes were identified belonging to the *HMAD* gene family and divided into five classes. Additionally, 84, 76 and 159 *HMAD* genes were identified in each *G. arboreum, G. raimondii and G. barbadense*, respectively. Furthermore, conserved sequence analysis found the conserved catalytic center containing an anion binding (CXXC) box. The *HMAD* gene family showed a differential expression levels among different tissues and developmental stages in *G. hirsutum* with the different cis-elements and transcription factor binding sites (TFBS) for abiotic stress.

Conclusions: Current study provides important information about *HMAD* genes under salt-stress in *Gossypium* genome, which would be useful to understand its putative functions in different species of cotton.

Background

Halophytes are ideal candidate crop for soil reclamation of heavy metal polluted soils and moreover of that affected by salinity [1]. Heavy metals (HMs), on the one hand, as micronutrient elements Level (such as Fe, Cu, Zn, Co, Mn, Mo, Ni) is essential for the plant growth while become toxic in excess; on the other hand, other heavy metals (Ag^{+}, Cd^{2+}, Pb^{2+}, Hg^{2+}) even at low doses, are highly toxic because of no need for life and biological roles [2]. HMs contamination significantly affects not only the plant itself, but also the soil microbial community structure and function [3-5]. Heavy metal stress mainly concentrated in the signaling networks of calcium signaling, hormone signaling and MAPK (mitogen activated protein kinase) signaling and peroxide, which focused on ion detoxification and transport [6, 7]. Metal chelators is majorly Phytochelatins (PCs) and Metallothioneins (MTs), although MTs protects the plant from heavy metals by scavenging of the ROS and sequestration, even which is multi-resistant under abiotic stress such as cold, heat, salt, drought and so on [8, 9]. In compared to metal chelators, prominent groups of heavy metal ion transport families are P-type ATPases and the cation antiporters, for example, *HMA* (Heavy metal ATPase), *ABC* (the ATP-binding cassette), *NRAMP* (Natural resistance and macrophage protein), *CDF* (Cation Diffusion Facilitator), yellow-stripe-like (YSL) transporter, *ZIP* (the Zrt, Irt-like proteins), *CAX* (the cation exchanger), *CTR* (the copper transporters), pleiotropic drug resistance (PDR) transporters, and metal responsive transcription factor 1 (MTF-1), which distributed at plasma membrane or on tonoplast membrane of cell [10-14]. For *HMA* hyperaccumulators, vacuolar compartmentalization and HMs ion long-distance translocation that depends on P-type ATPases and a set of tonoplast transporters play important role in heavy metals homeostasis [15, 16, 17].

P-type ATPases have been subsided into 5 subfamilies, P1B ATPases (heavy metal pumps), P2A and P2B ATPases (Ca^{2+} pumps), P3A ATPases (plasma membrane H+ pumps), P4 ATPases (phospholipid-transporting ATPase) and P5 (no assigned specificity) subfamilies [18, 19, 20]. At least four P1B-ATPase subgroups with distinct metal selectivity: P1B-1 (include AtHMA5-8, OsHMA4-9), Cu^{2+}, P1B-2 (include AtHMA2-4), Zn^{2+}, P1B-3, Cu^{2+}, P1B-4 (include AtHMA1), Co^{2+}, which share a common catalytic mechanism with four important domains which are enzyme phosphorylation (P-domain), nucleotide binding (N-domain) and energy transduction (A-domain) and a transmembrane (TM) domain, respectively [21-26]. PIB-type ATPase lpg1024 (LpCopA) from L. *pneumophila* demonstrated that Cu^{2+} ion-entry path involves two ion-binding sites: one transient Met148-Cys382 site and one intramembranous site formed by trigonal coordination to Cys384, Asn689, and Met717 [27]. One nanobodies (Nbs) selected against the zinc-transporting PIB-2-ATPases ZntA from Shigella sonnei (SsZntA), significantly reduces the ATPase activity [28]. The multifunctional P1B-4-ATPase CzcP is part of the cobalt, zinc, and cadmium resistance system from the metal-tolerant, model organism *Cupriavidus metallidurans,* because of an evolutionarily adapted flexibility in the TM region likely afforded CzcP the ability to transport Cd^{2+} and Zn^{2+} in addition to Co^{2+} [29]. in *Mycobacterium tuberculosis*, replacement of the conserved Cys of P1B-4-ATPases at the metal binding pocket leads to a large reduction in Fe^{2+} but not Co^{2+} binding affinity [30]. In *Sphaerobacter thermophilus*, the P1B-1-and P1B-3-ATPase subfamilies both comprise Cu^{2+} transporters [31].

HMA (Heavy Metal ATPase) belonging to P1B-type ATPases (also called CPx-ATPases), is responsible for ion detoxification/transport [32, 33, 34] and vacuolar compartmentalization [35, 36]. It is interesting in double mutant that *HMA* not only affects the transport of heavy metals [37], but also affect the plant growth and development [34]. And in rice, the DNA methylation state was altered in response to the heavy metal stress and showed transgenerational inheritance [38]. In Sorghum bicolor, arsenic stimulates expression of the P1B-ATPase transporter through the abscisic acid signaling pathway. In addition, Antioxidant Protein1 (OsATX1), as a Cu chaperone in rice, interacts with the P1B-ATPases *HMA4*, *HMA5*, *HMA6*, and *HMA9*, resulting in Cu trafficking and distribution in order to maintain Cu homeostasis in different rice tissues [39]. In a model of semi-halophyte *M. crystallinum*, *HMA4* (heavy metal ATPase 4) and *IRT2* (iron-regulated protein 2) had a significantly higher expression level compared to the control between Cd-untreated and NaCl-untreated, and effects on *IRT2* expression were cumulative [40]. Moreover, salinity stress overlaps with HMs toxicity to some extent, as several integrated mechanical and chemical signals are responsible for stress-related responses [41]. For example, chloroplast and chlorophyll content can measure salt stress [42], also affect the transport of heavy metals [43, 44]. Even flavonols have shown the ability in alleviating toxic effect of Pb and improving the resistance of plants, because it activated anti-oxidative process [45].

Cotton (*Gossypium hirsutum* L.), as a moderately salt-tolerant economic crop, is a pioneer crop for soil reclamation of saline-alkaline land [46, 47]. And cotton is an important fiber crop which provides the natural fiber for the textile industry [48]. Previously, much progress has been made in the identification of *HMAD* (heavy-metal-associated domain) genes in different plants [49-52]. However, there are no detail study has been reported in the identification, functional characterization, conserved domain analysis and

expression profiles of the *HMAD* genes under salt-stress condition in cotton until now. The released genome sequence data of cotton and a publicly available database on Cottongen (https://www.cottongen.org/) allow us to comprehensively identify and analyze the *HMAD* gene family in cotton [48]. In this study, we conducted a comprehensive identification of *HMAD* genes in *G. hirsutum*, *G. barbadense*、*G. raimondii* and *G. arboretum*, with their chromosomal distribution, syntenic analysis, gene structure and conserved motifs analysis, as well as Ka/Ks values and expression pattern. In addition, predicted regulatory mechanism showed 111 *HMAD* genes were possibly regulated by salt-stress. This study will provide the basic information to further explore the specific functions of *HMAD* gene family in cotton under salt-stress.

Results

Genome-wide identification and phylogenetic analysis

We used the Hidden Markov Model (HMM) profile of *HMAD* domain (PF00403.26) from Pfam (http://www.pfam.sanger.ac.uk/) database as queries to search the *HMAD* members in *G. hirsutum*, *G. arboreum*, *G. raimondii and G. barbadense* by Hmmer software with default parameters. A total of 169 proteins were identified belonging to the *HMAD* gene family in *G. hirsutum* with the number of amino acids ranged from 56 to 1011. Furthermore, we identified 84, 76 and 159 *HMAD* proteins in each *G. raimondii*, *G. arboreum and G. barbadense*, respectively (Table S1).

In order to explore the evolutionary relationships of the *HMAD* gene family, an unrooted phylogenetic tree was constructed using the full length *HMAD* protein sequences from *G. arboreum*, *G. barbadense*,*G. raimondii*, *G. hirsutum* (Fig. 1). The *HMAD* proteins in the three *Gossypium* species were divided into five groups (I, II, III, IV, Va, Vb, Vc), which the *HMA5-8* belongs to IV group (Table S2). Additionally, 87 orthologs of *HMAD* genes (Table 2) were identified in four *Gossypium* species (I account for 18.39%, II account for 18.39%, III account for 1.15%, IV account for 10.34%, Va account for 1.15%, Vb account for 20.69%, Vc account for 29.89%) (Figure 1), such as genes *Gh_D08G1950* and *Gh_A08G2387* of *G. hirsutum* are orthologs of the *Gorai.004G210800.1* and *Cotton_A_25987* gene in *G. raimondii* and *G. arboreum*, respectively. In addition, 15 genes were lost during evolution, among which 4 in A genome (*Cotton_A_04626*, *Cotton_A_25931*, *Cotton_A_00150*, *Cotton_A_35231*), 11 in D genome (*Gorai.001G250300.1*, *Gorai.005G218500.1*, *Gorai.005G220100.1*, *Gorai.007G134300.1*, *Gorai.007G295300.1*, *Gorai.008G005700.1*, *Gorai.009G162900.1*, *Gorai.009G199900.1*, *Gorai.009G414800.1*, *Gorai.012G027800.1*, *Gorai.008G245900.1*).

Chromosomal distribution and syntenic analysis

Physical mapping of the 169 *G. hirsutumHMAD* genes were showed that 79 and 77 *HMA* genes were variably distributed on 26 chromosomes of the A and D sub-genomes, respectively (Fig. 2), among which 13 genes localized in scaffold. Additionally, a maximum of 17 and 16 genes were located on the paralogous chromosome 12 of the A sub-genomes and D sub-genomes. Moreover, there were nine pairs and two gene clusters were marked as tandemly duplicated based on the criteria of less than five

intervening genes. Among these tandemly duplicated genes, five pairs and two clusters belonged to group Vb except of *Gh_D05G1684* - *Gh_D05G1685* and *Gh_A05G1510* - *Gh_A05G1511*pairs, which belonged to group III. To study the locus relationship of orthologs between the A and D sub-genomes, we also performed synteny analysis. The result of synteny analysis indicated that most of the *HMAD* gene loci were highly conserved between the A and D sub-genomes (Fig. 3) respectively. We also found that the *HMAD* genes located on A02 and A03 chromosomes while their corresponding orthologs were located on D03 and D02 (Table 2), respectively. These results are consistant with the previous research [53], which might be due to the chromosomal translocation between Chr02 and Chr03 before cotton polyploidization forming an allotetraploid [53].

Analysis of gene structure and conserved motifs

Gene structure is important to determine its role in showing the phylogenetic relation between the *HMAD* genes. A NJ tree was generated with MEGA using all the *HMAD* protein sequences from *G. hirsutum* and gene structure were determined (Fig. 4). Though the number of genes used for generating this phylogenetic tree was different from the phylogenetic tree shown in Figure 1, the gene members within the subclades were nearly same. As shown in the Fig. 4, *HMAD* genes from *G. hirsutum* were divided into five subclades (group I, group II, group III, group IV, group Va and group Vb, among which, group I contained 13 genes while group II to group Va and group Vb contained 66, 29, 14, 22 and 25 genes, respectively. Furthermore, the analysis of gene structure showed that just 4 genes (*Gh_D01G1640*, *Gh_Sca011408G01*, *Gh_A05G3385* of group I and *Gh_A08G0990* of group Vb) contained no intron.

To investigate the presence of domain sequence and the degree of conservation of the *HMAD* domain in *G. hirsutum*, we performed multiple sequence alignment. The result of different *HMAD* protein groups indicated that a highly conserved motif presence in the *HMAD* domain, as the metal binding, with two conserved cysteines. Consistent with previous studies [54, 55], an anion binding box (CXXC) was found in the catalytic centre (Fig. 5a).

Based on the Ka/Ks ratio, it can be assumed that Darwinian positive selection was linked with the *HMAD* gene divergence after duplication [56, 57]. In our study, we found that 79 genes pairs had low Ka/Ks ratios (smaller than 0.5) and 24 gene pairs had the Ka/Ks ratios between 0.5 and 1.0. And 13 genes pairs had Ka/Ks larger than 1, might be due to relatively rapid evolution following duplication (Table 2). As most of the Ka/Ks ratios were smaller than 1.0, we presumed that the cotton *HMAD* gene family had undergone strong purifying selection pressure with limited functional divergence that occurred after segmental duplications and whole genome duplication (WGD).

Expression profile of *HMAD* genes across different tissues and different stress condition in TM-1

To understand the temporal and spatial expression patterns of different *HMAD* genes, a publicly deposited RNA-seq data was used to assess the expression profile across different tissues and developmental stages. Results showed that *HMAD* genes were not widely expressed in tissues as well as under stress condition (cold, salt, PEG), indicating their critical role in different tissues and stress

condition. *Gh_D03G0414* highly expressed in root and 1DPA, and *Gh_D04G0001* and *Gh_Sca013298G01* highly expressed in the petals and stamens. *Gh_D09G0521* highly expressed in the ovule and in pistil, especially in 3DPA, 5DPA and 35DPA. *Gh_D05G1684* highly expressed in the 10 DPA in fiber. *Gh_A01G1399*, *Gh_D01G1640*, *Gh_Sca011408G01* highly expressed in the ovule after -1DPA.

Interestingly, we found that some *HMAD* genes highly expressed under stress condition. For example, *Gh_D08G0132* and *Gh_A05G1510* highly expressed after 12 hours of the salt stress condition, while *Gh_A01G1576* highly expressed after 1 hours of the stress condition (cold, salt, PEG). *Gh_A09G1374*, *Gh_D09G1375*, *Gh_D10G0078* expression level increased under stress condition (cold, salt, PEG). Even *Gh_A08G1780*, *Gh_D08G2126* highly expressed not only in torus, ovule and fiber after 25DPA, but also after 12 hours under stress condition.

Core promoter element analysis

To further explore why *HMAD* gene family highly expressed under biotic stress condition except heavy metal, the core promoter element of *HMAD* genes from *G. hirsutum* were divided into four types (hormone, stress, tissue and others) (Fig. 8), among which, element involved in hormone-responsiveness mainly contained ABA (abscisic acid), GA (gibberellins), IAA/auxin, SA (salicylic acid), MeJA (Methyl jasmonate). Element involved in defense and stress responsiveness mainly contained drought, low-temperature dehydration, salt stress, anaerobic, among which, 72 genes involved in drought, 51 genes involved in low-temperature responsiveness, 55 genes involved in defense and stress responsiveness with TC-rich repeats element, and 1 gene (*Gh_D04G1066*) both involved in salt and low-temperature responsiveness. In total, there were 111 genes of 169 *HMAD* genes with core promoter element responding to stress. As described above in TM-1 RNA-seq data, 12 of the 18 genes were highly expressed with at least one abiotic stress-related promoter element (Table S1). Element involved in tissues including the palisade mesophyll cells, meristem, endosperm, seed-specific. And element involved in other's function, such ascircadian control, cell cycle, flavonoid biosynthetic. It's interesting that 9 of 12 genes with element of flavonoid biosynthetic were along with other's stress element. In previous study, anthocyanins, as secondary metabolites, may respond to stress resistance through osmotic equilibrium [58, 59, 60]. For example, *Gh_A01G1576* highly expressed after 1 hours of the stress condition (cold, salt, PEG), whose core promoter element contained drought-inducibility, low-temperature responsiveness and MYB binding site involved in flavonoid biosynthetic genes regulation with MBSI promoter element.

The expression level of *HMAD* gene in different tissues under Na_2SO_4 stress

To further identify the function of *HMAD* genes under other abiotic stress condition, we take advantage of the material Zhong 9835 which was more resistant to Na_2SO_4 than TM-1. Based on the *HMAD* gene family of RNA-seq data (Fig. 8) in Zhong 9835 (Table S5), 14 genes significantly expressed differentially in roots, stems and leaves between control and treatment with 300mM Na_2SO_4 (Fig. S1), in which 10 genes with at least one core promoter element about stress (Table S1). It is interesting to note that 3 of 4 flavonoid biosynthetic element were along with the stress element. More important, some genes highly

expressed in both TM-1 and Zhong 9835 under stress condition, such as *Gh_D04G0145*, *Gh_D10G0078*, *Gh_Sca011408G01*, *Gh_A01G1576* and so on.

Discussion

Cotton is half halophytes, and Zhong 9835 was resistance to salt [61], including Na_2SO_4. Based on the transcriptome data of TM-1, we found that heavy metal transport protein highly expressed under adversity abiotic stress condition. Further, through gene sequences and promoter element analysis found that *HMAD* evolution speed is quickly, which divided into five types of *HMAD* family, and some of those genes with responding to stress element had a highly expression under adversity abiotic stress condition. According to the analysis of the root, stem and leaf between Na_2SO_4 treatment and control, 14 genes with stress element significantly expressed differentially (Fig. S1). *HMAD* highly expressed under salt condition, probably because of ROS caused by ion balance [6, 7]. For example, on the one hand, gene expression in ROS way and ion balance maintenance, such as Ca^{2+} signaling pathway and MAPK, MYB transcription factor [62, 63, 64], programmed cell death [65, 66, 67]. And then the GSH, as the main way to remove ROS under the condition of high concentration, can not only response to heavy metal ions [68], also can response to salt stress ion [69]. At last, the balance of ions, such as anthocyanins were associated with the salt stress [58, 59]. *HMAD* with anthocyanins related promoter elements highly expressed under Na_2SO_4 condition, similar to previous study that anthocyanins involved in resistance to salt, at the same time involved in heavy metal transport [60]. On the other hand, the transfer of heavy metals and salt stress are vacuole segregation [70, 71], such as the P-type ATP as an important role, can not only balance the salt ions and also can balance of heavy metal ions [72-75]

HMA genes can selectively absorb and transport metal ions [76]. CtpB, as a plasma membrane copper (I) transporting P-type ATPase of Mycobacterium tuberculosis, is different from copper detoxification [77]. In *Mycobacterium tuberculosis*, Cu^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+} stimulate the ATPase activity of the putative P1B-type ATPase CtpG in the plasma membrane, while Cd^{2+} more efficiently than other heavy metal cations across the mycobacterial plasma membrane [78]. Chaperone is an important way in delivering Cu to heavy metal P1B-ATPases [41]. In general, *HMA* contain approximately 6-8 transmembrane helices, a soluble nucleotide binding domain, phosphorylation domain, and a soluble actuator domain, of which *HMA1-4* belonging to $Zn^{2+}/Co^{2+}/Cd^{2+}/Pb^{2+}$ transporting, although *HMA1* conserved amino acids is different from the *HMA2*, *HMA3* and *HMA4* [33], whereas *HMA5-8* belong to the Cu^{+}/Ag^{+} subclass [51].

The sequences of *HMA* (Heavy Metal ATPase) of P1B-ATP from *G. hirsutum* based on the sequences of *HMA* in *Arabidopsis thaliana*, also contained P-ATPases (E1-E2 ATPases) and halo acid dehydrogenase (HAD) domain and HMA (heavy-metal-associated domain) domain. In this study, *HMAD* gene family contained *HMA5-HMA8* (except *Gh_A08G2387*) (Table S2). *HMA5* localized in the plasma membrane, of which *Gh_A05G0564*, *Gh_A08G2388*, *Gh_D05G0693* with 8 TMHs, while *Gh_D08G1950* with 6 TMHs. In *HMA6*, *Gh_A03G1525* with 7 TMHs localized in the plasma membrane whereas *Gh_A04G0969* and

Gh_D04G1512 without TMHs localized in the chloroplast. *HMA7* and *HMA8* localized in the plasma membrane with 8 TMHs and 5 TMHs, respectively. Obviously, in cotton *HMA* genes evolutionarily adapted quickly in the TM region through the analysis of the sequence, gene structure, Ka/Ks ratio and the phylogenetic tree [29, 30].

Conclusions

In summary, we identified a total of 169, 159, 76 and 84 full-length putative *HMAD* genes in *G. hirsutum*, *G. barbadense*, *G. arboreum* and *G. raimondii,* which were much larger than that of the other gene families. We also found that *HMAD* gene family, although evolutionarily adapted quickly and regulated by MYB transcription factors, plays important roles in abiotic stress. Our results provide a foundation to further explore the crosstalk of molecular mechanism of *HMAD* genes under abiotic stress and heavy metal condition.

Methods

Cotton genome and RNA-seq resources

The sequenced genome data and annotation information of four *Gossypium* species including *G. raimondii*, *G. arboreum*, *G. hirsutum and G. barbadense* were downloaded from the Cottongen (https://www.cottongen.org/). RNA-seq data for gene expression analysis in *G. hirsutum* was downloaded from ccNET database (http://structuralbiology.cau.edu.cn/gossypium/), which mainly includes the gene expression data under some stress condition such as cold, salt, PEG and in tissues such as root, stem, leaf, petal, stamen, pistil and fibers at 5, 10, 20 and 25 days of post anthesis (DPA).

Identification of *HMAD* domain-containing genes

To identify the *HMAD* domain-containing genes, the hidden Markov Models (HMM) of *HMAD* domain (PF00403.26) was downloaded from Pfam 29.0 database (http://pfam.xfam.org/), then used to retrieve the whole genome database of four cotton species by HMMER 3.0 software [79] and further identified gene family by pfamscan website (https://www.ebi.ac.uk/Tools/pfa/pfamscan/) and (http://smart.emblheidelberg.de/) SMART (Simple Modular Architecture Research Tool) for confirmation of results. The presence of the *HMAD* domains in the protein structure was further validated using SMART software (http://smart.embl-heidelberg.de/). The redundant sequences without *HMAD* domain were manually checked and then removed. Molecular weight (MW), theoretical isoelectric point (pI), Signal peptide and size of the *HMAD* were investigated with the online tool ExPASy (http://expasy.org/tools/). Subcellular locations were predicted by software WoLF PSORT (http://wolfpsort.org/). The putative transmembrane helixes were predicted using TMHMM Server V.2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

Phylogenetic analysis

The multiple sequence alignment of *HMAD* domain sequence containing genes of four cotton species was accomplished by ClustalX2 software [80] with default parameters. The unrooted phylogenetic tree was constructed by the neighbour joining tree (NJ) in MEGA 6 software (http://www.megasoftware.net/history.php) with the bootstrap analysis for 1000 iterations and ggtree packages [81].

Chromosomal mapping and gene duplication

The physical location data of *HMAD* genes were retrieved from genome sequence data of four cotton species, and was subsequently used to map these genes using Mapchart software [82]. Synonymous and non-synonymous rates of evolution were computed using the maximum likelihood method by the Ka/Ks calculator2.0 software [83].

Gene structure and domain analysis

The exon and intron organizations of *HMAD* genes were inferred in the gene structure display server (http://gsds.cbi.pku.edu.cn/) through comparison of genomic and CDS sequences. The conserved motifs in *HMAD* genes were identified by MEME (http://meme-suite.org/tools/meme) and Tbtools [84].

Genome wide synteny analysis of *HMAD* genes

A BLASTP comparison was used to obtain the pair wise gene information between two allotetraploid cotton species (*G. hirsutum* and *G. barbadense*) and two diploid cotton species (*G. raimondii* and *G. arboreum*). According to the BLASTP output, the synteny analysis was constructed using circos-0.69-3 software package (http://circos.ca/software/) with default parameters.

Analysis of cis-elements in the promoters

Promoter element sequences extracted from upstream 2000bp of genes cis-element and transcription factor binding sites (TFBS) were found through Plant CARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

RNA-seq between control and treatment with Na_2SO_4

Zhong 9835, a preserved self-bred line from cultivar of *G. hirsutum* by our lab, was used for this study. Seeds were sown in sand soil pots. The sand was washed cleanly and sterilized at 121 °C for 8 h. Four seedlings in each pot were cultivated in a 28 °C/16 h light and 25 °C/8 h dark cycle with a light intensity of 150μmol·m-2·s-1 and 75% relative humidity for approximately 30 days. Then, 300 mM Na_2SO_4 after 12 h was chosen as the applicable stress concentration and time. Seedlings transplanted into normal water were used as controls. After exposure for 12 h, leaf, stem and whole root samples were collected. Each sample was tested three times. Samples were frozen in liquid nitrogen and stored at -80 °C for physiological measurement and transcriptome analysis.

RNA extraction and qRT-PCR analysis

Total RNA was isolated from root, stem and leaf between control and treatment with 300mM Na_2SO_4 in the Zhong 9835 by the EASY spin Plant RNA Kit (TIANGEN). Afterwards, first-strand cDNA was synthesized using Prime Script TM II 1st strand cDNA Synthesis Kit (TaKaRa) according to the manufacturer's instructions. qRT-PCR was carried out in 20 μL volume containing 1.4 μL cDNA, 0.8 μL of 10 μM forward and reverse primer, 10 μL SYBR® Premix Ex Taq II (2×), and 7.8 μL ddH_2O. PCR amplification was performed under the denaturation at 95 °C for 30 sec; 40 cycles at 95 °C for 5 sec and 60 °C for 30 sec; followed by 95 °C for 15 sec, 60 °C for 1 min by Bio-Rad@CFX96 Real-Time PCR system. qRT-PCR was carried out by the gene-specific primers, Histone3 (AF024716240) (F: TCAAGACTGATTTGCGTTTCCA, R: GCGCAAAGGTTGGTGTCTTC) was employed as an internal control. In the end, relative gene expression was quantified using the $2^{-\triangle\triangle Ct}$ method.

Abbreviations

HMAD: heavy-metal-associated domain; HMA: heavy metal ATPase; DPA: days post anthesis;

MAPK: mitogen activated protein kinase; TFBS: transcription factor binding sites; PCs: Phytochelatins; MTs: Metallothioneins; ABC: ATP-binding cassette; NRAMP: Natural resistance and macrophage protein; CDF: Cation Diffusion Facilitator; YSL: yellow-stripe-like; HMA4: heavy metal ATPase 4; IRT2: iron-regulated protein 2; WGD: whole genome duplication

Declarations

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Authors' Contributions

Qinqin Wang and Wuwei Ye conceived the research; Xuke Lu and Xiugui Chen prepared the plant materials; Lanjie Zhao and Mingge Han performed the experiments and data analysis; Shuai Wang, Yuexin Zhang and Yapeng Fan conducted the bioinformatic analysis. Qinqin Wang wrote the manuscript; Wuwei Ye revised the manuscript. All authors read and approved the manuscript.

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Availability of data and materials

All of the data and materials supporting our research findings are contained in the methods section of the manuscript. Details are provided in the attached Additional files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

Author details

[1]Engineering Research Centre of Cotton, Ministry of Education / College of Agriculture, Xinjiang Agricultural University / Xinjiang research base, State Key Laboratory of Cotton Biology, 311 Nongda East Road, Urumqi, 830052, China; wqq4024@163.com (Q.W).

[2]State Key Laboratory of Cotton Biology / Institute of Cotton Research of Chinese Academy of Agricultural Science (ICR-CAAS), Anyang, Henan 455000 China; Luxuke198761@163.com (X.L); pycxg-007@163.com (X.C); zhaolj0707@163.com (L.Z); hmgg138@126.com (M.H);

wangshui_19871201@163.com (S.W); zzuzyx0410@163.com (Y.Z); fanyp0605@126.com (Y.F).

***Correspondence:**

Wuwei Ye. Tel.: 86-0372-2562283; Email: yew158@163.com.

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Tables

Due to technical limitations, table 1-2 is only available as a download in the Supplemental Files section.

Figures

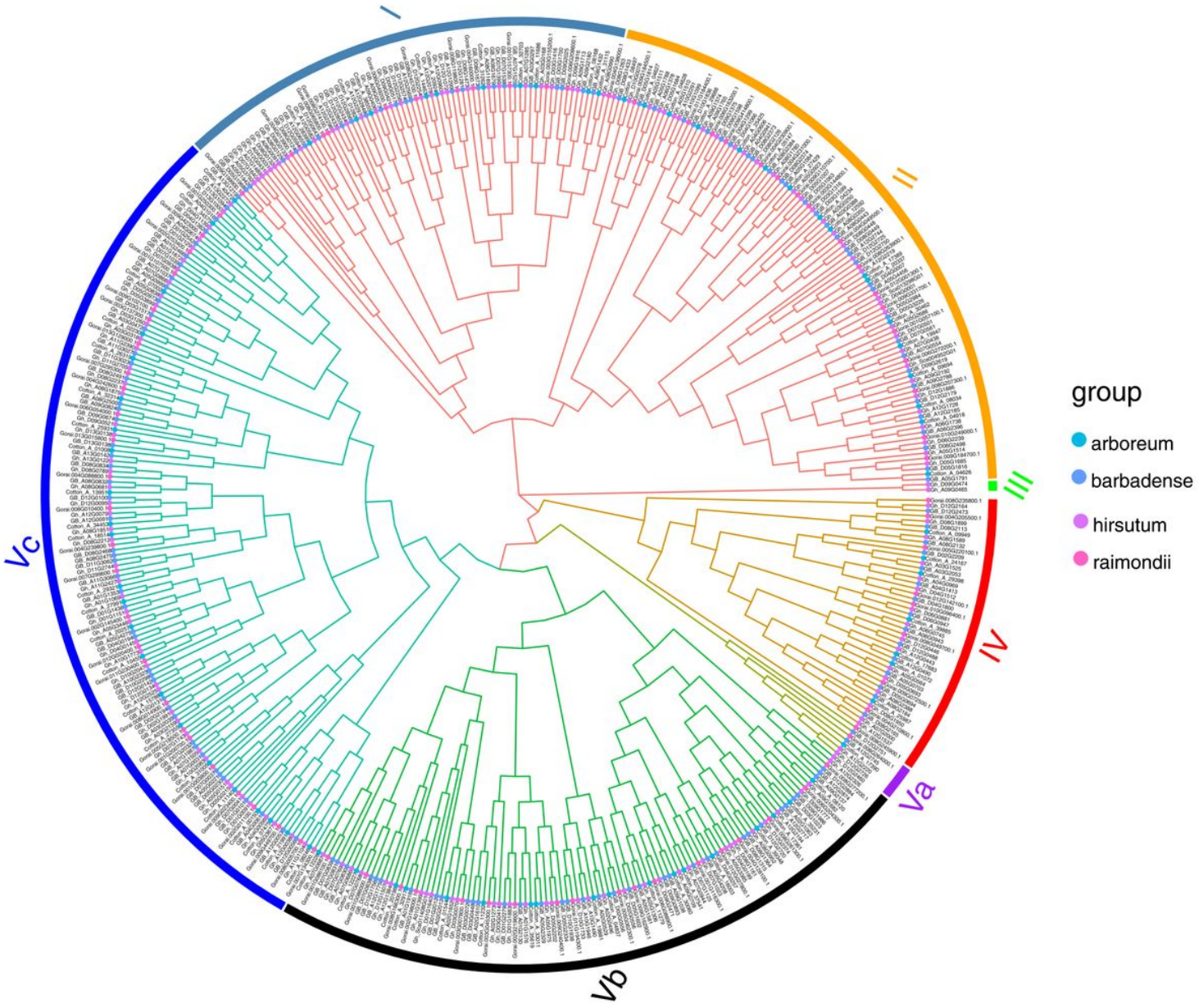

Figure 1

A phylogenetic tree of HMAD genes in four Gossypium species

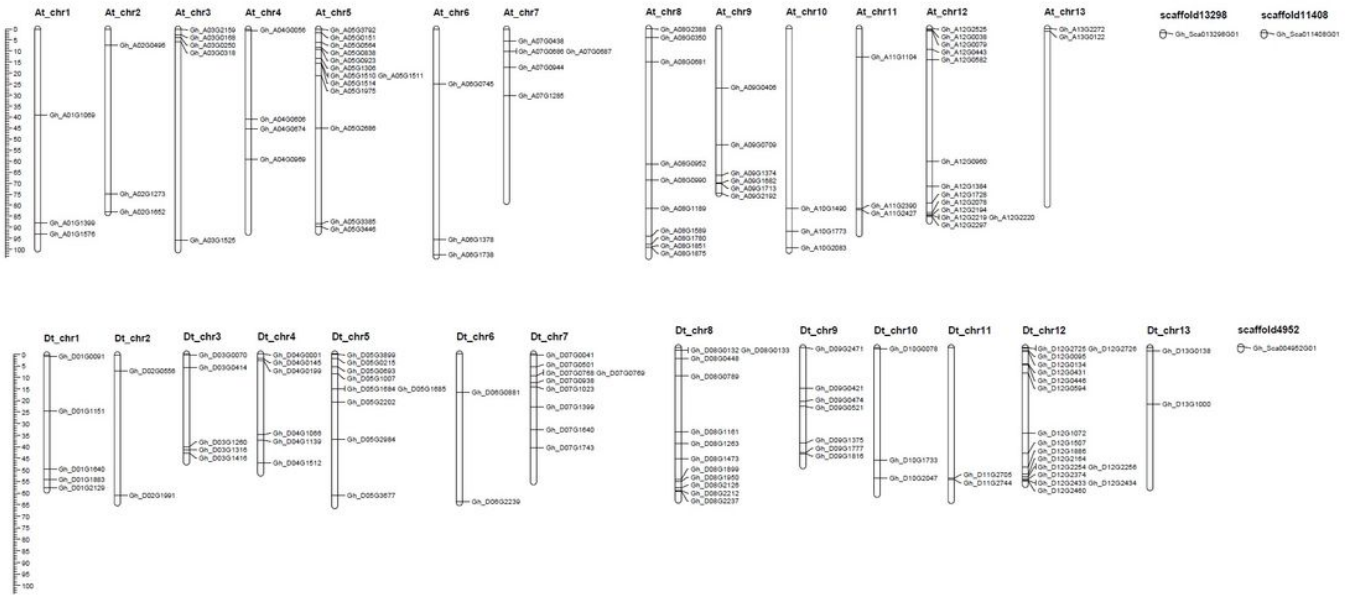

Figure 2

Mapping of the HMAD genes in the chromosomes. Partial HMAD genes localized in scaffolds

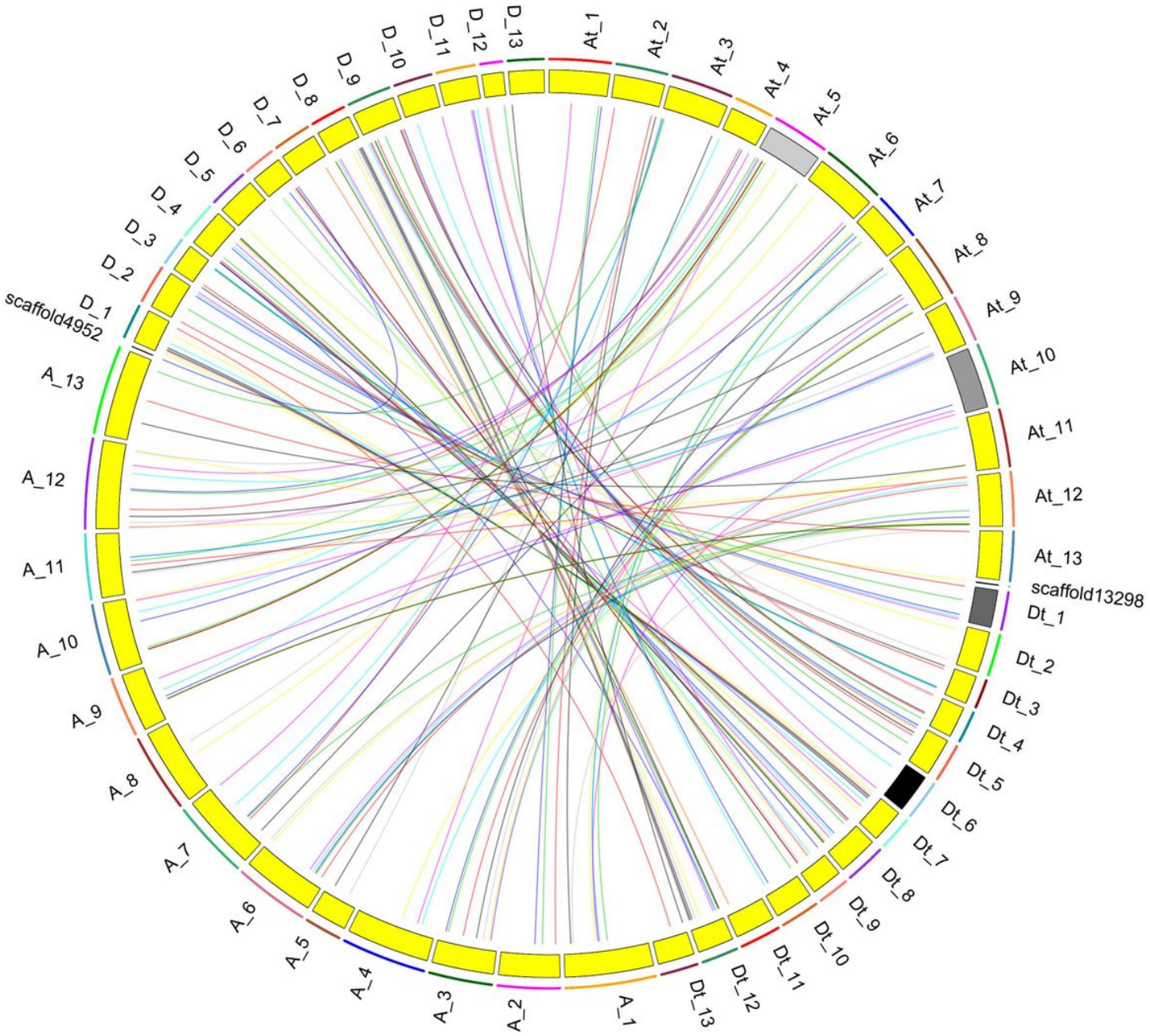

Figure 3

Genome wide synteny analysis of HMAD genes between G. hirsutum and two diploid cotton species. Blue lines indicate syntenic regions between G. arboreum and G. hirsutum, red lines indicate syntenic regions between G. raimondii and G. hirsutum

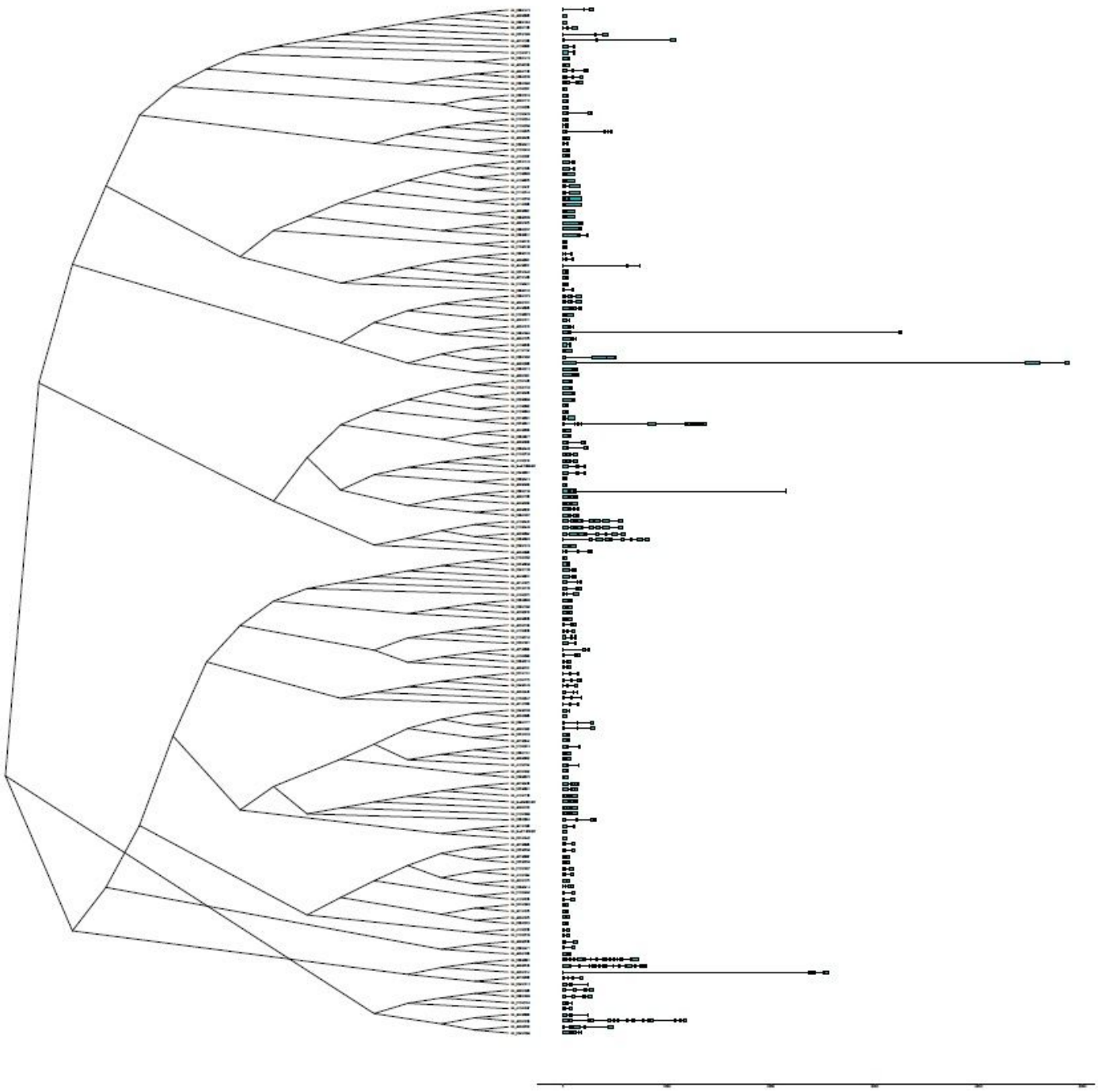

Figure 4

Gene structures of HMAD genes in G. hirsutum. Yellow boxes show exons and lines show introns

Figure 5

Logo of conserved motifs in HMAD domain in G. hirsutum. a: conserved motifs; b: Logo of conserved motif

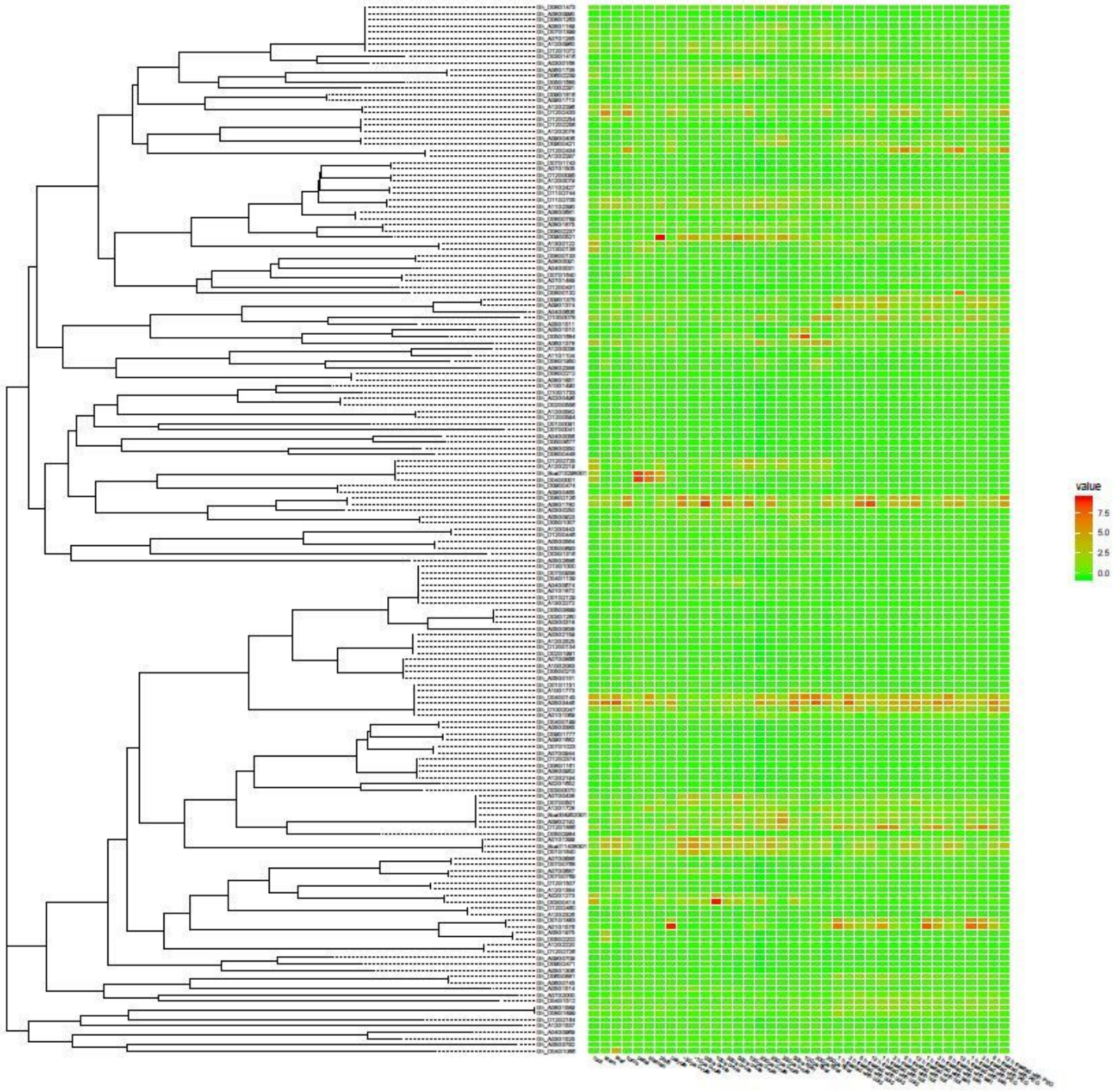

Figure 6

Expression levels of HMAD genes in different tissues and different stress. The color bar represents the expression values

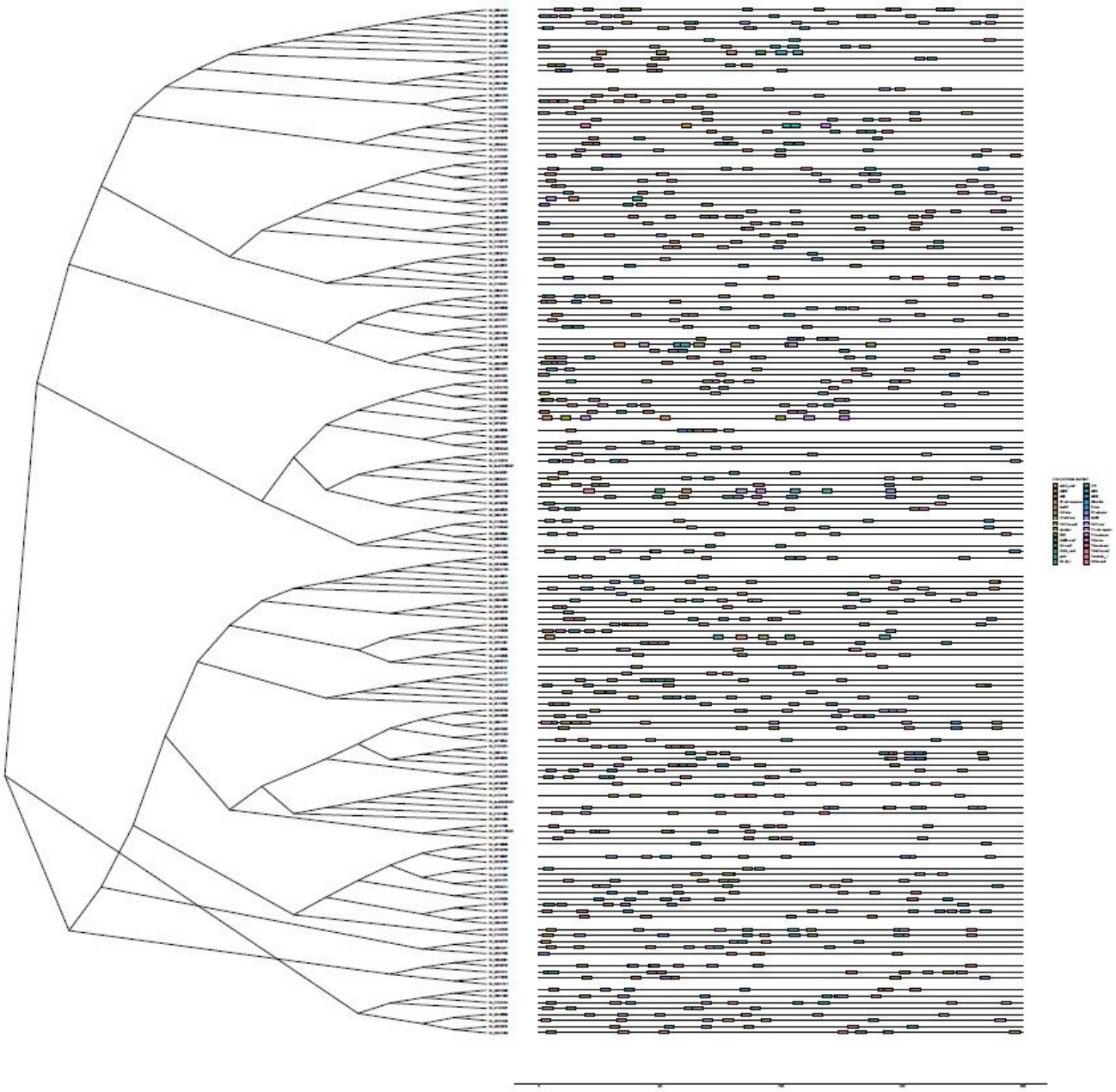

Figure 7

Core promoter element AACA_motif: involved in endosperm-specific negative expression ABRE: cis-acting element involved in the abscisic acid responsiveness ARE: cis-acting regulatory element essential for the anaerobic induction AT−rich sequence: element for maximal elicitor-mediated activation (2copies) AuxRE: part of an auxin-responsive element CAT−box: cis-acting regulatory element related to meristem expression CCAAT−box: MYBHv1 binding site CGTCA−motif: cis-acting regulatory element involved in the MeJA-responsiveness Circadian: cis-acting regulatory element involved in circadian control DRE: cis-acting element involved in dehydration, low-temp, salt stresses GARE−motif: gibberellin-responsive

element GC–motif: enhancer-like element involved in anoxic specific inducibility GCN4_ motif: cis-regulatory element involved in endosperm expression HD–Zip 1: element involved in differentiation of the palisade mesophyll cells LTR: cis-acting element involved in low-temperature responsiveness MBS: MYB binding site involved in drought-inducibility MBSI: MYB binding site involved in flavonoid biosynthetic genes regulation MSA–like: cis-acting element involved in cell cycle regulation P–box: gibberellin-responsive element RY–element: cis-acting regulatory element involved in seed-specific regulation SARE: cis-acting element involved in salicylic acid responsiveness TATC–box: cis-acting element involved in gibberellin-responsiveness TC–rich repeats: cis-acting element involved in defense and stress responsiveness TCA–element: cis-acting element involved in salicylic acid responsiveness TGA–box: part of an auxin-responsive element TGA–element: auxin-responsive element TGACG–motif: cis-acting regulatory element involved in the MeJA-responsiveness Unnamed_1: 60K protein binding site WUN–motif: wound-responsive element

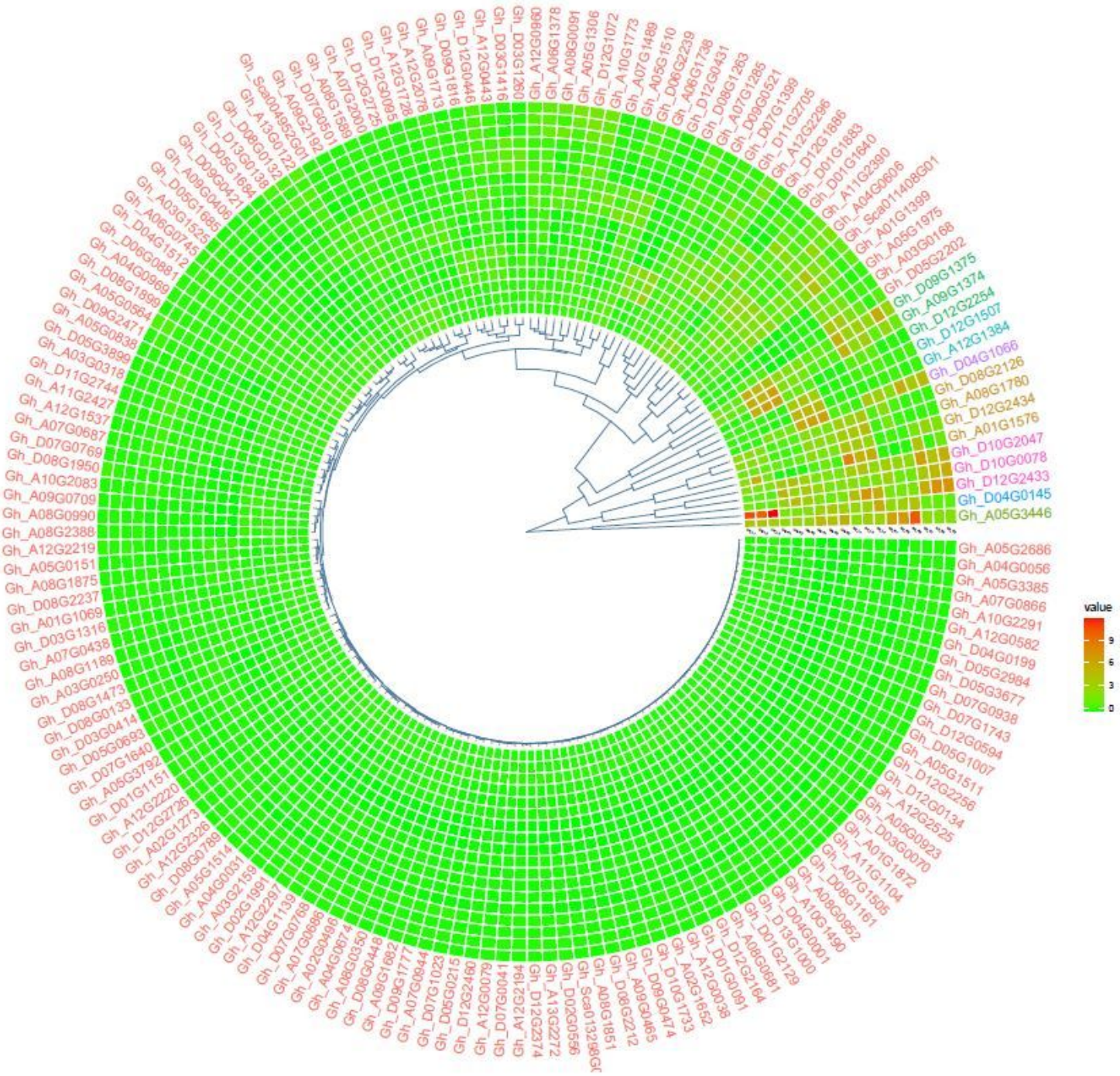

Figure 8

Expression levels of HMAD genes in different tissues (root, stem, leaf) between control and treatment with 300mMol Na2SO4. The color bar represents the expression values.

Supplementary Files

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